Transfer of colostrum components to newborn calves

By

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Thesis declaration

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Hue Thi Do

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List of publications

1. Do T. Hue, Rebel Skirving, Tong Chen, John L. Williams, Cynthia D. K. Bottema, and Kiro R Petrovski. **Colostrum source and passive immunity transfer in dairy bull calves.** *Journal of Dairy Science 104(7):8164-8176. DOI:https://doi.org/10.3168/jds.2020-19318.*

2. Do T. Hue, John L. Williams, Kiro R Petrovski and Cynthia D. K. Bottema. **Predicting colostrum and calf blood components based on refractometry.** *Journal of Dairy Research 88(2):194-200. DOI:10.1017/S0022029921000340.*

Conference presentations relevant to the thesis

1. Hue Thi Do, Tong Chen, John Williams, Kiro Petrovski, and Cynthia Bottema (2018). Transferring immune-related miRNAs from dam to newborn calves. "7th International Symposium on Animal Functional Genomics & Functional Annotation of Animal Genomes Workshop 2018", p.36, 12-15 November 2018.

2. H. T. Do, J. L. Williams, T. Chen, K. Petrovski, and C. D. K. Bottema (2019). Immune-related microRNA absorption in newborn calves. "37th International Society for Animal Genetics Conference (2019), 7-12 July 2019, Spain. (https://www.isag.us/2019/scientific-program.aspx).

List of abbreviations

Group A	calves fed own dam's colostrum
Group B	calves fed foster colostrum
Group C	calves fed pooled colostrum
Brix%	colostral total solids by refractometer
BGG	Bovine Gamma Globulin
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DE	differentially expressed
ELISA	enzyme-linked immunosorbent assay
FPIT	failure of passive immunity transfer
FT-MIR	Fourier-transform mid-range infrared analyser
GGT	gamma-glutamyl transferase
GO pathway	Gene Ontology pathway
HAP	high abundance protein
IgG	immunoglobulin G
KEGG pathway	Kyoto Encyclopedia of Genes and Genomes pathway
LAP	low abundance protein
MFGM	milk fat globule membrane
miRNA	microRNA
miR	refers to mature form of the miRNA
mir	refers to the pre-miRNA and the pri-miRNA
NGS	Next Generation sequencing
nt	nucleotide
TP-B	total protein by Bradford
TP-R	total soluble protein by refractometer
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
RNA	Ribonucleic acid

Technical terms in this thesis

- Dam colostrum: colostrum and transition milk of individual cows (n = 12) collected after calving to 3 days post-partum.
- Pooled colostrum: a mix of colostrum and transition milk from multiple cows 0-4 days post-partum.
- Bulk tank milk: a mix of milk harvested from multiple cows greater than four days post-partum.
- Reject milk: milk collected from cows greater than four days post-partum but not suitable for human consumption due to antibiotic residue or mastitis, etc.

Abstract

Bovine colostrum provides essential nutrition for newborn calves, and contains not only nutrients and growth factors but also various immune and bioactive components that impart passive immunity to help neonates resist infection during the first days of their life. The main objectives of this project were 1) to determine if different calves that receive identical colostrum from the same cow will absorb the colostrum components equally, 2) to investigate whether calves fed colostrum and transition milk pooled from cows 0-4 days postpartum receive adequate passive immunity, and 3) to explore whether immune-related microRNAs in the colostrum are absorbed by neonate calves.

For this project, 35 Holstein-Friesian bull calves were randomly allocated into three groups. Group A calves were fed colostrum from their own dam and Group B calves were fed foster cow colostrum. Group A and B calves were paired and received identical colostrum from each milking of the Group A dams for 3 days. The Group C calves were fed only one bottle of pooled colostrum (2 L), and then bulk tank milk thereafter.

The components in dam colostrum were higher than the pooled colostrum (except lactose), and consequently, the Group A calves that received their own dam colostrum for 3 days had a higher total serum protein and IgG concentration during the first week after birth than the Group C calves that received only one bottle of pooled colostrum. The paired Group A and B calves that received identical colostrum had similar levels of serum protein and IgG, although the levels in the Group B calves were slightly lower than the Group A calves. The Group C calves had less serum protein and IgG than the Group A and B calves, but did have adequate passive immunity transfer based on the accepted threshold (> 10 g/L IgG). Only two calves, one in Group B and one in Group C, did not receive adequate IgG. So no differences were detected in the proportion of calves that had failure of passive immunity transfer (FPIT) in the three calf Groups based on the IgG threshold.

MicroRNAs were in high concentrations in dam and pooled colostrum as well as in the calf blood based on RNA sequencing. There were 296 known miRNAs and 7 novel microRNAs identified in the colostrum. There was only 4 in total of 303 miRNAs detected in dam

colostrum that showed higher levels than pooled colostrum. The pathway analysis of the top 100 highly expressed microRNA in the colostrum indicated that the microRNAs in the colostrum were more related to mammary gland function than calf passive immunity or development. In the calf blood, there were 1,004 known miRNAs and 194 novel microRNAs identified. Calves were born with complex microRNAs and at high levels. There were only 22 microRNAs differentially expressed between day 0 before colostrum feeding and day 1 after the calves received colostrum. Of these 22 miRNAs, three miRNAs had higher levels at day 1 after two colostrum feeds. However, there was no evidence that the microRNAs in the dam colostrum were absorbed into the calf blood.

Immune-related microRNAs, namely miR-142-5p, miR-150, miR-155, miR-181a, and miR-223, in dam colostrum were shown to decrease steadily within 1 week postpartum by RT-qPCR. The levels of some of these microRNAs in the calf blood differed during the first 7 days after birth. Some microRNAs were high at birth and day 1 after birth, and then declined (miR-150 and miR-223). Other microRNAs increased by day 1 after birth, and then decreased thereafter (miR-155 and miR-181a). Overall, the results suggest that the calves are synthesizing most of their own microRNAs, and the microRNAs are not absorbed from the colostrum into their blood.

In conclusion, calves appear to absorb colostrum components equally. So it is possible to provide calves adequate passive immunity using a single bottle of pooled colostrum if the pooled colostrum is of reasonable quality and fed shortly after birth. While most colostrum components are absorbed by the calves, the microRNAs are an exception.

Chapter 1

Introduction and literature review

Chapter 1. Introduction and literature review

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1.1. Introduction

Bovine colostrum is produced by the mammary gland of the cow during the last weeks of pregnancy and is available during the first few days following parturition (Langer, 2009; Puppel et al., 2019). Colostrum not only includes nutrients and growth factors, but also contains a variety of immune components such as immunoglobulin, lactoferrin, lysozyme, lactoperoxidase, immune-related microRNAs (miRNAs), immune cells, cytokine, etc. which help the neonate resist infection (Hernandez-Castellano et al., 2014; McGrath et al., 2016; Godden et al., 2019; Puppel et al., 2019).

Most newborn mammals receive immunity from their mother via the placenta and colostrum; bovine newborn animals, however, only receive immunity by consuming colostrum. This is because ruminants have a synepitheliochorial placenta with eight membranes separating fetal blood from the maternal circulation (Barrington and Parish, 2001; Castro et al., 2011) that prevent the transfer of immunoglobulins from the dam to calf during pregnancy. Therefore, calves have little or no passive immunity when they are born (Barrington and Parish, 2001; Castro et al., 2011). Newborn calves receive passive immunity after birth by the absorption of immune proteins, primarily immunoglobulin G (IgG), from colostrum through the gut. The gut only remains “open” for the absorption of these components for approximately the first 24 hours after birth (reviewed by Godden et al. (2019)). To resist pathogens during the first weeks of life, newborn calves must receive a sufficient quantity of good quality colostrum as soon as possible after birth.

If calves receive too little colostrum prior to the gut closure, or if the colostrum quality is poor, without sufficient immunoglobulins, this may result in a failure of passive immunity transfer **(FPIT)**, which is correlated with increased calf morbidity and mortality (Barrington and Parish, 2001; Moran, 2002; Furman-Fratczak et al., 2011; Vandeputte et al., 2011). Avoiding FPIT and ensuring good calf health is critical to maximize profits for the dairy cattle industry. Thus, investigating the colostrum components that can be transferred to newborn calves is very important.

1.2. Literature review of bovine colostrum

1.2.1. Bovine colostrum composition

Bovine colostrum is the initial milk produced by the female cattle and the first nutrition for newborn calves. It provides key nourishment to the neonate and helps protect newborn mammals against pathogens. Bovine colostrum has been reported to be produced for up to 4 days (Dzik et al., 2017), 5 days (Moran, 2012), or to 5 or 7 days (Moran, 2002; Marnila and Korhonen, 2011b) after calving. In fact, bovine colostrum gradually changes into transition milk and subsequently to mature milk during the first 5 days postpartum (Van Hese et al., 2020).

Colostrum provides essential nutrients for the newborn, including proteins, fats, lactose, water, vitamins and minerals, but it also contains other vital factors that are crucial for growth and health (Figure 1.1 and Table 1.1). These colostrum components change over the first few days postpartum and the initial colostrum has a very different composition from the mature milk collected after day 4 post-partum. Colostrum collected at the first milking is the most crucial and contains the highest levels of components, such as protein, fat and IgG. These components decrease dramatically during the first few days of lactation though, so the colostrum collected after 24 hours to day 4 postpartum is often referred to as transition milk. In general, colostrum contains less lactose and more fat, protein, peptides, dry matter, ash, vitamins, minerals, hormones, growth factors, nucleotides and cytokines than milk (Marnila and Korhonen, 2011b; McGrath et al., 2016). Cow colostrum at calving contains, on average, 24.2% total solids, 13.5% total protein, 11.9 whey proteins, 8.0% fat, 1.9% lactose, 1.0% ash, 32.3 mg/mL IgG and 3.2 mg/mL IgM (Abd El -Fattah et al., 2012).

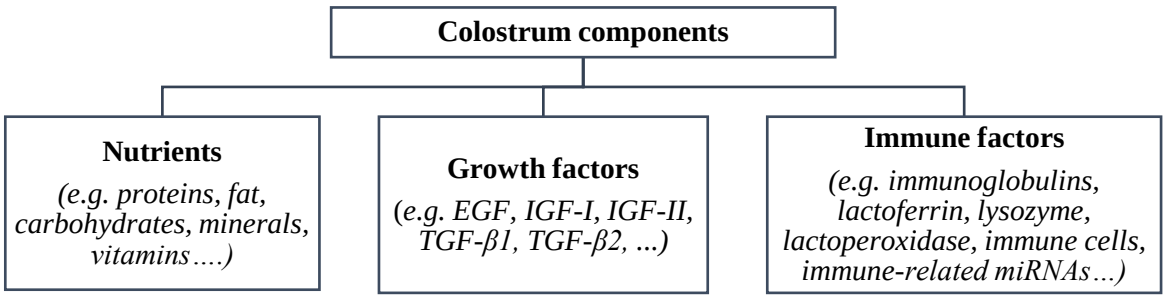

Figure 1.1. Main components in colostrum and milk. EGF: epidermal growth factor; IGF: insulin-like growth factor; TGF: transforming growth factor. *Source: Adapted from Gauthier et al., 2006; McGrath et al., 2016; Godden et al., 2019; Puppel et al., 2019.*

Table 1.1. Composition of colostrum, transition milk and milk of Holstein cows.

Parameter	Colostrum	Transition milk (milking postpartum)		Milk
	1	2	3	6
Specific gravity	1.056	1.040	1.035	1.032
Total solids (%)	23.9	17.9	14.1	12.9
Fat (%)	6.7	5.4	3.9	4.0
Total protein (%)	14.0	8.4	5.1	3.1
+ Casein (%)	4.8	4.3	3.8	2.5
+ Albumin (%)	6.0	4.2	2.4	0.5
+ Immunoglobulin (%)	6.0	4.2	2.4	0.09
+ IgG (g/100 mL)	3.2	2.5	1.5	0.06
Lactose (%)	2.7	3.9	4.4	5.0
IGF-1 (µg/L)	341	242	144	15
Insulin (µg/L)	65.9	34.8	15.8	1.1
Ash (%)	1.11	0.95	0.87	0.74
Calcium (%)	0.26	0.15	0.15	0.13
Magnesium (%)	0.04	0.01	0.01	0.01
Zinc (mg/100mL)	1.22	-	0.62	0.30
Manganese (mg/100mL)	0.02	-	0.01	0.004
Iron (mg/100mL)	0.2	-	-	0.05
Cobalt (µg/100g)	0.5	-	-	0.10
Vitamin A (mg/100mL)	295	190	113	34
Vitamin E (mg/g fat)	84	76	56	15
Vitamin B12 (mg/100mL)	4.9	-	2.5	0.6
Folic acid (mg/100mL)	0.8	-	0.2	0.2
Choline (mg/mL)	0.7	0.34	0.23	0.13

Source: Godden et al. (2019)

1.2.2. Colostrum and milk fractions

Colostrum and milk are complex fluids, which can be separated by centrifugation into different fractions (lipids, skim milk and cells) (Alsaweed et al., 2015a). The skimmed colostrum fraction contains two protein sub-fractions, soluble and insoluble (Hernandez-

Castellano et al., 2014). The fat fraction (known as the milk fat globule membrane; MFGM) of bovine colostrum appears to play a special role in transferring passive immunity from the dam to the neonate. MFGM are globules that contain proteins, glycoproteins, and various fats within a lipid membrane; it is secreted by the epithelial cells of the mammary gland (Fong et al., 2007). MFGM is an important fraction because it is rich in bioactive proteins (Mather, 2000; Affolter et al., 2010). Moreover, microRNAs were found in all three different fractions of milk (cell, skim milk and lipids), however, the highest miRNA concentration found in the cell and lipid fractions (Alsaweed et al., 2015b).

1.2.3. Colostrum quality

The quality of colostrum is usually evaluated by measuring the immunoglobulin concentration. It has been classified as excellent, good, moderate and poor if immunoglobulins were > 90; 65 – 90; 40 – 65 and < 40 g/L, respectively (Moran, 2002). For field conditions, it is general agreed that colostrum can be considered as high quality if the IgG concentration is above 50 g/L (McGuirk and Collins, 2004; Baumrucker et al., 2014; Bartier et al., 2015). There are many factors that affect the concentration of the immune components in colostrum, including breed, individual genetics, diet, the age of dam, parity, the lactation period, environment and management (Godden et al., 2019). Cows that are vaccinated at drying off will produce colostrum that can provide resistance to the specific pathogens targeted by the vaccine (Moran, 2002).

There are many different on-farm tools to test colostrum quality as well as laboratory assays to quantify IgG in colostrum (Table 1.2). It is difficult to measure IgG on-farm as most assays to measure IgG directly in colostrum are expensive and time consuming. Brix refractometry has been proposed as an accurate on-farm tool to estimate colostrum IgG (Quigley et al., 2013; Morrill et al., 2015; Cabral et al., 2016). Bovine colostrum is considered high quality if the Brix % is > 18-23% which equates to > 50 g/L of IgG (Quigley et al., 2013; Bartier et al., 2015; Morrill et al., 2015). In a meta-analysis by Buczinski and Vandeweerd (2016), the authors classified good quality colostrum as greater than 22 Brix % and poor quality colostrum as less than 18 Brix %.

Table 1.2. Different methods for measuring immune components in colostrum.

Method	References
Colostrum – on farm tools	
Hydrometers, weight of first milking colostrum; and electronic refractometer	(Chigerwe and Hagey, 2014)
Colostrometer	(Cabral et al., 2016)
Optical refractometer	(Quigley et al., 2013; Morrill et al., 2015; Cabral et al., 2016)
Brix digital refractometer	(Weaver et al., 2000; Quigley et al., 2013)
Colostrum – laboratory assays	
Enzyme-linked immunosorbent assays (ELISA)	(Arthington et al., 2000)
Radial immunodiffusion assays (RIA)	(Arthington et al., 2000)
Radial immunodiffusion (RID)	(Quigley et al., 2013; Elsohaby et al., 2017)
Transmission infrared (IR) spectroscopy	(Elsohaby et al. 2017)
Turbidimetric immunoassay	(Quigley et al., 2013)

1.2.4. Passive immunity transfer

In 1892, based on various experiments, Paul Ehrlich defined the concept of passive and active immunity, and published a paper that demonstrated immunised mice transferred immunity to newborn pups *in utero* and in the milk (Silverstein, 1996; Wheeler et al., 2007). Ehrlich compared the neonatal immunity of mice born from an immunized father and a non-immunized mother with mice born from a non-immunized father and an immunized mother. He concluded that immunity in newborn animals is owed to the transfer of immunity from the mother (Silverstein, 1996).

Newborn animals have the unique ability to absorb protein and macromolecules during first 24 to 36 hours, as the gastrointestinal tract is still permeable. Thereafter, the gut closes and macromolecules can no longer pass the intestinal barrier. The time of closure differs between species. Calf gut closure occurs approximately 24 hours after birth but can vary depending on the time of the first feed (Stott et al., 1979). Newborn calves have little or no passive immunity and require the passive transfer of immunity from the dam in order to adapt to

their new external environment (Godden, 2008). Most research on colostrum has focused on immune function, as colostrum has an essential role in building immunity in newborn calves.

Failure of passive immunity transfer **(FPIT)** occurs if insufficient good-quality colostrum is consumed soon after birth. Calves with FPIT are 4 times more likely to die and have twice a greater risk of infection (Moran, 2002), and 2-times greater odds of pneumonia (Virtala et al., 1999). In general, calves have increased morbidity and mortality if there is FPIT (Barrington and Parish, 2001; Furman-Fratczak et al., 2011; Vandeputte et al., 2011). Factors that affect the successful passive immunity transfer in calves include the colostrum quantity, quality, timing of feeding colostrum, age of the dam, parity, the presence of the dam, as well as colostrum management (Weaver et al., 2000; Jaster, 2005; Dawes and Tyler, 2007; Godden, 2008; Costa et al., 2017).

Receiving good quality colostrum is crucial to ensure that sufficient immunoglobulin can be absorbed into the circulation of the neonate. To avoid failure of passive immunity transfer, calves should receive 10% to 12 % body weight of colostrum with at least 50 g/L of IgG within a few hours of birth (McGuirk and Collins, 2004; Bartier et al., 2015; Godden et al., 2019). As it is difficult to measure IgG on-farm, it has been suggested that calves should receive 3 to 4 L of colostrum at their first feed (Moran, 2002; McGuirk and Collins, 2004; Godden et al., 2019). Other authors investigating the transfer of passive immunity to calves fed different colostrum volumes at different times after birth have suggested specific colostrum volumes and specific feeding times (Williams et al., 2014). For instance, Osaka et al. (2014) recommended that calves consume ≥ 3 L of colostrum with IgG concentration > 40 mg/mL within 6 h after birth and Jaster (2005) suggested that calves should receive 2 L of high quality colostrum at birth and every 12 hours to maximize the absorption of IgG from colostrum into calf blood.

Failure of passive immunity transfer is most commonly detected in calves by measuring IgG in the calf serum or by estimating the total serum protein using refractometry 24 hours after birth. Thresholds for FPIT indicators can vary depending on factors such as the environment, infection level, and breed (Vandeputte et al., 2011) (Table 1.3). However, the generally accepted thresholds for FPIT in calves are defined as less than 10 g/L of serum IgG at 24 -

48 hours postpartum (Weaver et al., 2000; Godden et al., 2019), less than 52 g/L of total protein (TP) in serum (McGuirk and Collins, 2004; Cuttance et al., 2017b), or as less than 8.1 - 8.5 Brix % (Hernandez et al., 2016; Godden et al., 2019). Lombard et al. (2020) suggested that the serum IgG levels ≥ 25.0, 18.0–24.9, 10.0–17.9, and < 10 g/L of IgG are categorized as excellent, good, fair, and poor, respectively.

Table 1.3. Threshold for the failure of passive immunity transfer in calves.

Indicators	Threshold of FPIT	Reference
Serum IgG	< 10 g/L at 24 h after birth	(Weaver et al., 2000; McGuirk and Collins, 2004); Godden et al. (2019); (Oliveira et al., 2019)
	<16 g/L at 24 h after birth	Vandeputte et al. (2011)
	20-25 g/L at 24 h after birth	Chigerwe et al. (2015)
Serum total protein	< 52 g/L at 24 h after birth	McGuirk and Collins (2004); (Trotz-Williams et al., 2008)
	58 – 63 g/L at 24 h after birth	Chigerwe et al. (2015)
	< 51 g/L at 24 h after birth	Godden et al. (2019)
Total solid by Brix refractometer	< 8.1 Brix % at 24 h after birth	Godden et al. (2019)
	< 8.5 Brix % at 24 h after birth	Hernandez et al. (2016)
GGT activity	< 200 IU/L at day 1; 100 IU/L at day 4; 75 IU/L at day 7; or 50 IU/L at day 14 after birth	Parish et al. (1997)
	200 IU GGT/L at 1 day after birth	Perino et al. (1993)
	100 IU GGT/L at 2 days after birth	Hogan et al. (2015)
	250 IU GGT/L for calves less than 5 days old and 210 IU GGT/L for GGT calves 5–8 days old	Cuttance et al. (2017a)

1.2.5. Immune components in colostrum

There are many immunologically active components in colostrum that can protect newborn animals passively against infection including immunoglobulins, lysozyme, cytokines, lactoferrin, different types of immune cells, such as leukocytes, and immune-related

miRNAs. All of these can be absorbed by the newborn calf when the gut epithelium is open during the first days of life.

1.2.5.1. Proteins

The total protein in the colostrum of cows is at highest at first milking after parturition but then declines dramatically. Total protein concentration in bovine colostrum a few hours after parturition is between 15 and 26%, approximately three times greater than blood plasma protein in the cow (Smith, 1946). The average total protein in bovine colostrum within 1 day postpartum is 11% to 17%, which is roughly three times higher than in milk depending the season of calving, parity, dry period, time to colostrum collection (Dunn et al., 2017; Godden et al., 2019). Cow colostrum contains 130 g/kg protein, whereas there are 33 g/kg protein in mature milk (reviewed by Langer (2009)). Immune proteins account for 50 – 60% of the total protein in colostrum and 85 – 90% of the total protein in colostrum whey (Smith, 1948).

a. Immunoglobulins

The most important immune protein in colostrum is immunoglobulin (Ig) which is a Y-shape protein generated mainly by the plasma B cells. Immunoglobulins provide the neonate with protection from specific pathogens when they are passed from the dam via colostrum (Hurley and Theil, 2011). There are five types of immunoglobulin, IgA, IgD, IgE, IgG and IgM. The type and amount of the immunoglobulin subclasses in colostrum differ between species, breed of the dam. While IgA is a main immunoglobulin in human colostrum, IgG is the predominant immunoglobulin in bovine colostrum (Hurley and Theil, 2011). Immunoglobulins in bovine colostrum are predominantly IgG1 and IgG2 and account for 80% of the immunoglobulins (Bourne, 1977; Shivley et al., 2018; Godden et al., 2019).

In bovine colostrum, the IgG1 is derived mainly from blood, with transport across mammary alveolar cells, and IgG2 is either derived from blood or is synthesised in the mammary gland (Gapper et al., 2007). Immunoglobulins from the mammary gland are transferred to colostrum by the mammary epithelial cells (Wheeler et al., 2007) (Figure 1.2). Immunoglobulins are stable in the gastrointestinal tract of neonate, and are absorbed into the vascular system of the newborn animals when the gut is open and promote protection against specific pathogens (Hurley and Theil, 2011). Total immunoglobulin concentration,

particularly IgG differs between cattle breeds, the parity, milking number or milking time postpartum (Oyeniyi and Hunter, 1978; Kehoe et al., 2011) (Tables 1.4 and 1.5).

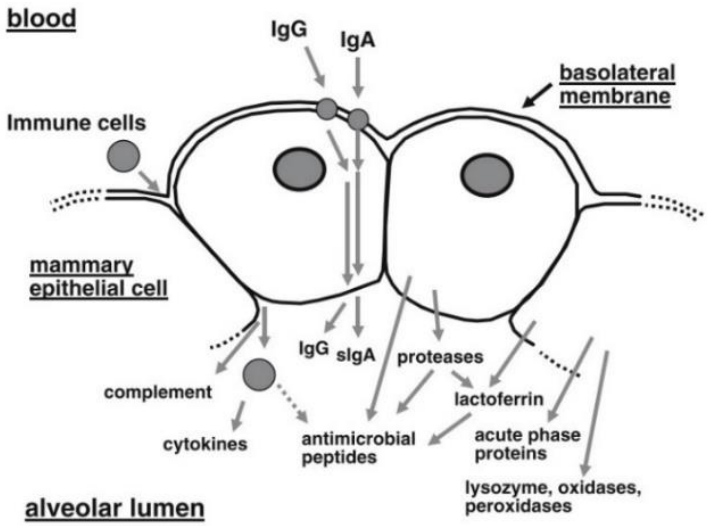

Figure 1.2. **Components of the host defence system in milk and colostrum.**

Source: Wheeler et al. (2007)

Table 1.4. IgG concentrations (mg/mL) in Holstein colostrum from the first three milking postpartum.

Lactation cow beginning (n)	IgG in colostrum from					
	Initial milking (0h)		Second milking (12h)		Third milking (24h)	
	Mean	SE	Mean	SE	Mean	SE
First (28)	29.8	2.4	23.5	2.3	14.3	2.0
Second (22)	30.5	3.3	22.4	3.0	11.4	1.8
Third (10)	33.9	3.4	26.6	3.3	16.8	3.2
Fourth – seventh (11)	41.6	6.1	36.3	5.6	24.9	3.9
First – seventh (71)	32.4	1.8	25.4	1.6	15.4	1.3

Note: Values are different (P<0.01) between 3 times milking by Student's t-test.

Source: Oyeniyi and Hunter (1978)

Table 1.5. Least squares mean of IgG concentration and mass and colostrum volume by lactation.

Measure	Lactation				
	1	2	3	4+	SEM
n	172	130	94	93	
IgG (mg/mL)	83.5[b]	92.9[b]	107.4[a]	113.3[a]	3.8
IgG	532.8[c]	579.0[bc]	619.6[ab]	690.2[a]	28.2
Volume (L) [(1)]	6.2	6.1	6.8	6.5	0.5

n = 507 Holstein colostrum samples in total. [a-c] Means in the same row with different superscripts differ (p<0.05). [(1)] n = 466, first-milking colostrum volume was not known for 41 samples.
Source: Kehoe et al. (2011)

b. Other proteins (Non-specific immunological substances)

Proteomics is the study of proteins, often in complex biological samples, and combines techniques, such as two-dimensional gel electrophoresis and mass spectrometry, to analyse hundreds of different proteins at the same time (Hernandez-Castellano et al. (2014). Proteomics has been used to identify the various proteins in colostrum and milk. The most immunologically active proteins in colostrum are found in the MFGM and can be classified as high abundance proteins (HAP) or low abundance proteins (LAP). There are 7 HAP in MFGM that are involved in immunity (Mather (2000); Table 1.6). The LAP can be classed into 8 immune related categories (Hernandez-Castellano et al., 2014) (Table 1.7).

Table 1.6. High abundance protein fraction (HAP) found in MFGM.

Proteins	Roles	Amount in colostrum
Mucin-1 (MUC-1)	Present in epithelial cells from different organs Protects body surface from physical damage or microorganism	40 mg/L
Xanthine dehydrogenase/oxidase (XDH/XO)	Immunomodulator	Goat: 2.77 mg/mL Cow: 2 mg/mL Sheep: 2.3 mg/mL
Lactoferrin	Binds and transports iron ions Provides antimicrobial activity (bactericide and fungicide) Increases in most inflammatory reactions	Camel: 5.1 mg/mL Cow: 0.84 mg/mL Goat: 3.09 mg/mL Sheep: 1.56 mg/mL
Caseins (αs1, αs2, β and ƙ)	Have a role in pathogen defence Stimulate the innate immune system ofmammary gland and prevent udder infections	

Source: adapted from Mather (2000) and Hernandez-Castellano et al. (2014)

Table 1.7. Low abundance protein fraction (LAP) of colostrum.

Proteins	Roles	Amount in colostrum
Plasmin	Prevents thrombosis by dissolution of blood clots. Migrates the neutrophils to infection sites	Cow: 0.49 µg/mL (in colostrum > 10 times in milk)
Serum amyloid A (SAA)	Essential role in the innate immune system Participates in acute phase of inflammation SAA-3 is involved host defence	SAA-3 concentration: Cow: 267.45 µg/mL (in colostrum > 10 times in milk) Sheep: 62.83 µg/mL
Fibrinogen	Binds integrins in the immune cells (monocytes and macrophages)	
Trypsin inhibitor (TI)	α1-antitrypsin Reduces biologically active trypsin Protects against the proteolytic cleavage and help neonates to absorb immune components	
Lipopolysaccharide -binding protein (LBP)	Generated during infections with Gram-negative bacteria	Cow: 0.85 mg/mL
Antimicrobial peptides (AMPs)	Inactivate infectious agents	
Defensins	Adapts immune response β-defensin family members are often found in mammary epithelial cells during mastitis	
Cathelicidin family	Part of innate immune defence	

Source: adapted from Hernandez-Castellano et al. (2014)

1.2.5.2. MicroRNAs (miRNAs)

a. Definition and history of miRNA

MicroRNAs are single-strand molecules with 19 to 24 nucleotides that were first reported by Rosalind Lee and colleagues in the worm *Caenorhabditis elegans* (Lee et al., 1993). They described 22-61 nucleotide lin-4 RNAs that do not encode a protein but do contain sequences that can bind the 3' untranslated region (UTR) of target mRNA and prevent transcription or initiate mRNA degradation. These lin-4 RNAs were found to play an important role in early larval development of the worm *Caenorhabditis elegans* (Lee et al., 1993). Subsequent studies defined an entire new class of RNAs, which are small like lin-4, and do not encode proteins. These so-called microRNAs (miRNAs) have regulatory functions by controlling gene expression post-translationally (Lee and Ambros, 2001).

Many miRNAs have been identified and have been shown to be involved in regulating a range of biological processes in many animal species, from stem cell differentiation, immune response, metabolism of cholesterol and insulin, development of neurogenesis and haematopoiesis, to cardiac and skeletal muscle development (Wienholds and Plasterk, 2005; Williams, 2007; Liu et al., 2010; Wang et al., 2013). MicroRNAs also implicated in oocyte maturation and the early stages of embryonic development (Wang et al. (2013) and apoptosis (Bartel, 2004). However, many of the functions of miRNAs remain unknown. The standard nomenclature system for miRNA is the prefix "miR" followed by a dash and a number, which indicates order of naming (Appendix 1).

b. Generation of mature miRNAs

MicroRNAs are transcribed from miRNA genes and three stages are required for their processing to maturation (Wang et al., 2013) (Figure 1.3).

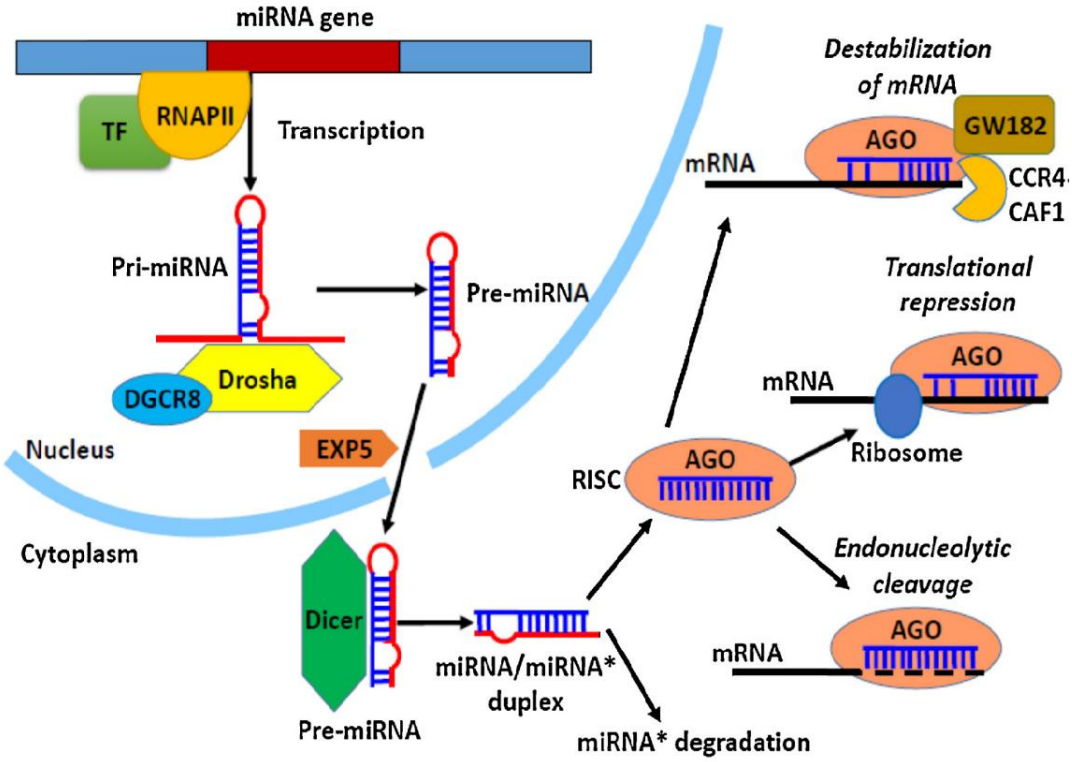

Figure 1.3. Generation of mature miRNAs.

Source: Liang et al. (2015b)

The first stage is the transcription of the miRNA gene to create the primary miRNA (pri-miRNA), which is processed in the second stage to a 70 nucleotide pre-miRNA with a stem-

loop structure. The last stage occurs when the pre-miRNA in nucleus is transported to the cytoplasm and is cleaved by RNase III Dicer to form the mature miRNA (around 22 nucleotides), which represses translation.

Depending on the degree of complementarity between the miRNA and its target mRNA, there are three mechanisms whereby miRNAs can affect gene expression: translational repression of mRNA, direct mRNA cleavage and mRNA de-adenylation (Wang et al., 2013) (Figure 1.3). While the functions of most miRNAs are unknown, microRNAs have been predicted to regulate the expression of up to 30% of the protein coding genes by binding to the corresponding mRNA (Wienholds and Plasterk, 2005; Izumi et al., 2012).

c. MicroRNA quantification

MiRNA quantification can be achieved by microarray analysis, quantitative RT-PCR (qRT-PCR) and Next Generation sequencing (NGS) (Table 1.8). Microarray assays can be hampered by cross-hybridization preventing the identification of individual members of miRNAs sequence families or mutant variants of miRNAs, whereas qRT-PCR methods are more precise. However, both these methods are limited to a pre-selected subset of miRNAs. In comparison with the other two approaches, NGS is expensive but produces an unprecedented amount of data including the identification of new miRNA following bioinformatic analysis.

d. MicroRNAs in colostrum and milk

MicroRNAs have been shown to be stored in exosomes within the colostrum and milk of cattle (Chen et al., 2010; Hata et al., 2010; Izumi et al., 2012; Izumi et al., 2015; Sun et al., 2015), pig (Gu et al., 2012b), rat (Izumi et al., 2014) and humans (Zhou et al., 2012). These exosomes are endosome-derived membranous vesicles found in various body fluids that can mediate intercellular communication (Zhou et al., 2012; Sun et al., 2013; Sun et al., 2015; Ross et al., 2016). Exosomes are tiny membrane vesicles (~40-100 nm in diameter) and are released into the extracellular environment from a variety of different cells (Qin and Xu, 2014).

Table 1.8. Advantages and disadvantages of miRNA quantification technology.

Technology	Advantages	Limitations
qPCR	Current gold standard for sensitivity and specificity	No genome-wide coverage
Microarray	Commercially available reagents Genome-wide coverage	Specific probes Specialized equipment Lack of reproducibility between platforms
NGS (Next-Generation sequencing)	Genome-wide coverage Multiple samples may be run in parallel Promotes novel miRNA discovery Can detect polymorphisms	Complicated, non-standardized data analysis
Isothermal amplification	No need to themocyling equipment Can improve existing qPCR, microarray, and NGS methods	Disadvantages are technique specific (see below)
Exponential amplification	High sensitivity	May require a nicking enzyme, which complicates primer design
Rolling circle amplification	1 primer Can be optimized for linear or exponential amplification	Requires 2 enzymes (polymerase and ligase) Initial denaturation not performed at room temperature
Duplex-specific nuclease signal amplification	High specificity	Enzyme is not readily available
Hybridization chain reaction Near-infrared technology	No polymerase No autofluorescence Minimal photobleaching No treatment of sample before or after the test	Linear amplification only Lanthanide probes are not yet commercially available and must be optimized
Near-infrared technology	No autofluoresence Minimal photobleaching No treatment of sample before or after the test	Lanthanide probes are not yet commercially available and must be optimized

Source: Moody et al. (2017)

Over the last decade, many miRNAs have been identified which are stable in colostrum and milk (Chen et al., 2010; Izumi et al., 2012; Sun et al., 2013). MicroRNAs are very stable in harsh conditions. Baddela et al. (2016) investigated miRNA stability by boiling at 100°C for 10 min, storing at 4°C for 24 h, and subjecting milk to repeated freezing and thawing and there was no loss of exosomal miRNA. In another study, Izumi et al. (2012) tested the stability of milk miRNA and determined that milk miRNAs were resistant to acidic conditions and RNase treatment.

The number of miRNAs detected in colostrum and milk depends on the method of miRNA assay, however, most studies have found that the number of different miRNA types and levels are higher in colostrum than in milk (Chen et al., 2010; Izumi et al., 2012; Sun et al., 2013). For instance, there were 230 known miRNAs in colostrum, 213 known miRNAs in mature milk, and 198 miRNAs in both bovine colostrum and milk found by Solexa sequencing (Chen et al., 2010) (Figure 1.4); 100 miRNAs are found in bovine colostrum, 53 miRNAs in mature milk and 51 miRNAs were detected in both colostrum and milk by microarray analysis (Izumi et al., 2012) (Figure 1.5). Many miRNAs are found in both bovine colostrum and milk, but have higher levels in colostrum (e.g. miR-24, miR-30d, miR-93, miR-106a, miR-181a, miR-200a and miR-451) (Chen et al., 2010; Sun et al., 2013). The unique expression of miRNAs in milk can provide novel indicators of the quality of milk and milk-related products, and seven miRNAs have been suggested as markers for quality control of bovine milk (miR-26a, miR-26b, miR-200c, miR-21, miR30d, miR-99a, and miR-148a) (Chen et al., 2010).

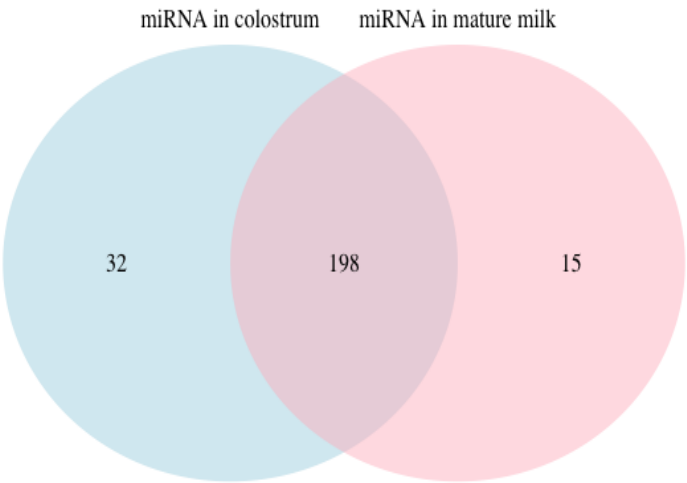

Figure 1.4. Number of miRNA species identified in bovine colostrum and mature milk by Solexa sequencing.

Blue circle: colostrum (7 days postpartum). Pink circle: mature milk (9 months postpartum). *Source: Adapted from Chen et al. (2010)*

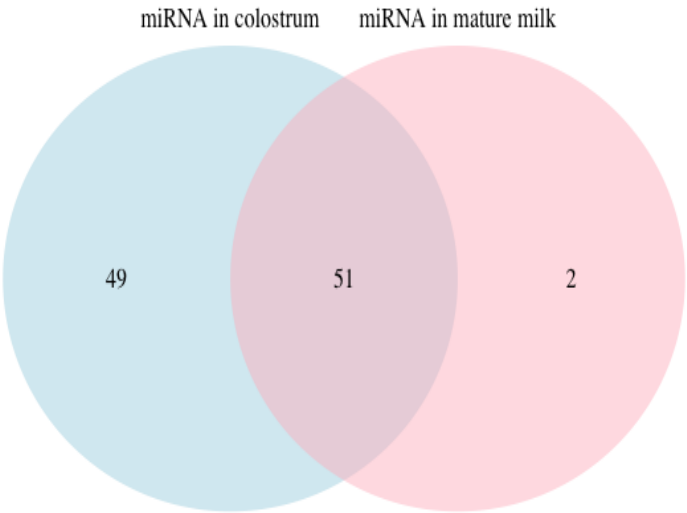

Figure 1.5. Number of miRNA species identified in bovine colostrum and mature milk by microarray analysis.

Blue circle: colostrum (within 3 days postpartum). Pink circle: mature milk (after 8 days postpartum). *Source: Adapted from Izumi et al. (2012)*

Table 1.9. MicroRNAs concentrations in bovine colostrum and mature milk.

miRNA species	**Colostrum(molar/mL)**	**Mature milk(molar/mL)**
let-7b	$2.40 \pm 0.11 \times 10^{-12}$	$3.21 \pm 0.47 \times 10^{-12}$
miR-16	$1.09 \pm 0.21 \times 10^{-12}$	$6.03 \pm 1.00 \times 10^{-13}$
miR-18a	$3.18 \pm 0.38 \times 10^{-15}$	$7.94 \pm 1.65 \times 10^{-16}$
miR-19a	N/A	$4.11 \pm 0.90 \times 10^{-14}$
miR-20a	$6.78 \pm 2.07 \times 10^{-13}$	$6.72 \pm 1.03 \times 10^{-13}$
miR-21	$4.48 \pm 0.26 \times 10^{-14}$	$3.05 \pm 0.49 \times 10^{-14}$
miR-24	$5.70 \pm 0.26 \times 10^{-14}$	$1.37 \pm 0.20 \times 10^{-14}$
miR-27a	$7.52 \pm 0.39 \times 10^{-14}$	$7.26 \pm 0.63 \times 10^{-14}$
miR-29a	$1.75 \pm 0.02 \times 10^{-13}$	$8.04 \pm 1.34 \times 10^{-14}$
miR-30d	$1.74 \pm 0.12 \times 10^{-13}$	$2.83 \pm 0.43 \times 10^{-14}$
miR-31	$5.39 \pm 0.70 \times 10^{-15}$	$4.21 \pm 1.02 \times 10^{-15}$
miR-32	$1.95 \pm 0.03 \times 10^{-14}$	$1.07 \pm 0.17 \times 10^{-14}$
miR-93	$2.36 \pm 0.25 \times 10^{-13}$	$3.25 \pm 0.36 \times 10^{-14}$
miR-100	$1.14 \pm 0.03 \times 10^{-15}$	$2.17 \pm 0.31 \times 10^{-15}$
miR-106a	$3.51 \pm 0.24 \times 10^{-13}$	$8.47 \pm 1.63 \times 10^{-14}$
miR-125b	$9.70 \pm 2.87 \times 10^{-16}$	N/A
miR-142-5p	$6.41 \pm 0.705 \times 10^{-15}$	N/A
miR-146a	$2.14 \pm 0.12 \times 10^{-14}$	$3.18 \pm 0.45 \times 10^{-14}$
miR-150	$2.64 \pm 0.09 \times 10^{-14}$	$4.05 \pm 0.38 \times 10^{-14}$
miR-155	$7.83 \pm 0.27 \times 10^{-15}$	$1.42 \pm 0.10 \times 10^{-14}$
miR-181a	$7.88 \pm 0.74 \times 10^{-14}$	$1.64 \pm 0.18 \times 10^{-14}$
miR-200a	$3.97 \pm 0.25 \times 10^{-13}$	$9.21 \pm 1.31 \times 10^{-14}$
miR-210	$2.84 \pm 0.06 \times 10^{-14}$	$4.40 \pm 1.10 \times 10^{-14}$
miR-214	N/A	$3.77 \pm 0.54 \times 10^{-14}$
miR-221	$2.70 \pm 0.07 \times 10^{-14}$	$7.33 \pm 1.67 \times 10^{-14}$
miR-223	$7.05 \pm 0.24 \times 10^{-13}$	$7.66 \pm 1.02 \times 10^{-13}$
miR-451	$2.07 \pm 0.42 \times 10^{-13}$	$1.05 \pm 0.26 \times 10^{-14}$

Molar abundance of each miRNA was calculated on the standard curve from different concentration of synthetic miR-16. Source: Sun et al. (2013)

Table 1.10. Immune-related miRNA reads in colostrum and mature milk.

Immune related miRNA	Solexa reads in mature milk	Solexa reads in colostrum
miR-155	110	121
miR-181a	1,023	6,255
miR-142-5p	69	204
miR-223	407	5,650
miR-146a	4	2
miR-150	123	1,232

Source: Chen et al. (2010)

In addition to embryonic development, miRNAs have been shown to play important roles mammary gland and adipose tissue function. They have been implicated in the regulation of production traits such as milk yield, milk quality and response to diseases including mastitis and Johne's disease (Do et al., 2018). Many miRNAs in milk and colostrum related to immune function have been also detected (Table 1.11 and 1.12) (Matukumalli, 2006; Sonkoly et al., 2008; Chen et al., 2010; Dilda et al., 2012; Izumi et al., 2012; Sun et al., 2013; Oh et al., 2015).

Table 1.11. Immune-related miRNAs in colostrum and milk.

Species	Immune-related miRNAs	Reference
Bovine	miR-155, miR-181a, miR-142-5p, miR-223, miR-146a, miR-150	Chen et al. (2010)
Bovine	Let-7b, miR-16, miR-18a, miR-19a, miR-20a, miR-21, miR-24, miR-27a, miR-29a, miR-30d, miR-31, miR-32, miR-93, miR-100, miR-106a, miR-125b, miR-142-5p, miR-146a, miR-150, miR-155, miR-181a, miR-200a, miR-210, miR-214, miR-221, miR-223, miR-451	Sun et al. (2013)
Bovine	miR-15b; miR-27b; miR-34a; miR-106b; miR-130a; miR-155; miR-223	Izumi et al. (2012)
Bovine	miR-9, miR-125b, miR-155, miR-146a and miR-223,	Dilda et al. (2012)
Goat, human and dairy cattle	miR-146; miR-150; miR-155; miR-181a; miR-223	Na et al. (2015)
Human	miR-181a, miR-181b, miR-155, miR-17, miR-92a, miR-125b, miR-146a, miR146b, miR-223, let-7i, and miR-150	Zhou et al. (2012)

Table 1.12. Role of miRNAs in immune cell populations.

Immune cell	MiRNAs	Functions
T cells	miR-17, miR-146	T-cell differentiation
	miR-181a	Enhances T cell receptor signalling by targeting protein
	miR-181a, miR-9	CD4+ T cell activation
	miR-17-92	CB8+ T cell activation during viral infections
	miR-29	Suppress Th1 cell responses
	miR-126	Supress Th2 responses
	miR-326, miR-301	Increase h17 cell responses
	miR-10	Increase Treg cell population
B cells	miR-150, mi-34a	Inhibit B cell differentiation from pro-B cells to pre-B cells
	miR-155	Inhibit activation of B cells present in germinal centres
Monocytes/ Macrophages	miR-146a, miR-155, miR0342, miR-338, miR-17-92	Inhibit differentiation of monocytes
Dendritic cell (DC)	miR-221, miR-222	Decrease plasmacytoid DCs population
	miR-21, miR-34a,	Inhibit monocyte-derived DCs differentiation
	miR-148, miR-152	Decrease expression of MHC class II on conventional DCs and decrease DC-medicated CD4+ T cell activation
Natural killer cells (NK)	miR-150	Increase natural NK population
	miR-181	Induce NK cell development
	miR-30, miR-27, miR-378, miR-223	Increase NK cytotoxic activity

Source: Liang et al. (2015b)

Different miRNAs have different immune functions. For instance, miR-181a and miR-223 have been implicated in establishing and maintaining the immune cells, while miR-146 is involved in innate immunity and miR-155 regulates central elements of the adaptive immune response. MicroRNAs, like miR-203 and miR-146, are implicated in immune-mediated

diseases, and miR-196 is implicated in viral immune escape and anti-viral defence (Sonkoly et al., 2008). A study by Dilda et al. (2012) revealed that miR-9, miR-125b, miR-146a, miR-233, and miR-155 were closely related with inflammation after stimulation of bovine monocytes with lipopolysaccharide and S. *aureus enterotoxin B.* MicroRNA-142-5p and miR-223 may be up-regulated by infections because they were highly expressed in cows with mastitis and could be potential biomarkers for the early detection of bacterial infection of the mammary gland (Sun et al., 2015; Cai et al., 2018).

MicroRNA-155 (miR-155) appears to be necessary for normal immune function, as mice lacking this miRNA are immunodeficient (Rodriguez et al., 2007). MicroRNA-155 regulates many genes including chemokines, cytokines and transcription factors in CD4+ cells. Other microRNAs have been shown to modulate the innate immune system, such as miR-223 which is involved in the adaption of responses to pathogens, and miR-146, which affects essential elements for inflammation. MicroRNA-181a is implicated in the development of both B and T lymphocytes (Sonkoly et al., 2008). The levels of most of the miRNAs change over the course of lactation and the miRNA targets and their function remain largely unknown (Floris et al., 2016).

1.2.6. Effects of colostrum components on calf health, growth, fertility and production

Of all the components in bovine colostrum, the main focus has been on IgG because of the obvious effects of immunoglobulins on neonate passive immunity. IgG is the immunoglobulin at highest concentration in bovine colostrum and the concept of failure of passive immunity transfer (FPIT) is based on the IgG concentration in the neonatal calf blood. If neonatal calves have more than 10 g/L of IgG in their serum 24 hours after birth they are considered to have received sufficient passive immunity transfer, which helps protect them from pathogens (Weaver et al., 2000; Barrington and Parish, 2001; Godden et al., 2019; Oliveira et al., 2019). As measuring of IgG is difficult, total serum protein is often used as an indicator of FPIT with a threshold of 52 g/L total protein (Weaver et al., 2000; Cuttance et al., 2017a).

Studies on IgG, and total protein in calf blood have considered whether the calf received maternal colostrum, colostrum placer, colostrum supplement (Quigley et al., 2001; Jones et

al., 2004), pasteurized and non-pasteurized colostrum (Armengol and Fraile, 2016), cell-free colostrum (Langel et al., 2016), heat-treated or unheated colostrum (Gelsinger and Heinrichs, 2017). In general, neonatal calves fed maternal colostrum had higher levels of IgG in their serum within the first few hours of life and greater passive immunity than those receiving colostrum supplement and colostrum replacer (Quigley et al., 2001; Fidler et al., 2011; Priestley et al., 2013). Other factors affecting IgG or total protein concentration included colostrum volume, the timing of colostrum feeding, and the type of colostrum, milking time, parity and method of feeding (Franklin et al., 2003; Jaster, 2005; Chamorro et al., 2017; Godden et al., 2019). Calves that received colostrum with high IgG levels or a large colostrum volume soon after birth had higher total protein and IgG in their blood than those receiving small volumes of poor colostrum a long time after birth (Jaster, 2005; Conneely et al., 2014). Interestingly, it has been observed that calves fed colostrum by bottle had higher total protein than calves that suckled from their dam (Franklin et al., 2003).

In addition to the link between different types of colostrum and passive immunity, the relationship between different sources of colostrum and health, growth and future production of calves has been investigated (Furman-Fratczak et al., 2011; Priestley et al., 2013; Chamorro et al., 2017; Lago et al., 2018). Calves that received pasteurized colostrum had significantly decreased morbidity and mortality in comparison with calves that received non-pasteurized colostrum for 21 days after birth (Armengol and Fraile, 2016). Feeding heat-treated colostrum reduces potential pathogens in the calf intestine (Malmuthuge et al., 2015). Heat-treating colostrum does not seem to prevent it stimulating an immune response to pathogens, although the average daily gain for 35 days was higher in the calves fed unheated rather than heat-treated colostrum (Gelsinger and Heinrichs, 2017). Calves that received colostrum and milk from their dams had higher plasma total protein, immunoglobulin G and insulin-like growth factor-1 than those receiving bulk milk which correlated with a greater body weight (Schiessler et al., 2002). Good passive immune transfer has been also associated with earlier first insemination, meaning earlier puberty (Furman-Fratczak et al., 2011).

Even if calves receive colostrum from the same source, there are other factors that may affect passive immunity transfer in calves and growth performance, in particular the volume of the colostrum. A significant difference in weight gain has been reported in calves receiving higher colostrum volumes (Faber et al., 2005). In addition, the manner by which calves

receive colostrum has an effect on social behaviour as an adult. Suckled calves receiving colostrum from their dam had a positive effect on the daily gain and better social behaviour compared to those fed colostrum from a bucket without their dam present (Krohn et al., 1999).

Proteins with anti-microbial activity are present in colostrum, such as lactoferrin, mucin-1 and plasmin. Pakkanen and Aalto (1997) found bovine lactoferrin, for example, can inhibit the growth of several kinds of bacteria by binding with iron, which is important for the growth of microbes. It is not known if the presence of these proteins in colostrum is to reduce bacterial growth in the colostrum or if the transfer of these immune proteins from the dam to newborn calf has a beneficial effect on the calf.

MicroRNAs are stable in colostrum and raw milk (Chen et al., 2010; Sun et al., 2013). The miRNAs are present in microvesicles in colostrum, where they are at a higher concentration than in milk (Chen et al., 2010; Sun et al., 2013; Izumi et al., 2015; Sun et al., 2015), and may be transferred from the colostrum to neonates (Sun et al., 2013). However, a more recent study has suggested that miRNAs are not all readily transferred from colostrum to newborn calves (Kirchner et al., 2020).

1.3. Gaps in knowledge

Research since the 19th century has confirmed the role of colostrum in neonate health and has explored different colostrum fractions and components, but the primary focus has been on the immunoglobulins. Other immune components in colostrum may affect calf health and in the long term such as growth and production, but these have not been fully explored. In particular, the research carried out to-date has not explored the miRNA profiles in individual cow colostrum and pooled colostrum, and whether these miRNAs are absorbed by the neonate calves. It is also not known if different calves can absorb the same amount of nutrients and immune components from the same colostrum source or if there are any differences should calves receive colostrum from their own dam or from another cow.

1.4. Objectives and hypothesis

The hypotheses tested in this research were:

1) Calves will absorb equal amounts of the components in colostrum whether or not that colostrum is from their own dam or a different cow.
2) If colostrum is fed shortly after birth, then poorer quality pooled colostrum will still provide adequate passive immunity for neonate calves.
3) MicroRNAs in the colostrum will be absorbed by neonate calves.
4) MicroRNAs in the colostrum will have immune-related functions in the calves.

To test these hypotheses, the goal of this research was *1)* to investigate the main components in individual cow colostrum, pooled colostrum and bulk tank milk, and *2)* to study the transfer of these components from different colostrum sources. The specific colostrum components that were investigated were protein, lactose, fat, IgG concentration, GGT, and immune-related miRNAs. The specific objectives were:

Objective 1: Quantify colostrum components, including total solids, total protein, IgG, fat and lactose in colostrum from individual cows, pooled colostrum and bulk tank milk and measure the transfer of these components into calf blood for one week after birth in calves fed colostrum from three sources.

Objective 2: Determine the relationship between the colostrum components and calf blood parameters one week after birth.

Objective 3: Investigate the miRNA profile of dam colostrum and pooled colostrum by Next Generation sequencing (NGS), and explore the transfer of miRNA to newborn calves before and after receiving their own dam colostrum, foster cow colostrum and pooled colostrum.

Objective 4: Quantify specific immune-related miRNAs (namely, miR-142-5p, miR-150, miR-155, miR-181a and miR-223) in individual cow colostrum, pooled colostrum, bulk tank milk and the transfer of these miRNAs to calves fed their own dam, foster dam colostrum and pooled colostrum.

Chapter 2

Experiment design and general methods

Chapter 2. Experiment design and general methods

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2.1. Experimental design

This study, funded by Davies Research Centre, The University of Adelaide and all animal experiments were approved by the Animal Ethics Committee (S-2017-060) prior to the start of animal work. Thirty-five Holstein Friesian bull calves, born from 18th February 2018 to 1st March 2018, were obtained from a farm with 1800 healthy cows in Mount Gambier, South Australia.

Cow and calf management at birth

Calves were born singletons to multiparous Holstein dams in the same milking group of a single farm with 1800 cows. The cows were kept as a cohort and had similar genetics, environment, nutrition and parity (2 to 4). The birth information was recorded, specifically calving difficulty score (Table 2.1), calf ID, cow ID, and time of birth. Calves were taken from their dam after being licked clean (about 30 min after birth), but not allowed to suckle, and were scored for calf vigour (1 = weak, 2 = lazy, 3 = vigorous, 4 = very vigorous) and health (Based on Calf Vitality sheet from the University of Guelph, Canada, https://www.progressivedairycanada.com/downloads/2012/09/0912ca_lee_vigor.pdf).

Table 2.1. Calving ease scoring system (6 point scale).

Score	Code	Description
1	Unassisted	Cow calved unassisted/no difficulty
2	Easy pull	One person without mechanical assistance
3	Hard pull	Two people without mechanical assistance One person with mechanical assistance
4	Surgical assistance	Veterinary intervention required
5	Mal-presentation	E.g. breech
6	Elective surgical	Surgical removal of calf before cow had opportunity to calve

The calving ease score of all calves in this study was 1 or 2, so that the dam colostrum volume and quality or the vitality of the calves should not have been affected by a difficult birth. The calves were randomly block allocated to 3 groups (the first calf was allocated in Group A (tagged as A1, the second and third calf were tagged as B1 and C1, respectively) (Figure 2.1). Three calves (A1, B1 and C1) were placed in the same pen. Group A dams were tagged with necklace numbered 1 to 12 corresponding to their calves, then kept for

three days postpartum for colostrum collection. Newborn calves were weighed by using portable animal weight scales and crate (kg), and then the calf girth was measured using a tape measure (cm). The Group A calves were fed colostrum from their own dam and Group B calves were fed colostrum from one of the group A cows. The Group C calves were fed a single bottle of pooled colostrum (2 L) obtained from the dairy's other cows 0 to 4 days post-partum. All calves were offered ad lib calf concentrate, lucerne or medic, and water from day 1. Calves were blood sampled prior to feeding (within 4 hours of birth, day 0) and again on days 1, 2, 3 and 7 after birth.

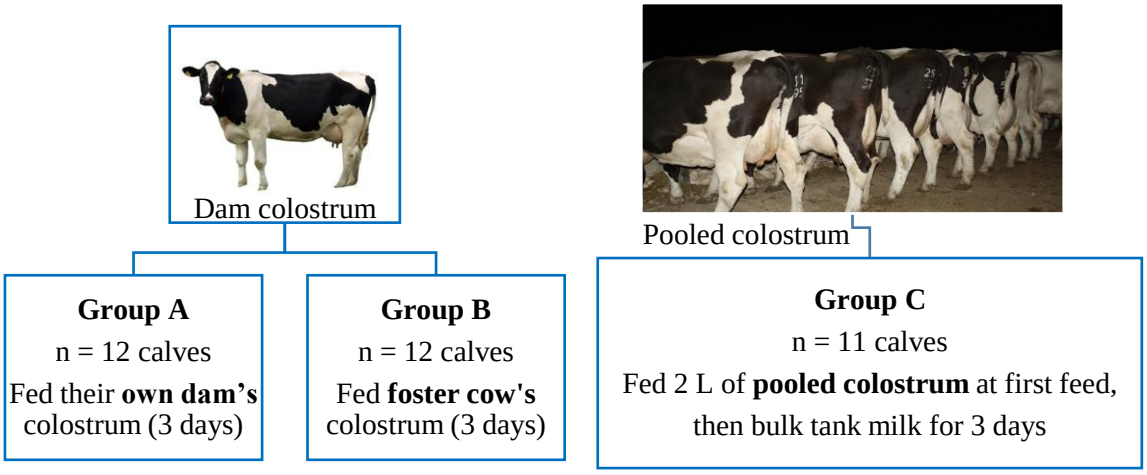

Figure 2.1. Experimental design.

Each pair of A_i and B_i calves was fed colostrum from the same cow (cow_i) for 3 days after birth and then bulk tank milk from day 4 to 7 after birth. Group C calves were fed 1 bottle (2 L) of pooled colostrum and then bulk tank milk thereafter until day 7 after birth.

The dams of Group A calves were milked within 2 hours post-partum using a portable milking machine for the first milking. Thereafter, the cows were milked in the dairy using a rotary milking parlour from the 2nd to 7th milking every 12 hours and the individual colostrum collected. The dam colostrum from each Group A cow was measured for volume, samples kept for analysis and the remaining colostrum divided into bottles for the Group A and B calves and tags with cow number using a permanent marker. All Group A and B calves were fed 5% of the birth weight of dam colostrum twice a day until day 3 by bottle with a calf teat. Pooled colostrum was collected from approximately 100-120 dairy cows 0-4 days

postpartum once per day for Group C calves, which was given for the first feed (2 L/calf/feed) using a bottle with a calf teat. It is a frequent practice for Australasia dairy farms to pool colostrum and transition milk from cows 0-4 days postpartum to feed the newborn calves (Denholm et al., 2017; Cuttance et al., 2018; Phipps et al., 2018). All calves were fed the first time within 4 hours after birth and second feed was approximately 12 hours after birth. The bulk tank milk was collected from dairy cows more than 5 days postpartum twice daily to be fed to Group C calves from 2nd feed until day 7 after birth and Group A, B calves from day 4 to 7 after birth. Bulk tank milk was fed 2 L per calf per feed twice daily using a drinking bucket. The sample of colostrum, pooled colostrum and bulk tank milk were collected into sterile 50 mL-tubes and then assayed for total solids using a Brix refractometer immediately after milking.

The volume of colostrum fed to calves each feed was 5% birth weight every 12 hours being the practice followed on many dairy farms (Cummins et al., 2016; Cuttance et al., 2018; Phipps et al., 2018; Abuelo et al., 2019; Inabu et al., 2019). The group C calves replicated the protocol used by many Australian commercial dairy farms (Vasseur et al., 2010; Vasseur et al., 2012; Cummins et al., 2016).

2.2. Sampling

2.2.1. Colostrum and milk samples

The dam colostrum (n = 12) from Group A cows was sampled twice daily until 3 days postpartum, and pooled colostrum, bulk tank milk were collected from the commercial dairy twice daily during collection time from 18th February 2018 to 8th March 2018. These colostrum and milk samples were immediately assayed for total solid by Brix refractometer, which is a digital optical refractometer DBR-1 (Starr Instruments, Dandendong, VIC, Australia). The Brix refractometer measured proteins, carbohydrates, and other soluble molecules. Additional colostrum, milk samples were collected into four sterile 50 mL-tubes, frozen at -20°C, then transported on dry ice to The University of Adelaide, Roseworthy Campus and stored at -80°C until analysed.

2.2.2. Cow blood and calf blood samples

Cows were blood sampled within 2 hours after parturition. Calves were blood sampled within 4 hours after calving (day 0), at days 1, 2, 3, and 7 after birth. Blood was drawn from the jugular vein using BD vacutainer (BD-Plymouth. PL6 7BP, UK) with multi-sample needles 20G or 18 G depending on calf age, into 6 mL – BD vacutainers with no anticoagulants (red-top tube), 6 mL – BD vacutainers with heparin (green-top tube) and 6 mL- BD vacutainers with EDTA (purple-top tube). Depending on calf age and the purpose of the sample at each time point, 6 mL or 10 mL was collected.

Fresh whole blood was collected into a vacutainer without anticoagulant and a drop was immediately absorbed onto an Accu-check Performa test strip. The glucose concentration was determined using a glucose meter (Accu-check Performa, Roche Diabetes Care, Basel, Switzerland).

Serum was separated from blood with no anticoagulants by allowing the blood to clot for 45 to 60 min at 4 °C then centrifugation (2000 x *g*, 5 min at room temperature). Serum samples were immediately tested for total soluble protein concentration **(TP-R)** by the digital refractometer (Digital Refractometer, ATAGO, Saitama, Japan) with a measurement range for protein from 0 to 12 g/100 mL. The leftover serum was stored at -20°C, transported to the University of Adelaide, Roseworthy Campus and stored at -80°C until analysed for total protein by refractometer, total protein by Bradford assay and IgG concentration.

Plasma was prepared from fresh blood with heparin anticoagulant (green-top tubes) at the time of collection by centrifugation at 2000 x *g* for 15 minutes at room temperature. Plasma samples were initially stored at -20°C, transported for to the University of Adelaide, Roseworthy Campus and stored at -80°C until analysed for GGT activity levels.

One mL of whole blood from the EDTA (purple-top) tubes for RNA extraction, was mixed with 3 mL Trizol LS reagent (Catalog Numbers 10296010 and 10296028, Invitrogen, Thermo Fisher scientific) and incubated at room temperature for 20 minutes then frozen at -20°C. The sample in Trizol LS was transported to the University of Adelaide, Roseworthy Campus and stored at -80°C until analysed for miRNA.

2.3. Colostrum and blood assay

2.3.1. Colostrum total solids measured by Brix refractometer

The concentration of the soluble solids, which include sugar, salts, proteins and acids (Brix%), in colostrum and milk samples, was measured in fresh colostrum and milk immediately after collection using a digital optical refractometer DBR-1 (Starr Instruments, Dandendong, VIC, Australia) with a measurement range of Brix 0 to 50%. The Brix refractometer was zeroed using ultrapure water (Milli Q).

2.3.2. Nutrient colostrum components

Protein (%), fat (%) and lactose (%) were determined using a Fourier-transform mid-infrared (FT-MIR spectrometer, Foss Analytics) at the National Herd Development Co-operative (Kyabram VIC 3620, Australia).

2.3.3. Total protein by Bradford assay (TP-B) (g/L)

Total protein in colostrum, pooled colostrum, bulk tank milk and calf serum was assayed in a 96 well-plate using a Quick Start Bradford Protein assay kit following the manufacturer's instructions (Quick Start, Bio-Rad Laboratories, Inc., USA). The colostrum samples (10 µL) at days 1, 2 and 3, pooled colostrum and bulk tank milk were diluted with ultrapure water at a ratio of 1:100 and colostrum at day 0 was diluted at a ratio of 1:200 or 1:300. The diluted samples (5 µL) were mixed with Coomassie Brilliant Blue G-250 dye (250 µL) and colour change was measured using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA) at 595 nm (OD595). The total protein by Bradford assay **(TP-B)** in the samples was calculated based on a standard curve for a serial dilution of bovine serum albumin.

Total protein in the calf serum was assayed by the same Bradford assay for colostrum and bulk tank milk samples. Before adding to a 96-well plate, serum samples (5 µL) were diluted in ultrapure water at a ratio of 1:100.

2.3.4. IgG concentration (g/L)

Immunoglobulin G concentration in colostrum, pooled colostrum, and bulk tank milk was quantified by a sandwich ELISA (enzyme-linked immunosorbent assay) in 96-well plates

(Coat Nunc F96 Maxisorp plates, Thermo-Fisher Scientific) using bovine gamma globulin (Bio-Rad Laboratories, Inc., USA) serially diluted for a standard curve (Appendix 2). Two bovine IgG specific antibodies (Life Technologies, USA), goat anti-bovine IgG antibody unconjugated, affinity purified (Novex Cat. #A18753) and goat anti-bovine IgG antibody conjugated with horseradish peroxidase (HRP), affinity purified (Invitrogen Cat. #18751). The colostrum samples from days 0 and 1 and the pooled colostrum samples were diluted in 0.05% Tween 20-PBS solution as appropriate (Appendix 2). The 3,3',5,5'-tetramethylbenzidine substrate (Ultra TMB-ELISA, Thermo Fisher Scientific) was added and the plates incubated at room temperature in the dark for 15 minutes before the reactions were stopped by the addition of 0.1 M H_2SO_4. Antibody binding was measured as the enzymatic colour change at 450 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA). The IgG concentrations in samples were calculated based on the standard curve.

Serum IgG was assayed by ELISA using the same protocol as for colostrum and milk, however, serum samples were diluted in 0.05%T-PBS solution at a ratio of 10^{-4} (calf serum day 0), or 10^{-6} for other time-points (days 1, 2, 3, 7).

2.3.5. Calf glucose concentration (mmol/L)

A drop of fresh whole blood was immediately absorbed onto an Accu-check Performa test strip after collection into a vacutainer without anticoagulant, and the glucose concentration was determined using a glucose meter (Accu-check Performa, Roche Diabetes Care, Basel, Switzerland).

2.3.6. Gamma-glutamyltransferase activity (GGT) (U/L)

Calf plasma was analysed for GGT activity using a kinetic colour test with a Beckman Coulter analyser (Coulter Manufacturing Company, Brea, CAL, USA) at the Veterinary Diagnostic Laboratory, The University of Adelaide, Australia.

2.3.7. Serum total protein by refractometer (TP-R) (g/L)

A digital refractometer with measurement range for protein from 0 to 12 g/100 mL (Digital Refractometer, ATAGO, Saitama, Japan) was used to determine total protein (**TP-R**)

concentration in the calf serum at days 0, 1, 2, 3 and 7 after birth. Serum was tested immediately after collection. The protein refractometer was zeroed using ultrapure water (Milli Q).

2.4. MicroRNA analysis

2.4.1. MicroRNA extraction

a. MicroRNA extraction using miRNeasy Mini Kits

Total RNA from colostrum and calf blood samples was extracted for miRNA sequencing by using miRNeasy Mini Kits (cat. No. 217004, Qiagen). Briefly, 250 µL of colostrum/calf blood mixed well with 750 µL Trizol LS (duplicate for each sample in order to increase RNA yield for miRNA sequencing). The mixed samples were incubated the homogenate at room temperature (15 - 25 ^{o}C) for 5 minutes. Chloroform (140 µL) was added, mixed for 15 seconds, incubated at room temperature for 3 minutes, and centrifuged for 15 minutes at 12,000 x *g* at 4 ^{o}C (~11,000 rpm). After centrifugation the samples were separated in 3 phases, and the upper aqueous phase was carefully transferred to a new collection tube. Then 700 µL of 100% ethanol was added and mixed thoroughly by pipetting. Up to 700 µL of the sample, including any precipitate, was pipetted into an RNeasy Mini column within a 2 mL collection tube, and the column was centrifuged at ≥ 8000 x *g* (10,500 rpm) for 20 second at room temperature. This step was repeated for the remainder of the samples, including the duplicate tubes of each sample. RWT Buffer (700 µL) was added into the RNeasy Mini column, centrifuged for 20 second at ≥ 8000 x *g* (10,500 rpm), and the flow-through was discarded. Then, Buffer RPE (500 µL) was pipetted onto the RNeasy Mini column and centrifuged for 20 second at ≥ 8000 x *g* (10,500 rpm), and the flow-through was discarded. This washing step with buffer RPE was repeated but the column was centrifuged for 2 minutes at ≥ 8000 x *g* (10,500 rpm) and the flow-through was discarded. The RNeasy Mini column was placed into a new 2 mL collection tube and centrifuged at full speed (14,000 rpm) for 1 min to further dry membrane. The RNeasy Mini column was transferred to a new 1.5 mL collection tube and RNase-free water (30 µL) directly added onto the RNeasy Mini column membrane, which was then centrifuged for 1 minute at ≥ 8000 x *g* (10,500 rpm) to elute. Note that the buffer RWT with 30 mL ethanol (96 - 100%) added and the buffer RPE with 44 mL ethanol (96-100%) added were stored at room temperature (15 – 25 ^{o}C) before starting the extraction steps.

b. MicroRNA extraction using Trizol LS reagent

Total RNA for RT-qPCR was extracted by using the Trizol LS method, according to the manufacturer's protocol with some modifications as detailed to increase RNA yield and quality. One µL of cel-miR-39-3p (5 pM/µL) (mirVana, miRNA mimic, ID: MC10956, Cat# 4464066, Lot# AS027ZX4) was added to a mix of colostrum (or milk or blood) and Trizol LS reagent with a ratio of sample in Trizol LS of 1:3 (250 µL sample: 750 µL Trizol LS). The sample and Trizol LS reagent was mixed well and incubated 5 minutes at room temperature. Chloroform (0.2 mL) was added into the sample for lysis, and incubated for 2-3 minutes at room temperature before centrifugation for 15 minutes at 12,000 × g (~10,500 rpm) at 4°C. The mixture separated into a lower red phenol-chloroform, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out. Isopropanol (0.5 mL) was added to the aqueous phase, shaken, and incubated for 10 minutes. Sample was centrifuged for 10 minutes at 12,000 × g (~10,500 rpm) at 4°C. The total RNA precipitate formed a white gel-like pellet at the bottom of the tube and the supernatant was discarded with a micropipettor. The pellet was re-suspended in 1 mL of 75% ethanol, centrifuged for 5 minutes at 7500 × g (~ 8,500 rpm) at 4°C, and the supernatant was discarded. The sample tube was centrifuged for 1 minute at 8500 × g (~ 10,500 rpm) at 4°C, and then the RNA pellet was vacuum or air dried for 5–10 minutes. The pellet was resuspended in 50 µL of RNase-free water by pipetting up and down, and the tube was placed immediately on ice.

2.4.2. MicroRNA profiling by Next Generation sequencing (NGS)

MicroRNA was extracted from dam colostrum (n = 4), pooled colostrum (n = 4), and calf blood from three calf Groups (A, B and C) (n = 4 per each group) before and after consuming colostrum (day 0 and day 1) using a miRNeasy Mini Kit (cat. No. 217004, Qiagen) as described in section 2.4.1a. The samples that had total RNA concentration of 1 µg in a maximum of 10 µL volume (1 ng per sample) with an OD 260/280 from 1.6 – 2.0 were used for the RNA library preparations if they were also deemed to be sufficient quality from Tape Station and Bioanalyzer analyses. The microRNAs were sequenced by using a NEXTflex small RNA-seq Kit v3, (Illumina, Bio scientific Corporation, USA) by ACRF Cancer Genomics Facility, SA Pathology, Adelaide, South Australia, as detailed in Chapter 5.

2.4.3. MicroRNA quantification by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Five different immune-related miRNAs, miR-142-5p, miR-150, miR-155, miR181a and miR-223, were quantified in colostrum days 0, 1, 2 and 3, pooled colostrum, bulk tank milk, cow blood at 2 hours postpartum and calf blood days 0, 1, 2, 3 and 7 by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using *Caenorhabditis elegans* cel-miR-39 as an internal standard for quantification.

Total RNA in colostrum, milk and whole blood was extracted using Trizol LS (Invitrogen, Catalog Numbers 10296010 and 10296028) as described in section 2.4.1b. Cel-miR-39-3p (5 picomole/µL) (mirVana, miRNA mimic, ID: MC10956, Cat# 4464066, Lot# AS027ZX4) was used as an exogenous control by adding 1 µL to the colostrum, milk or calf blood before starting the extraction steps. After total RNA isolation, the concentration and quality of total RNA were assessed by Nanodrop. All samples that had a ratio of A260/280 ranged from 1.6 to 2.0 were used for RT-qPCR. Total RNA was first transcribed into complementary DNA (cDNA) by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, product P/N 4366596 and 4366597, USA). The cDNA was then diluted at a ratio of 1:10 with RNase-free water and used as the template for the qPCR reaction. The cDNA from the cel-miR-39-3p was used for the standard curve. Quantitation using real-time PCR was performed by using Corbett Robotics CAS Robotics4 v4.9.1 system to load the PCR reagents into Gene-Disc 100 well rings, and the PCR ring was placed into a thermal cycler (Rotor-Gen 6000, Corbett Research, 2 PLEX). The reagents used for qPCR step were from TaqMan Universal Master Mix II, no uracil-N-glycoslyase (UNG) kit (Applied Biosystems) and all steps followed the protocol of the manufacturer with some modifications for the template amount. The details are described in Chapter 6.

2.5. Statistical and bioinformatics analyses

Statistical analyses were carried out in R (R version 3.6.3) and SAS (version 9.4). The details of the statistical analyses are provided in each chapter as appropriate. MicroRNA profiles and miRNA expression data were analysed by using a bioinformatics pipeline as described in Chapter 5.

Chapter 3

Colostrum source and passive immunity transfer in dairy bull calves

Chapter 3. Colostrum source and passive immunity transfer in dairy bull calves

This chapter is a paper published in Journal of Dairy Science:

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3.1. Authorship statement

Statement of Authorship

Title of Paper	**Colostrum source and passive immunity transfer in dairy bull calves**
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	**Do T. Hue**		
Contribution to the Paper	Designed and prepared for animal expreriment and sample collection, collected samples, optimised protocols and assayed samples, statistically analysed data, drafted and edited manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19-02-2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Rebel Skirving		
Contribution to the Paper =	Prepared for animal experiment and sample collection, collected samples, managed animals and edited manuscript.		
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Name of Co-Author	Tong Chen		
Contribution to the Paper	Prepared for animal expreriment and sample collection and assisted with benchwork.		
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Contribution to the Paper	Designed and prepared for animal expreriment, discussed data analysis and edited manuscript.		
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Name of Co-Author	Cynthia D. K. Bottema		
Contribution to the Paper	Designed and prepared for animal expreriment and sample collection, collected samples, developed protocols for different assays, discussed data analysis and edited manuscripts.		
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3.2. Original article

Colostrum source and passive immunity transfer in dairy bull calves

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Colostrum source and passive immunity transfer in dairy bull calves

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ABSTRACT

Colostrum is essential for good neonate health; however, it is not known whether different calves absorb the nutrients from colostrum equally well. In this study, the absorption of protein, IgG, and γ-glutamyl transferase was compared in newborn dairy bull calves for 1 wk after feeding colostrum from different sources. Thirty-five Holstein-Friesian bull calves were randomly allocated into 3 groups and fed colostrum within 4 h after birth. Group A calves (n = 12) were bottle fed colostrum from their own dam for 3 d. Colostrum from these group A cows was also used as foster cow colostrum for the group B calves (n = 12), such that each group A and B calf pair received identical colostrum from each milking of the respective group A dam (10% of birth weight per day). The group C calves (n = 11) were fed 1 bottle (2 L) of pooled colostrum and transition milk (referred to as pooled colostrum), as was the standard practice on the dairy farm. The pooled colostrum was collected from the other dairy cows on the farm 0 to 4 d postpartum and stored at 4°C for less than 12 h. Blood was sampled from calves before the first feeding and at 1, 2, 3, and 7 d after birth. Levels of total solids, total protein, and IgG were higher in the dam colostrum than in the pooled colostrum. At birth, there were no differences between the calf groups for any measurements, and all calves had very low IgG levels. After receiving colostrum, the glucose, plasma γ-glutamyl transferase, serum total protein, and IgG concentrations increased significantly in all calves. There were no differences in any blood measurements at any time point between the pairs of group A and group B calves that received colostrum from the same cow except for the IgG concentration 2 d after birth. However, the group A calves had a higher total serum protein level and IgG concentration than the group C calves for all the time points after the first feeding. The group B calves had a higher IgG concentration than the group C calves on d 1, 2, and 7 after birth. Compared with groups A and B, there was no difference in the proportion of calves in group C that failed to have passive immunity transferred adequately based on the IgG threshold (<10 g/L). Thus, the calves receiving identical colostrum from the same cow had the same levels of IgG, and even the pooled colostrum provided sufficient transfer of IgG as the calves were fed within 4 h after birth.

Key words: bovine, immunoglobulin G, passive transfer, failure of passive immunity transfer, passive immunity

INTRODUCTION

Management practices to ensure that calves receive adequate colostrum in the first day of life are critically important for calf health and future production (Quigley et al., 2001; Faber et al., 2005; Trotz-Williams et al., 2008; Furman-Fratczak et al., 2011). The placenta of ruminants is synepitheliochorial, which prevents the transfer of immunoglobulins from dam to calf during pregnancy; therefore, calves have little or no passive immunity when they are born (Barrington and Parish, 2001; Castro et al., 2011). Newborn calves receive passive immunity after birth by the absorption of immune-related cells and proteins, primarily IgG, from colostrum through the gut. The gut remains open for absorption only for approximately the first 24 h after birth (reviewed by Godden et al., 2019). To resist pathogens during the first weeks of life, newborn calves must receive a sufficient quantity of good-quality colostrum as soon as possible after birth. It is recommended that dairy calves be fed within 1 to 2 h of birth and receive colostrum with greater than 50 g/L IgG at a volume equivalent to 10 to 12% of their birth weight (Godden et al., 2019).

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If calves receive too little colostrum before gut closure or if the colostrum quality is poor (without sufficient immunoglobulins), then failure of passive immunity transfer (**FPIT**) may occur. Failure of passive immunity transfer is associated with increased calf morbidity and mortality (Barrington and Parish, 2001; Moran, 2002; Furman-Fratczak et al., 2011; Vandeputte et al., 2011; Lombard et al., 2020), and the suggested threshold to define FPIT in calves is less than 10 g/L IgG in the blood 24 to 48 h after birth (Barrington and Parish, 2001; Godden et al., 2019; Oliveira et al., 2019). In addition to serum IgG, the activity of γ-glutamyl transferase (**GGT**) in neonatal calf blood can be used as an indicator of colostrum ingestion (Braun et al., 1982; Weaver et al., 2000). Serum GGT activity greater than 200 IU/L at 1 d after birth or greater than 75 IU/L at 7 d after birth is considered to be indicative of successful passive immunity transfer, whereas a GGT level below 50 IU/L within the first 2 wk after birth is indicative of FPIT (Parish et al., 1997). As IgG and GGT concentrations are not measured routinely on farms, FPIT can be assessed in the field using refractometry to determine the total serum protein levels in calves. The refractometry threshold for FPIT is generally agreed to be less than 52 g/L total serum protein in neonate calves 1 d after birth (McGuirk and Collins, 2004; Cuttance et al., 2017; Godden et al., 2019), although 55 g/L may be a better threshold for ruling out FPIT (Buczinski et al., 2018).

Studies have shown that calves that receive sufficient colostrum shortly after birth and avoid FPIT have improved passive immunity and health status as well as weaning weights (Furman-Fratczak et al., 2011; Priestley et al., 2013; Lago et al., 2018), which allows first insemination earlier (Furman-Fratczak et al., 2011). Nevertheless, colostrum and transition milk are often pooled from cows 0 to 4 d postpartum to feed the newborn calves in New Zealand and Australia (Denholm et al., 2017; Cuttance et al., 2018; Phipps et al., 2018; Abuelo et al., 2019). The newborn calves are frequently fed pooled colostrum and transition milk on the first day after birth and are then fed fresh mature milk thereafter (Cuttance et al., 2018; Abuelo et al., 2019). Pooling colostrum from multiple cows postpartum has benefits for commercial dairy farms, as pooling increases the volume of colostrum available for all calves, saves labor, and simplifies colostrum management. However, the quality of colostrum, in terms of IgG concentration, decreases rapidly after the first 24 h postpartum (Morin et al., 2010; Conneely et al., 2014). Therefore, if the colostrum and transition milk are combined from cows 0 to 4 d postpartum, this pooled colostrum will be of lower quality with less IgG and protein than colostrum collected within the first day of calving (Godden et al., 2019). Poor-quality pooled colostrum with less than 50 g/L IgG has been shown to result in an increased risk of FPIT (Beam et al., 2009), whereas pooled colostrum of good quality with greater than 50 g/L IgG should provide adequate transfer of passive immunity to the calves (King et al., 2020).

The objectives of this study were to (1) establish whether different neonate dairy bull calves that are bottle fed colostrum (5% of birth weight every 12 h) from the same cow for 3 d will absorb equal amounts of the available components, including IgG; (2) examine whether the level of passive immunity transfer is comparable if calves are fed colostrum from their own dam or a foster cow; and (3) determine whether the level of passive immunity transfer is comparable if calves from the same cohort are fed a single bottle (2 L) of pooled colostrum and transition milk within 4 h of birth instead of being fed individual cow colostrum and transition milk for 3 d. It was hypothesized that calves that receive colostrum from individual cows would absorb similar amounts of the colostrum components and would have an adequate level of passive immunity. In contrast, calves that receive a minimal volume of pooled colostrum would have a lower level of passive immunity and a higher level of morbidity under the same environmental conditions.

MATERIALS AND METHODS

Experimental Design and Animals

All animal experimental work was approved by The University of Adelaide Animal Ethics Committee (approval number S-2017-060). A commercial farm with 1,800 cows located in Mount Gambier, South Australia, provided the 35 newborn Holstein-Friesian bull calves used in this study, which were singletons born in February 2018 from multiparous dams. The study power calculation with α of 80% and β of 0.05 estimated that 10.5 calves would be required per group to detect 5% variation in IgG. As a dropout rate of 10% was expected, 12 calves were chosen per group. The farm provided the facilities with 24-h access so that the samples could be processed immediately upon collection. Calving ease was recorded (Supplemental Table S1; https://adelaide.figshare.com/articles/journal_contribution/Do_et_al_2021_Colostrum_source_and_passive_immunity_transfer_in_dairy_bull_calves_Journal_of_Dairy_Science_paper_Supplementary_Files/14370269, Bottema et al., 2021) and calves were separated from their dam within 30 min of calving after being licked cleaned but before suckling. The calves were

tagged, measured for weight and girth, and scored for vigor and health within 4 h of birth (Supplemental File S1; Supplemental Figure S1; https://adelaide.figshare.com/articles/journal_contribution/Do_et_al_2021_Colostrum_source_and_passive_immunity_transfer_in_dairy_bull_calves_Journal_of_Dairy_Science_paper_Supplementary_Files/14370269, Bottema et al., 2021). The calves were randomly allocated to 3 groups using block randomization, with 6 blocks of 3 calf groups (groups A, B, and C). The calf serum GGT levels measured 1 d after birth indicated that one of the group C calves had suckled; therefore, this calf was removed from the study. All matched pairs of calves (e.g., A1 and B1) were born within 12 h of each other. The 12 dams of calves assigned to group A were milked within 2 h postpartum and twice daily thereafter for 3 d. The colostrum volume from each cow was recorded. The 12 group A calves were fed colostrum from their own dam, and the 12 matched group B calves were fed the identical colostrum from each milking of the respective group A dam (Supplemental File S1). Group A and B calves were bottle fed 5% of their birth weight of colostrum twice a day for 3 d, as recommended for maximum IgG absorption (Jaster, 2005). The first feeding was within 4 h after birth, and the second feeding was within 12 h after birth. After 3 d, the group A and B calves were fed bulk tank milk twice a day for the next 4 d (2 L/calf per feed). The feeding program used for the group C calves replicated the practice on the commercial dairy farm and represented a cohort-matched contrast for the calves fed the individual cow colostrum. The purpose of group C was to provide a benchmark for the expected morbidity under the local conditions on the farm. Within 4 h of birth, the 11 group C calves were fed a single bottle of mixed colostrum and transition milk (2 L), which was collected and pooled from the dairy transition cows between 0 and 4 d postpartum (referred to as "pooled colostrum" herein). Thereafter, the group C calves were fed bulk tank milk twice a day (2 L/calf per feed) for 7 d. All colostrum and milk was stored at 4°C until fed and was never stored more than 12 h.

Colostrum Samples

The group A cows were milked within 2 h of calving using a portable milking machine for the first milking, and then were milked in the dairy using a rotary milking parlor for the second to seventh milkings. Based on the definition of colostrum from the review of McGrath et al. (2016), the mammary secretions collected from these early milkings are referred to as "dam colostrum" herein. In addition to the bulk tank milk, the dairy provided the pooled colostrum and transition milk from multiple cows 0 to 4 d postpartum. These samples are referred to as "pooled colostrum" to distinguish from the bulk tank milk and the individual dam colostrum samples. The dam colostrum, pooled colostrum, and bulk tank milk were collected from the dairy twice a day and stored at 4°C for less than 12 h before feeding. Samples of dam colostrum, pooled colostrum, and bulk tank milk were collected into sterile 50-mL tubes immediately after each milking, frozen at −20°C, and then transported to The University of Adelaide, Roseworthy Campus, for storage at −80°C until analyzed.

Calf Blood Samples

Calf blood samples were collected within 4 h of calving before feeding (d 0) and on d 1, 2, 3, and 7 after birth. Blood was drawn from the jugular vein into 6-mL BD Vacutainers without anticoagulant and 6-mL BD Vacutainers with heparin. The blood samples in tubes without anticoagulant were allowed to clot for 45 to 60 min at 4°C and centrifuged (2,000 × g for 15 min at room temperature), and the serum was collected. Heparinized blood was centrifuged at 2,000 × g for 15 min at room temperature immediately after collection, and plasma was collected. Serum and plasma samples were stored at −20°C and then transported to The University of Adelaide, Roseworthy Campus, for storage at −80°C until analyzed.

Colostrum and Serum Refractometer Measurements

The concentration of the total soluble solids (Brix value) was measured in fresh colostrum and bulk tank milk immediately after milking using a digital optical refractometer (DBR-1, Starr Instruments). The refractometer measured proteins, carbohydrates, and other soluble molecules within a Brix value range of 0 to 50%. A different digital refractometer (Atago) with measurement range for protein from 0 to 12 g/100 mL was used to determine total soluble protein concentration by refractometer (**TP-R**) in the calf serum at d 0, 1, 2, 3, and 7 immediately after separating the serum by centrifugation. Both refractometers were calibrated using ultrapure water (Milli-Q).

Colostrum Fourier-Transform Midrange Infrared Analyzer Measurements

Total protein, lactose, and fat percentages in the thawed–frozen individual dam colostrum and pooled colostrum samples were determined using a Fourier-

transform midrange infrared spectrometer (Foss Analytics) at the National Herd Development Co-operative (Kyabram, VIC, Australia).

Bradford Assay for Total Protein

Total protein in colostrum, pooled colostrum, bulk tank milk, and calf serum was assayed in a 96 well-plate using a Quick Start Bradford protein assay kit following the manufacturer's instructions and bovine serum albumin for the standard curves (Quick Start, Bio-Rad Laboratories Inc.). Calf serum, the dam colostrum samples at d 1, 2, and 3, pooled colostrum, and bulk tank milk were diluted with ultrapure water at a ratio of 1:100, and colostrum at d 0 was diluted at a ratio of 1:200 or 1:300. The diluted samples (5 μL) were mixed with Coomassie Brilliant Blue G-250 dye (250 μL) and color change was measured using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories Inc.) at 595 nm. The total protein by Bradford assay (**TP-B**) in the samples was calculated based on the standard curve.

ELISA for IgG Concentration

The IgG concentration in colostrum, pooled colostrum, bulk tank milk, and calf serum was quantified by a sandwich ELISA in 96-well plates (Coat Nunc F96 Maxisorp plates, Thermo Fisher Scientific) using 2 bovine IgG-specific antibodies (Life Technologies): goat anti-bovine IgG antibody unconjugated, affinity purified (Novex cat. no. A18753), and goat anti-bovine IgG antibody conjugated with horseradish peroxidase, affinity purified (Invitrogen cat. no. 18751). Calf serum samples were diluted in 0.05% Tween 20-PBS solution at a ratio of $1{:}10^4$ for d 0 and a ratio of $1{:}10^6$ for all other time points. The colostrum samples from d 0 and 1 and the pooled colostrum samples were diluted at a ratio of $1{:}10^6$, and the colostrum samples from d 2 and 3 and the bulk tank milk samples were diluted at a ratio of $1{:}10^5$. Bovine γ-globulin (Bio-Rad Laboratories Inc.) was used as a standard with serial dilutions (0, 6.3, 12.5, 25, 37.5, 50, 75, and 100 ng/mL). The 3,3′,5,5′-tetramethylbenzidine substrate (Ultra TMB-ELISA, Thermo Fisher Scientific) was added and the plates were incubated at room temperature in the dark for 15 min before the reactions were stopped with the addition of 0.1 M H_2SO_4. Antibody binding was measured as the enzymatic color change at 450 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories Inc.). The IgG concentrations in samples were calculated based on the standard curve. The intra-assay variation was 7.2%, and the interassay variation was 9.4%.

Calf Blood Glucose Concentration

Fresh whole blood was collected into a Vacutainer without anticoagulant. A drop was immediately absorbed onto an Accu-Check Performa test strip, and the glucose concentration was determined using a glucose meter (Accu-Check Performa, Roche Diabetes Care).

Calf Plasma GGT Measurements

Calf plasma was analyzed for GGT levels using a kinetic color test with a Beckman Coulter analyzer (Coulter Manufacturing Co.) at the Veterinary Diagnostic Laboratory, The University of Adelaide, Australia. Because the data for GGT were not normally distributed, these data were log-transformed and reported as geometric means with confidence intervals.

Statistical Analyses

The preliminary statistical analyses were conducted in R (version 3.6.3; https://cran.r-project.org/bin/windows/base/old/3.6.3/). Descriptive statistics for the colostrum components (Brix value, TP-B, IgG, and protein, lactose, and fat percentages) and calf blood measurements (glucose, GGT, TP-R, TP-B, and IgG concentration) were calculated using the R "psych" package, and normality was determined using Shapiro-Wilk test in the R "nortest" package. T-tests were performed to compare differences between dam colostrum and pooled colostrum components when the data were normally distributed, whereas Kruskal-Wallis tests were used when the data were not normally distributed. The differences between the groups at birth were tested by 1-way ANOVA when the data were normally distributed and verified using Tukey's honestly significant difference test, whereas Kruskal-Wallis tests were used when the data were not normally distributed.

The effect of time was estimated using mixed models in SAS (PROC MIXED, SAS version 9.4, SAS Institute Inc.), which accounted for the repeated effect of individual dams on the means of the colostrum-dependent variables (Brix value, TP-B, IgG, and protein, lactose, and fat percentages) across the time points (d 0, 1, 2, and 3). The effect of time was also estimated using mixed models (PROC MIXED), which accounted for the repeated effect of individual calves on the means of the blood-dependent variables (glucose, GGT, TP-R, TP-B, and IgG concentration) across the time points (d 0, 1, 2, 3, and 7). Last, the effects of group, time point, and the interaction between the groups and time points were estimated by mixed models in SAS (PROC MIXED), which accounted for the repeated effect of individual calves on the means of dependent variables

Table 1. Descriptive statistics and comparison of the components in the dam colostrum (n = 12) and pooled colostrum (n = 6) at first feeding on d 0

	Dam colostrum		Pooled colostrum		
Component[1]	Mean	SE	Mean	SE	P-value[2]
Total soluble solids (Brix %)	25.4	1.4	19.4	2.1	0.04
Total protein (g/L)	170.8	16.0	88.7	11.6	0.001
IgG (g/L)	184.4	20.4	90.5	9.6	0.008
Protein (%)	16.6	0.9	12.4	1.3	0.02
Fat (%)	4.6	0.4	5.4	0.9	0.46
Lactose (%)	1.9	0.2	2.4	0.1	0.02

[1]Total protein was measured by Bradford assay; protein, fat, and lactose percentages were measured by Fourier-transform midrange infrared analysis; and IgG was measured by ELISA.

[2]$P < 0.05$ was considered significantly different.

(glucose, GGT, TP-B, TP-R, and IgG levels) for the 3 calf groups (A, B, and C) across time points (d 0, 1, 2, 3, and 7). Blood measurements taken on d 1 from the calves in groups A and B were compared for each calf pair by PROC GLM in SAS using a Bonferroni adjustment for nonparametric variables and Tukey adjustment for parametric variables. Colostrum components at d 0 and calf blood measurements at d 1 were compared using PROC CORR in SAS by estimation of the Pearson correlation coefficients.

The classification scheme of Lombard et al. (2020) was used to categorize the calf serum IgG levels at 48 h as excellent, good, fair, and poor, where the serum IgG levels were ≥25.0, 18.0 to 24.9, 10.0 to 17.9, and <10 g/L, respectively. The proportion of calves from the groups within each category and the proportion of calves in the groups that failed to reach the thresholds for adequate passive immunity transfer were compared pairwise using a Fisher's exact test (2 × 2 table) with a confidence level of 95%. Failure of passive immunity transfer was indicated by values below the thresholds of 52 g/L total protein at d 1 and 2, 10 g/L IgG at d 1 and 2, 200 U/L GGT on d 1, and 75 U/L GGT on d 7.

RESULTS AND DISCUSSION

To investigate the level of passive immunity transferred after feeding neonate calves colostrum from different sources and to establish whether calves that receive identical colostrum from the same milking of a cow will absorb similar amounts of the available nutrients, 3 groups of neonate calves were fed dam, foster cow, or pooled colostrum. Components in the colostrum were analyzed with respect to their absorption into the blood of the calves during the first 7 d after birth.

Colostrum and Bulk Tank Milk Measurements

The levels of the total solids (Brix value), lactose percent, protein percent, total protein (TP-B), and IgG were found to be different between the dam and pooled colostrum ($P < 0.05$; Table 1). The only exception was the concentration of lipid, as there was no difference in the fat percentage when measured by Fourier-transform midrange infrared analyzer between the dam and pooled colostrum.

Colostrum Total Soluble Solids and IgG. The total soluble solids of the dam colostrum were significantly higher than the pooled colostrum (mean ± SE Brix value: 25.4 ± 1.4% vs. 19.4 ± 2.1%, respectively; $P < 0.05$; Table 1). The Brix value for the dam colostrum observed in the present study ranged between 18.5 and 34% (95% CI = 22.4–28.4%), and pooled colostrum had a range of 12.3 to 25.9% (95% CI = 14.0–24.8%).

Colostrum is considered good quality if the concentration of IgG is greater than 50 g/L, and many studies have suggested that Brix values between 18 and 23% are equivalent to 50 g/L IgG (Bielmann et al., 2010; Quigley et al., 2013; Bartier et al., 2015; Morrill et al., 2015). In a meta-analysis by Buczinski and Vandeweerd (2016), the authors classified good-quality colostrum as having a Brix value greater than 22% and poor-quality colostrum as having a Brix value less than 18%, where these percentages ensure greater than 50 g/L IgG or less than 50 g/L IgG, respectively. On this basis, although the pooled colostrum generally had less total solids than the dam colostrum, it was not always considered poor quality.

The average IgG concentration in dam colostrum was 184.4 g/L (95% CI: 139.5–229.2 g/L). This was significantly higher than the pooled colostrum ($P < 0.05$), which had an average of 90.5 g/L (95% CI: 66.0–115.1 g/L). The minimum IgG concentration of the dam colostrum was 83.7 g/L, whereas the minimum of the pooled colostrum was 69.7 g/L.

The dam colostrum in this study had higher IgG levels than some studies (Marnila and Korhonen, 2011; Quigley et al., 2013; Dunn et al., 2017), most likely because it was collected within 2 h of calving and the cows were multiparous. The IgG concentrations

in pooled colostrum in the present study were in the ranges reported elsewhere (Lago et al., 2018: 22.7–96.9 g/L, mean of 63.6 g/L; King et al., 2020: 90.5–104.4 g/L, mean of 100.7 g/L).

Overall, the dam colostrum was good quality, whereas the pooled colostrum was clearly of lower quality. This was expected because total protein and IgG concentrations in colostrum are known to steadily decrease postpartum and are significantly higher in colostrum than in the mature milk produced by d 4 (McGrath et al., 2016).

Colostrum Lactose and Fat. The lactose and fat provided by colostrum and milk are essential energy sources for the neonate (Hammon et al., 2013). The dam colostrum collected within 2 h postpartum contained 1.9% lactose and 4.6% fat on average, whereas the pooled colostrum contained 2.4% and 5.4%, respectively. Both the lactose and fat concentrations were in the range reported in previous studies (Kehoe et al., 2007; Godden, 2008; Dunn et al., 2017). The lactose in dam colostrum was significantly lower than in pooled colostrum ($P < 0.05$), as expected, because lactose is known to be low in colostrum and increases over time as milk production increases (McGrath et al., 2016; Dunn et al., 2017). In contrast, fat concentration has been reported to decrease over time, declining from 7.0% to 6.0% within 24 h (Dunn et al., 2017) and from 6.7% to 3.9% between the first and third milkings (Godden, 2008). However, in the present study, the fat concentration in dam colostrum was similar to that in the pooled colostrum.

Dam Colostrum Components Day 0 to 3 Postpartum. Dam colostrum volume increased slowly between successive time points ($P < 0.05$ for colostrum volume between d 0 and 2 postpartum), whereas the concentration of colostrum components, as measured by Brix value, TP-B, and IgG, decreased ($P < 0.05$ between d 0 and all other time points), which is consistent with the observations of others (Yang et al., 2015). The mean Brix value measured in colostrum was 25.4% at the first milking postpartum (d 0) and then decreased considerably to 12.2, 10.6, and 10.5% by d 1, 2, and 3, respectively (Figure 1). Total protein, measured by Bradford assay, declined from 170.8 g/L at d 0 to 51.2 g/L on d 1 and 34.4 g/L for d 2 and 3. The mean IgG concentration in the dam colostrum was 184.4 g/L on d 0 and then rapidly declined to 29.4, 4.9, and 2.7 g/L on d 1, 2, and 3, respectively. Only the colostrum collected within 24 h of parturition had Brix value and IgG concentration above the thresholds of 22% and 50 g of IgG/L, respectively, recommended for the first calf feeding (Quigley et al., 2013; Bartier et al., 2015). Therefore, when pooling colostrum for the first feeding, it is advisable to use only colostrum from cows within 1 d postpartum.

Bulk Tank Milk Measurements. Twenty-five bulk tank milk samples, which were fed to the group C calves for 7 d and the group A and B calves from d 4 to 7 postpartum, were also analyzed. The average Brix value, TP-B, and IgG concentration of the bulk tank milk were 8.9 ± 0.2%, 28.6 ± 2.0 g/L, and 0.3 ± 0.1 g/L, respectively, which were all lower than the colostrum sample levels ($P < 0.05$).

Calf Birth Measurements

Within 4 h after birth and before feeding, no significant differences were observed in any of the physical or blood measurements for the 3 groups of calves (Table 2), and no health problems or calving difficulties were observed. The average BW at birth of the 35 calves was 38.3 ± 0.9 kg. At birth, the mean glucose, TP-R, and TP-B of 35 calves was 3.8 mmol/L, 40.4 g/L, and 52.5 g/L, respectively. The mean IgG concentration in calf serum was 0.3 g/L, indicating that the calves had not suckled from their dams.

Another good indication of whether a calf has suckled colostrum is the presence of GGT in the neonate blood (Braun et al., 1982; Parish et al., 1997). γ-Glutamyl transferase is found in very low concentrations in the blood of newborn calves until it is rapidly absorbed from the colostrum before gut closure (Weaver et al., 2000). The GGT level was used to exclude calves from this study that suckled from their dams before the initial sampling. The mean GGT level in the plasma of the newborn calves included in this study at d 0 was 11.7 U/L, with a range of 6.2 to 26.2 U/L, which is similar to the 10 to 31 U/L values reported before suckling by Braun et al. (1982).

Calf Measurements After Receiving Colostrum from Different Sources

Between 2 and 4 h after birth, the calves were fed colostrum from their own dam (group A calves), foster colostrum from one of the group A dams (group B calves), or pooled colostrum (group C calves). Each group A calf was paired with a group B calf that was born within 12 h (e.g., A1 and B1). For 3 d, these pairs of calves received identical colostrum from each milking of the respective group A dam. In contrast, the group C calves received 1 bottle (2 L) of a pool of colostrum and transition milk from the other dairy cows 0 to 4 d postpartum and bulk tank milk thereafter.

Twenty-four hours after consumption of colostrum, the glucose, TP-B, TP-R, IgG, and GGT concentrations

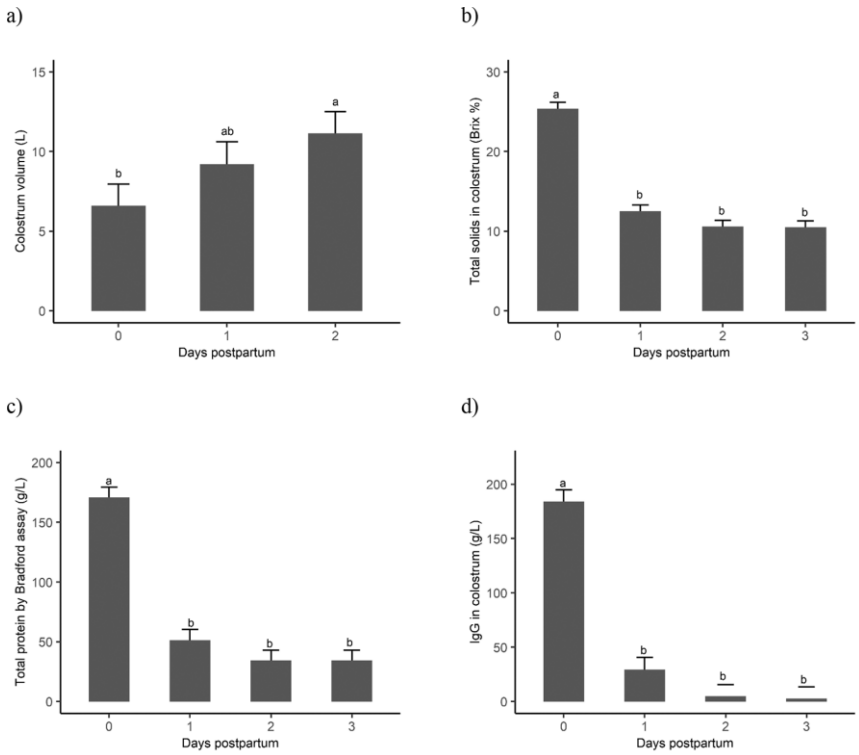

Figure 1. Dam colostrum data from d 0 to 3 postpartum (LSM ± SE, n = 12). Colostrum was milked from the dams of the group A calves within 2 h postpartum and then every 12 h. The colostrum was measured and sampled, and the data were analyzed for 4 time points (2, 24, 48, and 72 h postpartum). Colostrum volume (a), total solids measured by Brix refractometer (b), total protein measured by Bradford assay (c), and IgG concentration measured by ELISA (d). Different letters (a, b) indicate significant differences between means ($P < 0.05$).

Table 2. Descriptive statistics and comparison of birth measurements among calf groups[1]

	Group A		Group B		Group C		All			
Measure[2]	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Minimum–maximum	*P*-value[3]
Birth BW (kg)	38.0	1.5	36.8	1.8	40.1	1.3	38.3	0.9	25.0–48.0	0.36
Girth[4] (cm)	43.7	1.3	44.9	1.7	45.7	1.4	44.7	0.8	38.0–59.0	0.43
Glucose (mmol/L)	3.8	0.4	4.1	0.3	3.6	0.4	3.8	0.2	1.6–6.5	0.67
GGT[4] (U/L)	10.8	1.0	11.0	0.8	13.3	1.8	11.7	0.7	6.2–26.2	0.55
TP-R (g/L)	40.5	1.0	39.6	1.1	41.1	0.9	40.4	0.6	34.0–46.0	0.58
TP-B[4] (g/L)	55.0	3.5	52.1	2.7	50.1	1.7	52.5	1.6	38.3–80.0	0.66
IgG[4] (g/L)	0.3	0.2	0.3	0.1	0.2	0.1	0.3	0.1	0.0–1.7	0.78

[1]Group A = calves (n = 12) fed own dam's colostrum; group B = calves (n = 12) fed foster cow colostrum; group C = calves (n = 11) fed 1 bottle of pooled colostrum. All: n = 35 calves.

[2]GGT = γ-glutamyl transferase activity; TP-R = total serum protein measured by refractometer; TP-B = total serum protein measured by Bradford assay.

[3]$P < 0.05$ was considered significantly different.

[4]Data were nonparametric.

in calf blood had increased in all groups. Significant differences were observed between d 0 (before feeding) and all other time points (d 1, 2, 3, and 7; $P < 0.05$; Figure 2; Supplemental Table S2; https://adelaide.figshare.com/articles/journal_contribution/Do_et_al_2021_Colostrum_source_and_passive_immunity_transfer_in_dairy_bull_calves_Journal_of_Dairy_Science_paper_Supplementary_Files/14370269, Bottema et al., 2021).

Glucose Concentration. There was no difference in glucose concentration between the 3 calf groups, although there was a difference between the time points ($P < 0.05$; Figure 2a). The blood glucose concentration increased from a mean for all 3 groups of 3.8 ± 0.2 mmol/L before feeding to a mean of 6.9 ± 0.2 mmol/L on d 1 after receiving the dam colostrum or pooled colostrum ($P < 0.05$). This level was maintained from d 2 (6.6 ± 0.1 mmol/L) and d 3 (6.4 ± 0.1 mmol/L) to d 7 for all groups (6.0 ± 0.1 mmol/L). These glucose concentrations were in the same range as reported in other studies (Schiessler et al., 2002).

GGT Activity. The GGT activity in the calf plasma of all the groups increased 100-fold from d 0 before feeding to d 1 following colostrum feeding ($P < 0.05$; mean: 1,271.4 ± 94.8 U/L; range: 89.5–4,034.5 U/L). These GGT levels are similar to those reported by Braun et al. (1982), who found a range of 10 to 31 U of GGT/L at birth (mean: 18.2 U/L), which increased to 370 to 5,000 U/L by d 1. The GGT levels in the group A and B calves were higher than those in the group C calves on d 1 ($P < 0.05$; Figure 2b). However, the GGT level in all 3 groups declined by d 7 (mean: 200.8 ± 94.8 U/L), and there were no differences between the groups.

Serum Protein Measured by Refractometer. Serum protein refractometry is a tool that can be used on-farm to estimate serum total protein (TP-R) and detect possible FPIT in neonatal calves (Vandeputte et al., 2011; Deelen et al., 2014; Hernandez et al., 2016). The TP-R differed depending on the calf group and time point ($P < 0.05$; Figure 2c). There were large differences in TP-R between d 0 and all other time points (d 1, 2, 3, and 7). At birth, TP-R concentrations in group A, B, and C calves were similar. After receiving colostrum, TP-R increased dramatically by d 1 to 60.9 ± 1.9, 59.3 ± 1.9, and 52.4 ± 1.9 g/L for group A, B, and C calves, respectively, with a significant difference between the group A and B calves versus the group C calves ($P < 0.05$). The TP-R levels in the 3 calf groups remained at these levels for d 2 and 3, and then decreased slightly by d 7. Differences between the group A and C calves were observed for all time points, but there were no significant differences between the group A and B calves at any time point (Figure 2c). The total serum protein levels, as measured by refractometry in the present study, were similar to those in previous studies, where calf serum TP-R at 24 h postpartum was 52 g/L following feeding 2 L of pooled colostrum or 59 g/L following feeding 4 L (Williams et al., 2014).

Bradford Serum Total Protein. There was a difference in total protein in the calf blood, as measured by the Bradford assay (TP-B), across time points ($P < 0.05$), but no difference was observed between the groups at any of the time points (Figure 2d). The pattern in TP-B levels was similar to the TP-R, with a large increase by d 1 and declining by d 7. The protein concentration estimated by refractometry was lower than the total protein measured by Bradford assay ($P < 0.05$). The Bradford assay measures both soluble and nonsoluble proteins in the blood, whereas the refractometer measures the refractive index of the serum soluble components. Therefore, the refractometer underestimates total protein concentration. This has been observed in other studies where refractometer values were lower by 20 to 25 g/L compared with the Bradford assay or Biuret method (Braun et al., 2001).

Serum IgG. The group A and B calves received dam colostrum with very high IgG concentrations (184.4 g/L IgG for the first feed) and then dam colostrum twice daily for 3 d, whereas the calves in group C received only 1 bottle of lower quality pooled colostrum (94.4 g/L IgG for the first feed) and then bulk tank milk thereafter (0.3 g/L IgG). There were significant differences in the calf serum IgG concentrations between d 0 and all other time points for all calf groups ($P < 0.05$; Figure 2e). After 24 h, the IgG concentration in serum of group A and B calves was similar (27.9 ± 2.9 and 26.4 ± 2.9 g/L, respectively) and higher than that in the group C calves (15.5 ± 3.0 g/L), although the difference was significant only for the group A and C calves ($P = 0.004$).

The IgG concentration in the calf serum on d 2 was different among all groups (group A, B, and C mean: 35.0 ± 2.9, 25.7 ± 2.9, and 15.5 ± 3.0 g/L, respectively; $P < 0.05$). A difference in the IgG levels between the group A calves (fed their own dam's colostrum) and the group B calves (fed foster cow colostrum) was observed only at d 2 ($P = 0.03$). The mean IgG concentrations were higher in the group A and B calves than in the group C calves (fed only 1 bottle of pooled colostrum) at all of the time points ($P < 0.05$), except d 3, where there was no difference between the group B and C calves.

It is not clear whether the difference between the IgG levels in the group A and B calves and the levels in the group C calves was the result of the colostrum quality, the amount of colostrum provided, or both. A limitation of the experimental design is that the data from

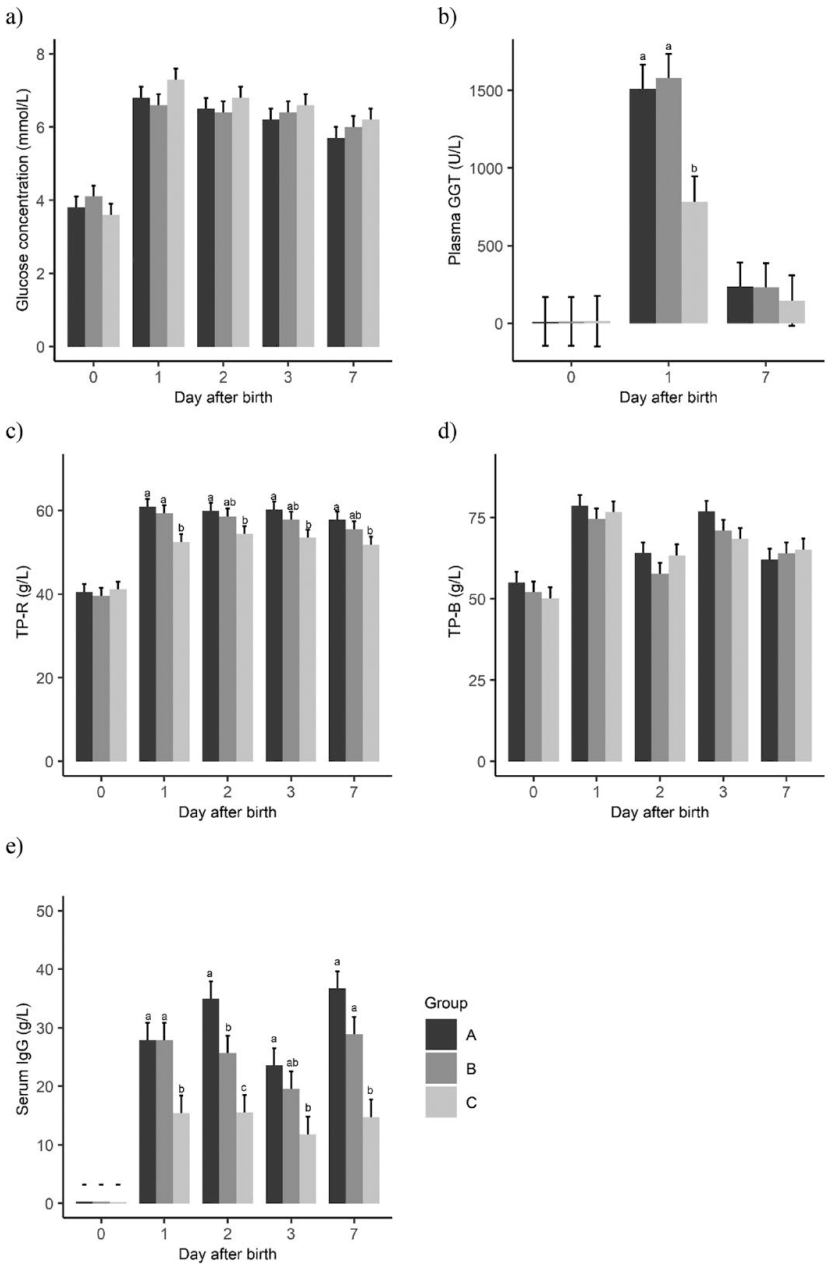

Figure 2. Blood measurements of the 3 calf groups from d 0 to 7 after birth (LSM ± SE). Blood was sampled before receiving colostrum (d 0) and after feeding colostrum and bulk tank milk (d 1, 2, 3, and 7 after birth). Group A = calves fed own dam's colostrum for 3 d and then bulk tank milk (black); group B = calves fed foster cow colostrum for 3 d and then bulk tank milk (dark gray); group C = calves fed 1 bottle of pooled colostrum and then bulk tank milk for 7 d (light gray). Blood glucose levels (a), plasma γ-glutamyl transferase (GGT) activity (b), serum total protein by refractometer (TP-R; c), serum total protein by Bradford assay (TP-B; d), and serum IgG concentrations (e). Different letters (a–c) indicate significant differences between means at each time point ($P < 0.05$).

the group C calves could not be compared directly with the data of the other groups in terms of the absorption of the colostrum components because the volume of colostrum fed to these calves differed from the group A and B calves. It was important to have a benchmark that reflected the conditions and the expected morbidity on the farm; however, the results from the group C calves were confounded and prevented additional conclusions regarding quality versus quantity.

The serum IgG concentrations in all calf groups were in the same range as previous studies with similar-quality colostrum (Jones et al., 2004; Poulsen et al., 2010; Priestley et al., 2013; Lago et al., 2018; King et al., 2020). The classification scheme of Lombard et al. (2020) was used to categorize the calf serum IgG levels at 48 h as excellent, good, fair, and poor (Supplemental Table S3; https://adelaide.figshare.com/articles/journal_contribution/Do_et_al_2021_Colostrum_source_and_passive_immunity_transfer_in_dairy_bull_calves_Journal_of_Dairy_Science_paper_Supplementary_Files/14370269, Bottema et al., 2021). The majority of group A and B calves had excellent levels of IgG (75 and 66.7%, respectively). The majority of the group C calves, which received 1 bottle of pooled colostrum, had only fair levels of IgG (63.6%). Thus, the proportions within the categories did not differ between the group A and B calves, but they were different for the group C calves ($P < 0.01$). One group B calf and 1 group C calf were classified as poor, with serum IgG levels below 10 g/L.

Pairwise Comparisons Between Calves Receiving the Same Cow Colostrum

In the pairwise comparison of group A and B calves receiving colostrum from the same cow, no differences were observed in the levels of plasma GGT, glucose, serum total protein, or serum IgG at d 1 between the calves receiving identical colostrum, whether the colostrum was from their own dam or a foster cow (e.g., calf A1 vs. calf B1; Table 3). The lack of any significant differences suggests that calves given the same colostrum will absorb similar levels of nutrients.

Interestingly, although each pair of A and B calves received the same colostrum and the mean IgG concentrations for these groups were not statistically different, the group A calves generally had higher average IgG concentrations after d 0 compared with the group B calves (27.9 and 26.4 g/L at d 1, respectively). The dissimilarity in IgG concentrations between group A and B calves was even greater by d 2 (35.0 and 25.7 g/L, respectively; $P = 0.03$). The IgG levels in the group B calves were consistently between the levels of the group A and group C calves for all time points. The correlation between calf serum IgG concentration at d 1 and d 0 colostrum IgG was also lower for the group A calves (r = 0.49) than for the group B calves (r = 0.76).

This suggests that factors other than IgG itself may be involved in its absorption in the calf. The speed of ingestion could play a role, as there is evidence that bioactive factors in colostrum improve gastrointestinal development and nutrient absorption (Blum, 2006; Hammon et al., 2020). The effect of cross-fostering on the absorption of IgG and other components may warrant further investigation.

Calf Health

Calf health was carefully monitored for 1 wk after birth, and diarrhea, fever, and respiratory infections were noted (Table 4). No health problems were detected in the group A calves, and only 2 calves in group B and 2 calves in group C were ill in the first 7 d. Consequently, the numbers were insufficient to assess the effects of colostrum source on calf health. A constraint in this study was the small number of calves in

Table 3. Pairwise comparison of calf blood components on d 1 between group A calves (n = 12) and group B calves (n = 12)[1]

	Group A			Group B			
Component[2]	Mean	SEM	95% CI	Mean	SEM	95% CI	P-value[3]
Parametric							
Glucose (mmol/L)	6.8	0.8		6.6	0.8		0.77
TP-R (g/L)	60.9	5.4		59.3	5.5		0.66
TP-B (g/L)	78.6	9.9		74.5	9.8		0.55
IgG (g/L)	27.9	8.1		26.4	8.1		0.79
Nonparametric							
GGT (U/L)	1,511.0		873.2–2,148.8	1,580.1		942.3–2,217.8	0.88

[1]Group A = calves fed own dam's colostrum; group B = calves fed foster cow colostrum. Paired group A and B calves received identical colostrum from the same milking of the dam of the group A calf.

[2]TP-R = total serum protein measured by refractometer; TP-B = total serum protein measured by Bradford assay; GGT = γ-glutamyl transferase activity. Nonparametric data are presented with 95% CI for the estimate rather than SEM.

[3]$P < 0.05$ was considered significantly different.

Table 4. Frequency and proportion of calves classified as having failure of passive immunity transfer (FPIT) by calf group[1]

FPIT threshold for measurement[2]	Time point	Frequency (no.)			Proportion (%)		
		Group A	Group B	Group C	Group A	Group B	Group C
TP-R <52 g/L	Day 1	0	4	5	0[b]	33.3[ab]	45.5[a]
	Day 2	1	4	3	8.3	33.3	27.3
TP-B <52 g/L	Day 1	0	1	0	0	8.3	0
	Day 2	0	3	2	0	25	18.2
IgG <10 g/L	Day 1	0	1	1	0	8.3	9.1
	Day 2	0	1	1	0	8.3	9.1
GGT <200 U/L	Day 1	0	1	1	0	8.3	9.1
GGT <75 U/L	Day 7	0	1	1	0	8.3	9.1
Illness within 7 d after birth		0	2	2	0	16.7	18.2

[a,b]Means within a row of FPIT proportion with different superscripts differ ($P < 0.05$), comparing pairwise using 2 × 2 tables with Fisher's exact test.

[1]Group A = calves (n = 12) fed own dam's colostrum; group B = calves (n = 12) fed foster cow colostrum; group C = calves (n = 11) fed 1 bottle of pooled colostrum.

[2]TP-R = total serum protein measured by refractometer; TP-B = total serum protein measured by Bradford assay; GGT = γ-glutamyl transferase activity.

each group. In particular, an accurate assessment of morbidity was not possible because there were very few cases of illness.

FPIT

Failure of the transfer of passive immunity is defined by thresholds that are indicative of the amount of IgG that has been absorbed from the colostrum into the blood of the newborn calf. All calves in group A, which were fed their own dam's colostrum, had total protein (TP-R), GGT, and IgG levels above the thresholds that indicated successful transfer of passive immunity. However, several calves in groups B and C had TP-R levels below the suggested threshold for d 1 (4 and 5 calves, respectively; Table 4). Compared with group A, group C had a significantly higher proportion of calves that failed to have adequate passive transfer of immunity based on the serum protein refractometry (TP-R <52 g/L; $P < 0.05$). Nevertheless, on d 1, only 1 calf from group B (fed foster cow colostrum) and 1 calf from group C (fed only 1 bottle of the lower quality pooled colostrum) had IgG concentrations and GGT levels below the suggested passive immunity transfer thresholds (<10 g/L and <200 U/L, respectively; Table 4).

Few studies have focused on feeding pooled colostrum or foster cow colostrum to calves. In general, feeding colostrum and transition milk pooled from multiple cows has been found to increase the risk of FPIT because the IgG concentration is diluted by the milk from those cows that calved 3 to 4 d earlier (Pithua et al., 2013). However, calves are less at risk of FPIT if the pooled colostrum is collected within the first 2 d postpartum and has an adequate IgG concentration (Williams et al., 2014; King et al., 2020). The results of our study indicate that calves fed pooled colostrum collected 0 to 4 d postpartum had adequate passive immunity transfer as defined by IgG and GGT thresholds, presumably because the pooled colostrum contained relatively high IgG concentrations (90.5 ± 9.6 g/L) and was fed within 4 h of birth. Given that additional factors besides IgG are transferred from the colostrum to the neonate, including other immunity-related proteins, microRNA, and cells (Gelsinger and Heinrichs, 2017; Godden et al., 2019), it would be of interest to examine other components that may be vital.

CONCLUSIONS

Calves receiving colostrum collected soon after birth from their own dam (group A) had significantly higher TP-R and IgG concentrations on d 1 compared with calves fed lower quality pooled colostrum (group C). The group C calves, which received only 1 bottle of pooled colostrum, had fair levels of IgG, with only 1 calf with poor IgG levels (below 10 g/L) and no demonstrable increased morbidity. Thus, the dairy farm practice of providing 1 bottle of pooled colostrum for the first feeding was generally adequate for the transfer of passive immunity in this study. However, it should be noted that the group C calves were fed within 4 h after birth and the quality of the pooled colostrum was good.

No significant concentration differences for any calf blood components (including glucose, GGT, total protein, and IgG) were observed between the calves that received their own dam's colostrum (group A) and the paired calves that received identical colostrum (group B). Moreover, there were no differences in the transfer of passive immunity based on IgG levels. This suggests that the calves receiving the same colostrum will ab-

sorb the colostrum components equally. Intriguingly, though, the results hint that the calves that receive their own dam's colostrum may absorb more IgG than those that receive foster cow colostrum; this should be explored in larger studies.

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REFERENCES

Abuelo, A., P. Havrlant, N. Wood, and M. Hernandez-Jover. 2019. An investigation of dairy calf management practices, colostrum quality, failure of transfer of passive immunity, and occurrence of enteropathogens among Australian dairy farms. J. Dairy Sci. 102:8352–8366. https://doi.org/10.3168/jds.2019-16578.

Barrington, G. M., and S. M. Parish. 2001. Bovine neonatal immunology. Vet. Clin. North Am. Food Anim. Pract. 17:463–476. https://doi.org/10.1016/s0749-0720(15)30001-3.

Bartier, A. L., M. C. Windeyer, and L. Doepel. 2015. Evaluation of on-farm tools for colostrum quality measurement. J. Dairy Sci. 98:1878–1884. https://doi.org/10.3168/jds.2014-8415.

Beam, A. L., J. E. Lombard, C. A. Kopral, L. P. Garber, A. L. Winter, J. A. Hicks, and J. L. Schlater. 2009. Prevalence of failure of passive transfer of immunity in newborn heifer calves and associated management practices on US dairy operations. J. Dairy Sci. 92:3973–3980. https://doi.org/10.3168/jds.2009-2225.

Bielmann, V., J. Gillan, N. R. Perkins, A. L. Skidmore, S. Godden, and K. E. Leslie. 2010. An evaluation of Brix refractometry instruments for measurement of colostrum quality in dairy cattle. J. Dairy Sci. 93:3713–3721. https://doi.org/10.3168/jds.2009-2943.

Blum, J. W. 2006. Nutritional physiology of neonatal calves. J. Anim. Physiol. Anim. Nutr. (Berl.) 90:1–11. https://doi.org/10.1111/j.1439-0396.2005.00614.x.

Bottema, C., H. Do, T. Chen, R. Skirving, K. Petrovski, L. Kiro, and J. Williams. 2021. Hue, et al. (2021) Colostrum source and passive immunity transfer in dairy bull calves. Journal of Dairy Science paper Supplementary Files. The University of Adelaide. Journal contribution. https://doi.org/10.25909/14370269.

Braun, J.-P., J.-F. Guelfi, and J.-P. Pagès. 2001. Comparison of four methods for determination of total protein concentrations in pleural and peritoneal fluid from dogs. Am. J. Vet. Res. 62:294–296. https://doi.org/10.2460/ajvr.2001.62.294.

Braun, J. P., D. Tainturier, C. Laugier, P. Bénard, J. P. Thouvenot, and A. G. Rico. 1982. Early variations of blood plasma gamma-glutamyl transferase in newborn calves—A test of colostrum intake. J. Dairy Sci. 65:2178–2181. https://doi.org/10.3168/jds.S0022-0302(82)82479-X.

Buczinski, S., E. Gicquel, G. Fecteau, Y. Takwoingi, M. Chigerwe, and J. M. Vandeweerd. 2018. Systematic review and meta-analysis of diagnostic accuracy of serum refractometry and Brix refractometry for the diagnosis of inadequate transfer of passive immunity in calves. J. Vet. Intern. Med. 32:474–483. https://doi.org/10.1111/jvim.14893.

Buczinski, S., and J. Vandeweerd. 2016. Diagnostic accuracy of refractometry for assessing bovine colostrum quality: A systematic review and meta-analysis. J. Dairy Sci. 99:7381–7394. https://doi.org/10.3168/jds.2016-10955.

Castro, N., J. Capote, R. M. Bruckmaier, and A. Argüello. 2011. Management effects on colostrogenesis in small ruminants: A review. J. Appl. Anim. Res. 39:85–93. https://doi.org/10.1080/09712119.2011.581625.

Conneely, M., D. P. Berry, J. P. Murphy, I. Lorenz, M. L. Doherty, and E. Kennedy. 2014. Effect of feeding colostrum at different volumes and subsequent number of transition milk feeds on the serum immunoglobulin G concentration and health status of dairy calves. J. Dairy Sci. 97:6991–7000. https://doi.org/10.3168/jds.2013-7494.

Cuttance, E. L., W. Mason, R. Laven, K. Denholm, and D. Yang. 2018. Calf and colostrum management practices on New Zealand dairy farms and their associations with concentrations of total protein in calf serum. N. Z. Vet. J. 66:126–131. https://doi.org/10.1080/00480169.2018.1431159.

Cuttance, E. L., W. A. Mason, R. A. Laven, J. McDermott, and C. V. C. Phyn. 2017. Prevalence and calf-level risk factors for failure of passive transfer in dairy calves in New Zealand. N. Z. Vet. J. 65:297–304. https://doi.org/10.1080/00480169.2017.1361876.

Deelen, S. M., T. L. Ollivett, D. M. Haines, and K. E. Leslie. 2014. Evaluation of a Brix refractometer to estimate serum immunoglobulin G concentration in neonatal dairy calves. J. Dairy Sci. 97:3838–3844. https://doi.org/10.3168/jds.2014-7939.

Denholm, K. S., J. Hunnam, E. Cuttance, and S. McDougall. 2017. Associations between management practices and colostrum quality on New Zealand dairy farms. N. Z. Vet. J. 65:257–263. https://doi.org/10.1080/00480169.2017.1342575.

Dunn, A., A. Ashfield, B. Earley, M. Welsh, A. Gordon, and S. J. Morrison. 2017. Evaluation of factors associated with immunoglobulin G, fat, protein, and lactose concentrations in bovine colostrum and colostrum management practices in grassland-based dairy systems in Northern Ireland. J. Dairy Sci. 100:2068–2079. https://doi.org/10.3168/jds.2016-11724.

Faber, S. N., N. E. Faber, T. C. McCauley, and R. L. Ax. 2005. Case study: Effects of colostrum ingestion on lactational performance. Prof. Anim. Sci. 21:420–425. https://doi.org/10.15232/S1080-7446(15)31240-7.

Furman-Fratczak, K., A. Rzasa, and T. Stefaniak. 2011. The influence of colostral immunoglobulin concentration in heifer calves' serum on their health and growth. J. Dairy Sci. 94:5536–5543. https://doi.org/10.3168/jds.2010-3253.

Gelsinger, S. L., and A. J. Heinrichs. 2017. Comparison of immune responses in calves fed heat-treated or unheated colostrum. J. Dairy Sci. 100:4090–4101. https://doi.org/10.3168/jds.2016-12010.

Godden, S. 2008. Colostrum management for dairy calves. Vet. Clin. North Am. Food Anim. Pract. 24:19–39. https://doi.org/10.1016/j.cvfa.2007.10.005.

Godden, S. M., J. E. Lombard, and A. R. Woolums. 2019. Colostrum management for dairy calves. Vet. Clin. North Am. Food Anim. Pract. 35:535–556. https://doi.org/10.1016/j.cvfa.2019.07.005.

Hammon, H. M., W. Liermann, D. Frieten, and C. Koch. 2020. Review: Importance of colostrum supply and milk feeding intensity on gastrointestinal and systemic development in calves. Animal 14(S1):s133–s143. https://doi.org/10.1017/S1751731119003148.

Hammon, H. M., J. Steinhoff-Wagner, J. Flor, U. Schönhusen, and C. C. Metges. 2013. Lactation Biology Symposium: Role of colostrum and colostrum components on glucose metabolism in neonatal calves. J. Anim. Sci. 91:685–695. https://doi.org/10.2527/jas.2012-5758.

Hernandez, D., D. V. Nydam, S. M. Godden, L. S. Bristol, A. Kryzer, J. Ranum, and D. Schaefer. 2016. Brix refractometry in serum as a measure of failure of passive transfer compared to measured immunoglobulin G and total protein by refractometry in serum from dairy calves. Vet. J. 211:82–87. https://doi.org/10.1016/j.tvjl.2015.11.004.

Jaster, E. H. 2005. Evaluation of quality, quantity, and timing of colostrum feeding on immunoglobulin G_1 absorption in Jersey calves. J. Dairy Sci. 88:296–302. https://doi.org/10.3168/jds.S0022-0302(05)72687-4.

Jones, C. M., R. E. James, J. D. Quigley III, and M. L. McGilliard. 2004. Influence of pooled colostrum or colostrum replacement on IgG and evaluation of animal plasma in milk replacer. J. Dairy Sci. 87:1806–1814. https://doi.org/10.3168/jds.S0022-0302(04)73337-8.

Kehoe, S. I., B. M. Jayarao, and A. J. Heinrichs. 2007. A survey of bovine colostrum composition and colostrum management practices on Pennsylvania dairy farms. J. Dairy Sci. 90:4108–4116. https://doi.org/10.3168/jds.2007-0040.

King, A., M. Chigerwe, J. Barry, J. P. Murphy, M. C. Rayburn, and E. Kennedy. 2020. Short communication: Effect of feeding pooled and nonpooled high-quality colostrum on passive transfer of immunity, morbidity, and mortality in dairy calves. J. Dairy Sci. 103:1894–1899. https://doi.org/10.3168/jds.2019-17019.

Lago, A., M. Socha, A. Geiger, D. Cook, N. Silva-del-Río, C. Blanc, R. Quesnell, and C. Leonardi. 2018. Efficacy of colostrum replacer versus maternal colostrum on immunological status, health, and growth of preweaned dairy calves. J. Dairy Sci. 101:1344–1354. https://doi.org/10.3168/jds.2017-13032.

Lombard, J., N. Urie, F. Garry, S. Godden, J. Quigley, T. Earleywine, S. McGuirk, D. Moore, M. Branan, M. Chamorro, G. Smith, C. Shivley, D. Catherman, D. Haines, A. J. Heinrichs, R. James, J. Maas, and K. Sterner. 2020. Consensus recommendations on calf- and herd-level passive immunity in dairy calves in the United States. J. Dairy Sci. 103:7611–7624. https://doi.org/10.3168/jds.2019-17955.

Marnila, P., and H. Korhonen. 2011. Milk: Colostrum A2. Pages 591–597 in Encyclopedia of Dairy Sciences. 2nd ed. J. W. Fuquay, ed. Academic Press.

McGrath, B. A., P. F. Fox, P. L. H. McSweeney, and A. L. Kelly. 2016. Composition and properties of bovine colostrum: A review. Dairy Sci. Technol. 96:133–158. https://doi.org/10.1007/s13594-015-0258-x.

McGuirk, S. M., and M. Collins. 2004. Managing the production, storage, and delivery of colostrum. Vet. Clin. North Am. Food Anim. Pract. 20:593–603. https://doi.org/10.1016/j.cvfa.2004.06.005.

Moran, J. 2002. Calf Rearing: A Practical Guide. Landlinks Press.

Morin, D. E., S. V. Nelson, E. D. Reid, D. W. Nagy, G. E. Dahl, and P. D. Constable. 2010. Effect of colostral volume, interval between calving and first milking, and photoperiod on colostral IgG concentrations in dairy cows. J. Am. Vet. Med. Assoc. 237:420–428. https://doi.org/10.2460/javma.237.4.420.

Morrill, K. M., K. E. Robertson, M. M. Spring, A. L. Robinson, and H. D. Tyler. 2015. Validating a refractometer to evaluate immunoglobulin G concentration in Jersey colostrum and the effect of multiple freeze–thaw cycles on evaluating colostrum quality. J. Dairy Sci. 98:595–601. https://doi.org/10.3168/jds.2014-8730.

Oliveira, L. C. C., S. Borchardt, W. Heuwieser, E. Rauch, M. Erhard, and F. Sutter. 2019. Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves. J. Dairy Sci. 102:557–566. https://doi.org/10.3168/jds.2018-15006.

Parish, S. M., J. W. Tyler, T. E. Besser, C. C. Gay, and D. Krytenberg. 1997. Prediction of serum IgG1 concentration in Holstein calves using serum gamma glutamyltransferase activity. J. Vet. Intern. Med. 11:344–347. https://doi.org/10.1111/j.1939-1676.1997.tb00478.x.

Phipps, A. J., D. Beggs, A. Murray, P. Mansell, and M. Pyman. 2018. A survey of northern Victorian dairy farmers to investigate dairy calf management: Colostrum feeding and management. Aust. Vet. J. 96:101–106. https://doi.org/10.1111/avj.12683.

Pithua, P., S. S. Aly, D. M. Haines, J. D. Champagne, J. R. Middleton, and S. E. Poock. 2013. Efficacy of feeding a lacteal-derived colostrum replacer or pooled maternal colostrum with a low IgG concentration for prevention of failure of passive transfer in dairy calves. J. Am. Vet. Med. Assoc. 243:277–282. https://doi.org/10.2460/javma.243.2.277.

Poulsen, K. P., A. L. Foley, M. T. Collins, and S. M. McGuirk. 2010. Comparison of passive transfer of immunity in neonatal dairy calves fed colostrum or bovine serum-based colostrum replacement and colostrum supplement products. J. Am. Vet. Med. Assoc. 237:949–954. https://doi.org/10.2460/javma.237.8.949.

Priestley, D., J. H. Bittar, L. Ibarbia, C. A. Risco, and K. N. Galvão. 2013. Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. J. Dairy Sci. 96:3247–3256. https://doi.org/10.3168/jds.2012-6339.

Quigley, J. D., A. Lago, C. Chapman, P. Erickson, and J. Polo. 2013. Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. J. Dairy Sci. 96:1148–1155. https://doi.org/10.3168/jds.2012-5823.

Quigley, J. D., R. E. Strohbehn, C. J. Kost, and M. M. O'Brien. 2001. Formulation of colostrum supplements, colostrum replacers and acquisition of passive immunity in neonatal calves. J. Dairy Sci. 84:2059–2065. https://doi.org/10.3168/jds.S0022-0302(01)74650-4.

Schiessler, G., A. Nussbaum, H. M. Hammon, and J. W. Blum. 2002. Calves sucking colostrum and milk from their dams or from an automatic feeding station starting in the neonatal period: Metabolic and endocrine traits and growth performance. Anim. Sci. 74:431–444. https://doi.org/10.1017/S1357729800052589.

Trotz-Williams, L. A., K. E. Leslie, and A. S. Peregrine. 2008. Passive immunity in Ontario dairy calves and investigation of its association with calf management practices. J. Dairy Sci. 91:3840–3849. https://doi.org/10.3168/jds.2007-0898.

Vandeputte, S., J. Detilleux, and F. Rollin. 2011. Comparison of four refractometers for the investigation of the passive transfer in beef calves. J. Vet. Intern. Med. 25:1465–1469. https://doi.org/10.1111/j.1939-1676.2011.00816.x.

Weaver, D. M., J. W. Tyler, D. C. VanMetre, D. E. Hostetler, and G. M. Barrington. 2000. Passive transfer of colostral immunoglobulins in calves. J. Vet. Intern. Med. 14:569–577. https://doi.org/10.1111/j.1939-1676.2000.tb02278.x.

Williams, D. R., P. Pithua, A. Garcia, J. Champagne, D. M. Haines, and S. S. Aly. 2014. Effect of three colostrum diets on passive transfer of immunity and preweaning health in calves on a California dairy following colostrum management training. Vet. Med. Int. 2014:698741. https://doi.org/10.1155/2014/698741.

Yang, M., Y. Zou, Z. H. Wu, S. L. Li, and Z. J. Cao. 2015. Colostrum quality affects immune system establishment and intestinal development of neonatal calves. J. Dairy Sci. 98:7153–7163. https://doi.org/10.3168/jds.2014-9238.

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Colostrum source and passive immunity transfer in dairy bull calves

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SUPPLEMENTARY FILE S1

Supplementary Methods

**** Bull calf selection and birth measurements.*** At birth, all birth information was recorded including calving difficulty score (Supplementary Table 1), calf ID, cow ID, and time of birth. Calves were taken from dam after licked clean (about 30 min after birth), but not allowed to suckle and scored for calf vigour (1 = weak, 2 = lazy, 3 = vigorous, 4 = very vigorous) and health (Supplementary Figure 1). The calves were randomly allocated to 3 groups in 6 blocks. The first calf was allocated in Group A and tagged as A1 and the first calves allocated in Groups B and C were tagged as B1 and C1, respectively. Three calves (A1, B1 and C1) were placed in the same pen. Only the Group A cows were tagged with necklace as number 1 to 12, sprayed with paint to be distinguished from other cows on the dairy, and kept for three days postpartum for milking. Newborn calves were weighed by using portable animal weight scales and crate (kg), and measured for girth by using tape measure (cm). Blood samples were taken and the calves given their first feeding of colostrum within 4 hours of birth.

****Calf feeding.*** Pairs of Group A and B calves were fed identical colostrum from the same milking of the dam of the Group A calf. The Group A dams were first milked within 2 hours postpartum and then milked again within 10 hours postpartum. The Group A and B calves were fed 5% of their birth weight within 4 hours after birth and another 5% of their birth weight within 12 hours of birth. Thereafter, the pairs of Group A and B calves were fed identical

colostrum from the same milking of the dam of the Group A calf twice per day (5% of their birth weight/feeding) for 3 days, and then were fed bulk tank milk twice per day (2 L/calf/feeding) for 4 days. The Group C calves were only fed 1 bottle of "pooled" colostrum for their first feeding (2 L) and then were fed bulk tank milk twice per day (2 L/calf/feeding) for 7 days. The "pooled" colostrum was the colostrum and transition milk collected from the other cows on the dairy 0-4 days postpartum. All calves were fed by nipple bottles until 3 days after birth and nipple buckets from day 4 to 7 after birth. All calves were given *ad lib* access to calf concentrate, lucerne or medic, and water from the beginning.

Colostrum, pooled colostrum and bulk tank milk samples. The Group A dams were first milked using a portable milking machine and then twice daily in the dairy. The colostrum and transition milk was collected for 3 days postpartum and the volume was measured. Dam colostrum was dispensed into bottles for 2 calves (Group A and B calves) based on calf birth weight (5% birth weight per feed) and each bottle clearly marked for the Group A and Group B calves with tags. Pooled colostrum was collected from the other dairy cows 0-4 days postpartum once per day for the first feeding of the Group C calves. The bulk tank milk was collected from dairy cows more than 5 days postpartum twice daily for feeding the Group C calves from their second feeding until day 7 after birth and for feeding the Group A and B calves from day 4 to 7 after birth. The samples of dam colostrum, pooled colostrum and bulk tank milk were collected into sterile 50 mL-tubes and total solids were measured by using Brix refractometer immediately after milking. The samples were frozen at -20^{0}C, transported on dry ice and stored at -80^{0}C for remaining analyses.

Calf blood sample samples. Calf blood was collected at day 0 (within 4 hours after birth, before any feeding) and day 1, 2, 3 and 7 after birth. The blood samples were taken into 2 red cap

vacutainer tubes (no coagulant) for serum and 2 green vacutainer tubes (heparin) for plasma. The glucose concentration was measured in whole blood immediately after blood collection. Total protein was measured by refractometry immediately after serum collection. The remaining serum and plasma were frozen at -20°C, transported on dry ice and stored at -80^{0}C for further analyses.

Calf health measurements. Calf health was monitored from birth until 7 days after birth by Dr Rebel Skirving from Gambier Vets, Mount Gambier, SA 5290, Australia. All illnesses were recorded including no or slow drinking, coughing, diarrhea, fever, and respiratory problems. If symptoms appeared, then these symptoms and temperatures were monitored twice daily, and treated as required.

Supplementary Tables

Supplementary Table S1. Calving ease scoring system (6 point scale).

Score	Code	Description
1	Unassisted	Cow calved unassisted/no difficulty
2	Easy pull	One person without mechanical assistance
3	Hard pull	Two people without mechanical assistance One person with mechanical assistance
4	Surgical assistance	Veterinary intervention required
5	Mal-presentation	E.g. breech
6	Elective surgical	Surgical removal of calf before cow had opportunity to calve

Supplementary Table S2. Descriptive statistics and comparison of the components in calf blood within 7 days after birth (Least Squares Means with Standard Errors (LSM ± SE), letter superscripts indicate significant differences of P < 0.05).

Component [1]	Time point [2]	Group A (n=12)	Group B (n=12)	Group C (n=11)	All calves [3] (n = 35)
Glucose (mmol/L)	Day 0	3.8 ± 0.3	4.1 ± 0.3	3.6 ± 0.3	3.8^{x} ± 0.2
	Day 1	6.8 ± 0.3	6.6 ± 0.3	7.3 ± 0.3	6.9^{z} ± 0.2
	Day 2	6.5 ± 0.3	6.4 ± 0.3	6.8 ± 0.3	6.6^{z} ± 0.2
	Day 3	6.2 ± 0.3	6.4 ± 0.3	6.6 ± 0.3	6.5^{yz} ± 0.2
	Day 7	5.7 ± 0.3	6.0 ± 0.3	6.2 ± 0.3	6.0^{y} ± 0.2
GGT (U/L)	Day 0	10.8 ± 155.8	11.0 ± 155.8	13.3 ± 162.8	11.7^{x} ± 94.8
	Day 1	1511.0^{a} ± 155.8	1580.1^{a} ± 155.8	783.1^{b} ± 162.8	1271.4^{y} ± 94.8
	Day 7	234.8 ± 155.8	231.0 ± 155.8	146.4 ± 162.8	200.8^{x} ± 94.8
TP-R (g/L)	Day 0	40.5 ± 1.9	39.6 ± 1.9	41.1 ± 1.9	40.4^{x} ± 1.2
	Day 1	60.9^{a} ± 1.9	59.3^{a} ± 1.9	52.4^{b} ± 1.9	57.1^{y} ± 1.2
	Day 2	60.0^{a} ± 1.9	58.6^{ab} ± 1.9	54.4^{b} ± 1.9	57.3^{y} ± 1.2
	Day 3	60.2^{a} ± 1.9	57.8^{ab} ± 1.9	53.5^{b} ± 1.9	56.8^{y} ± 1.2
	Day 7	57.8^{a} ± 1.9	55.5^{ab} ± 1.9	51.8^{b} ± 1.9	54.7^{y} ± 1.2
TP-B (g/L)	Day 0	55.0 ± 3.3	52.1 ± 3.3	50.1 ± 3.4	53.2^{x} ± 1.9
	Day 1	78.6 ± 3.3	74.5 ± 3.3	76.6 ± 3.4	75.8^{z} ± 1.9
	Day 2	64.1 ± 3.3	57.7 ± 3.3	63.3 ± 3.4	61.2^{y} ± 1.9
	Day 3	76.8 ± 3.3	70.9 ± 3.4	68.4 ± 3.4	71.7^{z} ± 2.0
	Day 7	62.1 ± 3.3	64.0 ± 3.3	65.1 ± 3.4	63.1^{y} ± 1.9
IgG (g/L)	Day 0	0.3 ± 2.9	0.3 ± 2.9	0.2 ± 3.0	0.3^{x} ± 2.0
	Day 1	27.9^{a} ± 2.9	26.4^{a} ± 2.9	15.5^{b} ± 3.0	23.4^{z} ± 2.0
	Day 2	35.0^{a} ± 2.9	25.7^{b} ± 2.9	15.5^{c} ± 3.0	25.1^{z} ± 2.0
	Day 3	23.6^{a} ± 2.9	19.5^{ab} ± 3.0	11.8^{b} ± 3.0	18.1^{y} ± 1.5
	Day 7	36.7^{a} ± 2.9	29.0^{a} ± 2.9	14.7^{b} ± 3.0	26.5^{z} ± 2.0

[a,b,c] LSM within each time point with a different superscript letter are significantly different (P < 0.05)

[x,y,z] LSM within an "All calves" column with a different superscript letter are significantly different (P < 0.05)

[1] GGT = gamma-glutamyl transferase; TP-R = Total protein by refractometer; TP-B = Total protein by Bradford assay; IgG = immunoglobulin G;

[2] Day 3, Group B had 11 samples instead of 12 samples.

[3] All calves = Group A + Group B + Group C calves; Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed one bottle of pooled colostrum

Supplementary Table S3. Frequency and proportion of calves in each Group categorized by serum IgG levels on day 2 after birth (Lombard et al., 2020). [1,2]

	Frequency			Proportion (%)			P-values of pairwise comparisons		
Category [1]	**Group A (n = 12)**	**Group B (n = 12)**	**Group A (n = 12)**	**Group B (n = 12)**	**Group C (n = 11)**	**Group C (n = 11)**	**Groups A & B**	**Groups A & C**	**Groups B & C**
Excellent	9	8	75.0 [a]	66.7 [a]	9.1 [b]	1	1.000	0.003	0.009
Good	1	0	8.3	0.0	18.2	2	1.000	0.590	0.217
Fair	2	3	16.7 [b]	25.0 [ab]	63.6 [a]	7	1.000	0.036	0.099
Poor	0	1	0.0	8.3	9.1	1	1.000	0.478	1.000

[a,b] Means within a row of a category proportion with different superscripts differ ($P < 0.01$), comparing pairwise by using 2 x 2 tables with Fisher's exact test.

[1] Categories based on Lombard et al. (2020) where calf serum immunoglobulin G (IgG) levels of ≥ 25.0, 18.0–24.9, 10.0–17.9, and < 10 g/L of IgG at 24-48 hours are classified as excellent, good, fair, and poor, respectively.

[2] Group A = calves fed own dam colostrum; Group B = calves fed foster cow colostrum; Group C = calves fed one bottle of pooled colostrum.

Supplementary Figures

Table 1 **University of Guelph: Calf vitality score sheet**

Visual Appearance				
1. Meconium Staining	Normal: no staining	Slight: around anal/tail head area	Moderate: extending over body	Severe: completely covered
	0	1	2	3
2. Tongue/Head	Normal (no swelling, tongue not protruding)	Tongue protruding but not swollen	Tongue protruding and swollen	Head and tongue swollen, tongue protruding
	0	1	2	3
Initiation of Movement				
3. Calf movement	Taken within:			
	0 - 30 min	30 min - 1.5 hr	1.5 hr - 3 hr	> 3 hr
	Standing/ walking	Attempts to stand	Sternal	On side, no efforts to rise
	0	1	2	3
General Responsiveness				
4. Head shake in response to straw in nasal cavity	Shakes head vigorously	Moves head away	Twitches or flinches	Does not respond
	0	1	2	3
5. Tongue Pinch	Actively withdraws tongue	Attempts to withdraw	Twitches tongue	Does not respond
	0	1	2	3
6. Eye Reflex (in response to touching eyeball)	Actively blinks and closes eye	Slow to blink	Does not respond	
	0	1	2	
Oxygenation				
7. Mucous Membrane Colour	Bright Pink	Light Pink	Brick Red	White/blue
	0	1	2	3
8. Length of Tongue*	< 50 mm	50 - 61 mm	> 62 mm	
	2	0	1	
Rates				
9. Heart Rate**	< 80 bpm	80 - 100 bpm	> 100 bpm	
	2	0	1	
10. Respiration (approximate)	SLOW (~ 24 rrpm)	NORMAL (~ 24 - 36 rrpm)	FAST (~ > 36 rrpm)	
	1	0	2	

***Length of Tongue:** Measure from lips. Record this measurement only if within 5 minutes of calving.
****Heart Rate:** Put your hand on the calf's chest. Take pulse for 15 seconds then multiply by 4.

Supplementary Figure S1. Scoring system to assess calf health at birth. (Based on the Calf Vigor Scoring System from the University of Guelph, Canada; https://www.progressivedairycanada.com/downloads/2012/09/0912ca_lee_vigor.pdf, accessed February 2018).

Chapter 4
Predicting colostrum and calf blood components based on refractometry

Chapter 4. Predicting colostrum and calf blood components based on refractometry

This chapter is a paper published in Journal of Dairy Research:

Do T. Hue, John L. Williams, Kiro R Petrovski and Cynthia D. K. Bottema. **Predicting colostrum and calf blood components based on refractometry.** *Journal of Dairy Research 88(2):194-200. DOI:10.1017/S0022029921000340.*

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4.1. Authorship statement

Statement of Authorship

Title of Paper	**Predicting colostrum and calf blood components based on refractometry**
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	**Do T. Hue**		
Contribution to the Paper	Designed and prepared for animal experiment and sample collection, collected samples, optimised protocols and assayed samples, statistically analysed data, drafted and edited manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19-02-2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.
iv.

Name of Co-Author	John L. Williams		
Contribution to the Paper	Designed and prepared for animal experiment, discussed data analysis and edited manuscript.		
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Contribution to the Paper	Designed and prepared for animal experiment, collected samples, supervised data analysis, and edited manuscript.		
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Contribution to the Paper	Designed and prepared for animal experiment and sample collection, collected samples, developed protocols for different assays, discussed data analysis and edited manuscript.		
Signature		Date	20-2-21

Please cut and paste additional co-author panels here as required.

4.2. Original article

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Predicting colostrum and calf blood components based on refractometry

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Abstract

Provision of good quality colostrum is essential for the passive immunity and nutrition of newborn calves. In order to better predict the quality of colostrum and the transfer of passive immunity, the relationships between colostrum components and between calf serum components were examined in this study. Samples of bulk tank milk, colostrum pooled from several cows 0–4 d postpartum, and colostrum collected from individual cows twice daily for 3 d postpartum were compared. With the exception of fat percentage, there were strong correlations between the levels of the components in the pooled colostrum and in the individual cow colostrum collected 0–1 d postpartum. The correlations between total solids as measured by Brix refractometry and total protein, immunoglobulin G (IgG), lactose % and protein % in colostrum within 1 d postpartum and pooled colostrum were 0.92, 0.90, −0.88 and 0.98, respectively. These high correlations enabled these colostrum components to be accurately predicted from Brix % and therefore, the volume of colostrum required to feed neonate calves can be optimised based on Brix refractometry to avoid failure of passive immunity transfer. To assess whether the components obtained from colostrum were correlated in calf blood, newborn calves were separated from their dams before suckling and blood sampled before feeding (day 0), and on days 1 and 7, after receiving colostrum or milk twice a day. The correlations between glucose, total protein, IgG, and gamma-glutamyl transferase (GGT) levels in the calf blood were lower than the correlations observed between the colostrum components. The highest correlation was between serum protein measured by refractometer and serum IgG within one week postpartum. GGT activity was not a good indicator of serum IgG levels. However, serum protein refractometer measurements predicted serum IgG level with high accuracy, providing an on-farm test to determine that calves have received sufficient passive immunity and colostrum components.

The morphology of bovine placenta prevents the transfer of immunoglobulins from dam to foetus during pregnancy, therefore, colostrum is essential for the transfer of passive immunity to newborn calves (Barrington and Parish, 2001; Castro *et al.*, 2011). Failure of passive immunity transfer (FPIT) may occur if a calf does not receive enough good-quality colostrum soon after birth and absorb sufficient immunoglobulins, specifically immunoglobulin G (IgG). Failure of passive immunity transfer is associated with increased calf morbidity and mortality (Barrington and Parish, 2001; Moran, 2002; Furman-Fratczak *et al.*, 2011; Vandeputte *et al.*, 2011), and avoiding FPIT is crucial to optimise calf health and productivity.

Failure of passive immunity transfer can be detected in calves by measuring serum IgG or serum protein or by estimating the total serum protein using refractometry. The generally accepted FPIT thresholds at 24 h postpartum are less than 10 g/l of serum IgG (Weaver *et al.*, 2000; Godden *et al.*, 2019; Oliveira *et al.*, 2019) or less than 52 g/l of serum total protein (TP) (McGuirk and Collins, 2004; Hernandez *et al.*, 2016; Cuttance *et al.*, 2017*b*). Other authors have estimated a Brix refractometry threshold for FPIT as less than 8.1–8.8% for calf serum at 24–48 h postpartum (Hernandez *et al.*, 2016; Cuttance *et al.*, 2017*a*; Godden *et al.*, 2019). It has been suggested that FPIT can be also inferred by the gamma-glutamyl transferase (GGT) levels in the calf blood with levels less than 200 IU/l of GGT at 24 h postpartum indicating FPIT (Perino *et al.*, 1993; Parish *et al.*, 1997).

To avoid FPIT, it is recommended that calves receive approximately 10% of their body weight of good quality colostrum (>50 g/l of IgG) within few hours of birth (McGuirk and Collins, 2004; Bartier *et al.*, 2015; Godden *et al.*, 2019). By quantifying the components in the colostrum before feeding the calf, the quality can be assessed so that a sufficient volume can be given to avoid FPIT. There are many laboratory-based methods for quantifying IgG to determine colostrum quality (Gapper *et al.*, 2007). However, these laboratory-based methods are time consuming, costly, and difficult to apply routinely as samples need to be transported to the laboratory.

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On-farm techniques for estimating the IgG concentration in samples that provide results rapidly can be used to manage the colostrum fed to neonatal calves and to monitor the transfer of colostrum components to the calves. Two such techniques are Brix refractometry for colostrum samples (Quigley *et al.*, 2013; Morrill *et al.*, 2015; Cabral *et al.*, 2016) and digital serum refractometry for blood samples (Vandeputte *et al.*, 2011; Deelen *et al.*, 2014; Hernandez *et al.*, 2016). Brix refractometry values between 18 and 23% are considered to be equivalent to 50 g/l IgG, the threshold for good quality colostrum (Bielmann *et al.*, 2010; Quigley *et al.*, 2013; Bartier *et al.*, 2015; Morrill *et al.*, 2015).

In addition to providing passive immunity, colostrum contains all the nutrients that are essential for the survival of the neonate calf, including proteins, lactose, fat, minerals, and vitamins (McGrath *et al.*, 2016; Godden *et al.*, 2019). However, colostrum quality is rarely considered in terms of these other components, and their assessment on-farm has not been studied. In the present study, the components of colostrum, milk and calf blood were quantified by both refractometry and laboratory assays and their correlations determined. The data were then used to develop linear regression models to determine the predictability of colostrum component levels or blood component levels based on refractometry measurements or the other components.

Materials and methods

Sample collection

The animal work was performed at a commercial dairy farm with over 1800 cows located in Mount Gambier, South Australia. All animal experimental work was approved by the University of Adelaide Animal Ethics Committee (approval number S-2017-060). Twelve Holstein-Friesian cows were milked within 2 h postpartum prior to being suckled (day 0), and then twice daily for 3 d (days 1, 2 and 3). The colostrum and transition milk samples were collected every 24 h during this time and are referred to as 'individual cow colostrum'. Twenty five (25) samples of bulk tank milk were also collected. The 'bulk tank milk' was from the all cows on the dairy farm at 5 d or more postpartum. In addition, six samples were collected from the pooled colostrum and transitional milk of the other cows on the farm at 0–4 d postpartum. These samples are referred to as 'pooled colostrum' to distinguish from the 'bulk tank milk' and 'individual cow colostrum' samples. Total solids in the colostrum and milk samples were measured immediately after collection by Brix refractometry (digital refractometer DBR-1, Star Instrument with a measurement range from Brix 0–50%). The colostrum and milk samples were aliquoted into sterile 50 ml-tubes, frozen at −20°C, and stored at −80°C until analysed.

Thirty-five Holstein-Friesian bull calves born in February and March 2018 were divided into 2 groups using block randomisation by birth order. The first group ($n = 24$) was fed colostrum from the individual cows (5% of body birth weight/feed) harvested at the milking just prior to feeding twice a day for 3 d, and then fed bulk tank milk for 4 d. The second group of calves ($n = 11$) was fed one bottle of pooled colostrum at the first feed (1.7 litres), and then fed bulk tank milk twice a day for 7 d.

Blood samples were collected into 6 ml – BD vacutainers without anticoagulant and BD vacutainers with heparin from all calves within 4 h after birth before their first feed (day 0) and at day 1 and day 7 postpartum. Glucose concentration in whole calf blood was measured using a glucose meter immediately after collection (Accu-check Performa, Roche Diabetes Care, Basel, Switzerland). The blood was then separated immediately by centrifugation (2000 ***g***) for 15 min at room temperature to obtain serum and plasma. The total protein was measured in serum immediately after centrifugation using a serum protein refractometer (TP-R) (ATAGO Digital Refractometer with a protein measurement range from 0 to 12 g/100 ml). The serum and plasma samples were frozen at −20°C and stored at −80°C until analysed.

Colostrum and calf blood component measurements

The IgG concentration in the individual cow colostrum, pooled colostrum, bulk tank milk samples and calf serum samples was quantified by enzyme-linked immunosorbent assay (ELISA) using two bovine IgG specific antibodies (Life Technologies, USA) as described in the Supplementary File. Total protein in the individual cow colostrum, pooled colostrum, bulk tank milk and calf serum samples was assayed in a 96 well-plate using a Quick Start Bradford Protein assay kit (TP-B) (Bio-Rad Laboratories, Inc., USA) following the manufacturer's instructions as detailed in the Supplementary File.

Percentages of total protein, lactose and fat concentration in the thawed colostrum and milk samples were determined using a Fourier-transform mid-range infrared analyser (FT-MIR spectrometer, Foss Analytics) at the National Herd Development Co-operative (Kyabram VIC 3620, Australia). Calf plasma was analysed for gamma-glutamyl transferase (GGT) levels using a kinetic colour test with a Beckman Coulter analyser at the Veterinary Diagnostic Laboratory, the University of Adelaide, South Australia.

Statistical analysis

Statistical analyses were conducted in R (R version 3.6.3) and independently verified in SAS (Edition 9.4, SAS Institute, Cary, NC, USA). Descriptive statistics for the total solids, total protein, IgG, protein %, fat %, and lactose % in colostrum, and the glucose concentration, the total protein, IgG and GGT in calf blood were calculated and the correlations between the various components in the colostrum and milk samples and in the calf blood samples were determined. The correlations were categorised as follows: negligible ($r < 0.30$); low ($r = 0.30$ to 0.49); moderate ($r = 0.50$ to 0.69); high ($r = 0.70$ to 0.89); or very high ($r \geqslant 0.90$) (Wilm *et al.*, 2018). To evaluate the association between any two components, simple linear regression analysis was performed using linear models for two continuous variables. The accuracy of the predictions was estimated using the coefficient of determination (R^2). The coefficient of determination (R^2), or goodness of fit, has a value between 0.0 and 1.0, where a value of 1.0 indicates a perfect fit and a value of 0.0 indicates that the model fails to accurately predict the data.

Results and discussion

Concentration of colostrum components

The constituents of individual cow colostrum collected days 0–3 postpartum, pooled colostrum and bulk tank milk were compared. The pooled colostrum replicated the dairy's practice of mixing colostrum and transition milk from all cows 0 to 4 d postpartum to feed to the newborn calves.

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Table 1. Descriptive statistics of different colostrum and milk components

Sample	IgG (g/l)	Total solids (Brix %)	TP-B (g/l)	Protein (%)	Fat (%)	Lactose (%)
Cow day 0 ($n = 12$)						
Mean ± SE	184.4 ± 20.4	25.4 ± 1.4	170.8 ± 16.0	16.6 ± 0.9	4.6 ± 0.4	1.9 ± 0.2
Range	84.7–330.7	18.5–34.0	88.7–260.5	11.0–21.2	2.2–6.6	1.1–3.0
Cow day 1 ($n = 11$)						
Mean ± SE	29.4 ± 6.6	12.2 ± 0.6	51.2 ± 5.8	6.3 ± 0.5	3.2 ± 0.4	4.2 ± 0.1
Range	5.5–63.5	9.0–16.1	23.6–80.3	3.9–8.7	1.6–6.1	3.7–4.9
Cow day 2 ($n = 12$)						
Mean ± SE	4.9 ± 0.6	10.6 ± 0.2	34.4 ± 2.1	–	–	–
Range	2.3–9.4	8.8–12.2	19.1–44.7	–	–	–
Cow day 3 ($n = 12$)						
Mean ± SE	2.7 ± 0.6	10.5 ± 0.3	34.4 ± 2.1	–	–	–
Range	1.2–8.0	8.7–11.9	23.1–51.0	–	–	–
Pooled colostrum ($n = 6$)						
Mean ± SE	90.5 ± 9.6	19.4 ± 2.1	88.7 ± 11.6	12.4 ± 1.3	5.4 ± 0.9	2.4 ± 0.1
Range	69.7–131.6	12.3–25.9	54.3–127.8	–	–	–
Bulk tank milk ($n = 25$)						
Mean ± SE	0.3 ± 0.1	8.9 ± 0.2	28.6 ± 2.0	–	–	–
Range	0.2–0.7	7.6–12.0	7.1–50.1	–	–	–

IgG = immunoglobulin G; TP-B = total protein measured by Bradford assay; Cow = individual cow colostrum at the time point (day) indicated; Pooled colostrum = colostrum and transition milk pooled from cows days 0–4 postpartum; Bulk tank milk = milk collected from cows more than 4 d postpartum.

The IgG concentration in the individual cow colostrum decreased dramatically from a mean value of 184.4 g/l at day 0 to 2.7 g/l by day 3 (Table 1). The level of IgG in the pooled colostrum was relatively high (90.5 g/l), but was very low in the bulk tank milk (0.3 g/l). Both the individual cow colostrum and pooled colostrum had IgG concentrations that were higher than the threshold of 50 g/L recommended to avoid FPIT (McGuirk and Collins, 2004; Baumrucker *et al.*, 2014; Bartier *et al.*, 2015).

Immediately postpartum, the level of total solids in the individual cow colostrum was high (25.4 ± 1.4 Brix %), but declined rapidly by day 1 (12.2 ± 0.6 Brix %). The Brix % observed in all the individual cow colostrum samples immediately postpartum was higher than the published thresholds of 18–23% for good quality colostrum. This is presumably because the colostrum was obtained from the individual cows within 2 h postpartum and the cows were multiparous. The mean Brix % was also high in pooled colostrum (19.4 ± 2.1%), although two of the samples were below 18%, the Brix threshold for good quality colostrum (Morrill *et al.*, 2015).

Total protein in the individual cow colostrum, as measured by Bradford assay and FT-MIR, had the same trend as the levels of IgG and total solids, with very high levels on day 0 (170.8 ± 16.0 g/l, 16.6 ± 0.9%), a sharp decline by day 1 (51.2 ± 5.8 g/l, 6.3 ± 0.5%) and a constant level thereafter (34.4 ± 2.1 g/l at days 2 and 3). Total protein levels in the pooled colostrum were roughly half of the values of the individual cow colostrum between days 0 and 1 (88.7 ± 11.6 g/l, 12.4 ± 1.3%), and the level of total protein in the bulk tank milk was similar to the day 2 and day 3 individual cow colostrum (28.6 ± 2.0 g/l).

The fat concentration in the individual cow colostrum at day 0 was slightly lower than the pooled colostrum (4.6 ± 0.4% and 5.4 ± 0.9% respectively) and declined by day 1 (3.2 ± 0.4%). The decline in colostrum fat percentage one day postpartum was also observed by Dunn *et al.* (2017), who found the fat percent in colostrum decreased from 7.0% to 6.0% within 24 h postpartum and from 6.7% to 3.9% between the first and third milking postpartum (reviewed by Godden *et al.* (2019)).

In contrast to the other components, the lactose concentration in the individual cow colostrum increased from day 0 to day 1 (1.9 ± 0.2% and 4.2 ± 0.1%, respectively). The pooled colostrum was intermediate (2.4 ± 0.1%). These lactose levels were comparable to those obtained in other studies where lactose was found to be low in colostrum but to increase in the milk as lactation is established (Kehoe *et al.*, 2007).

Correlations between colostrum components

The correlations between components in the individual cow colostrum from days 0 and 1 postpartum were high (Table 2). The correlations between total solids measured by Brix refractometer with total protein, IgG, lactose % and protein % were 0.92, 0.90, −0.88 and 0.98, respectively, for the combined data from the pooled colostrum and the individual cow colostrum days 0 and 1 postpartum samples ($n = 29$) (Table 2).

Correlations between Brix refractometer measurements and colostrum IgG measured by radial immunodiffusion (RID) or turbidimetric immunoassay (TIA), previously reported for day 0 and 1 post-partum, range between 0.64 and 0.75 (Bielmann *et al.*, 2010; Quigley *et al.*, 2013; Bartier *et al.*, 2015; Elsohaby *et al.*, 2017). In the present study, the IgG concentration was measured by sandwich ELISA, which has higher sensitivity and specificity for IgG than other methods, including RID or TIA (Li-Chan

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Table 2. Correlations between components in colostrum and milk

Correlated parameters	Individual cow colostrum				Bulk tank milk	Cow + pooled colostrum
	Day 0 ($n = 12$)	Day 1 ($n = 11$)	Day 2 ($n = 12$)	Day 3 ($n = 12$)	($n = 25$)	($n = 29$)
Total solids and TP-B	0.82	0.80	−0.02	0.50	0.15	0.92
Total solids and IgG	0.80	0.60	0	0.13	0.36	0.90
Total solids and fat %	0.62	0.08	[a]	[a]	[a]	0.54
Total solids and lactose %	−0.84	−0.71	[a]	[a]	[a]	−0.88
Total solids and protein %	0.94	0.90	[a]	[a]	[a]	0.98
TP-B and IgG	0.69	0.76	0.41	0.76	−0.13	0.90
TP-B and fat %	0.43	0.28	[a]	[a]	[a]	0.40
TP-B and lactose %	−0.66	−0.70	[a]	[a]	[a]	−0.82
TP-B and protein %	0.75	0.86	[a]	[a]	[a]	0.91
IgG and fat %	0.58	−0.08	[a]	[a]	[a]	0.40
IgG and lactose %	−0.80	−0.69	[a]	[a]	[a]	−0.88
IgG and protein %	0.84	0.87	[a]	[a]	[a]	0.92
Fat % and lactose %	−0.36	−0.16	[a]	[a]	[a]	−0.46
Fat % and protein %	0.60	−0.09	[a]	[a]	[a]	0.52
Lactose % and protein %	−0.87	−0.81	[a]	[a]	[a]	−0.92

Colostrum is taken d0 or d1 postpartum, milk is bulk tank milk from d5 or later. Total solids measured by Brix refractometer; IgG = immunoglobulin G; TP-B = total protein measured by Bradford assay.
[a]Data not available.

and Kummer, 1997), which may confound direct comparisons of the correlations. Notably the correlations between Brix refractometry and the components of individual cow colostrum from days 2 and 3 and in the bulk tank milk were low or negligible (Table 2). These results are similar to those of Rayburn *et al.* (2019), who found that refractometry was not useful for assessing IgG by the fourth or fifth milking. Therefore, using Brix refractometer or protein levels to estimate IgG in colostrum after 2 d postpartum is not advised.

IgG represents approximately 75% of the protein in colostrum (McGrath *et al.*, 2016; Godden *et al.*, 2019). However, other proteins are also important for calf health, including beta-lactoglobulin, alpha-lactalbumin, lactoferrin and other immunoglobulins (namely, IgA and IgM) (McGrath *et al.*, 2016). Although laboratory assays to measure colostrum components can be expensive and time consuming, they are highly accurate. Among these laboratory assays, the Bradford assay (TP-B) is a relatively easy and inexpensive method to measure total protein. The correlations between total protein, as measured by Bradford assay (TP-B) in the individual cow colostrum at day 0 and 1 postpartum, and IgG or protein % were high and positive ($r = 0.90$ and 0.91, respectively).

There was a strong and highly negative correlation between total protein (TP-B) and lactose ($r = -0.82$) (Table 2). The colostrum lactose concentration was also highly negatively correlated with protein % measured by FT-MIR ($r = -0.92$). This osmotic activity of lactose dilutes other colostrum components, specifically the protein concentration, resulting in an inverse relationship between the levels of lactose and protein in the colostrum. Consequently, the colostrum lactose concentration also had a high negative correlation with IgG concentration ($r = -0.88$), as has been observed by others (Dunn *et al.*, 2017).

Estimating colostrum components using Brix refractometer measurements

The very high correlations observed between each of the components of individual cow colostrum within 1 d postpartum and the pooled colostrum means that the concentration of one component can be potentially used to predict that of the others (Supplementary Figures S1–S4). The exception is fat percentage as it was not strongly correlated with the level of other colostrum components.

Simple linear model equations were used to estimate IgG, protein, and lactose concentrations based on the Brix refractometer measurements of individual cow colostrum day 0 and 1 postpartum and the pooled colostrum ($n = 29$). The very high correlations between the components were reflected in the coefficients of determination (R^2) (Supplementary Figure S1). Importantly, the formula for predicting IgG (IgG (g/l) = −103.1 + 10.9 × Brix %) had good predictive power ($R^2 = 0.80$, $P < 0.001$). Both r and R^2 in the present study were higher than reported other studies such as Bartier *et al.* (2015) (0.64 and 0.43, respectively) and Bielmann *et al.* (2010) (0.73 and 0.53, respectively). This may be a result of the method used to measure the IgG concentration in the present study as the ELISA is more sensitive and accurate than the RID assays used in the other studies.

Both lactose and protein levels in the individual cow colostrum 0–1 d postpartum and pooled colostrum could be estimated with reasonable accuracy from Brix % (Supplementary Figure S1). The lactose concentration could be estimated using the formula, Lactose (%) = 5.7–0.2 × Brix %, with a reasonable goodness of fit ($R^2 = 0.78$, $P < 0.001$). The total protein in colostrum could be estimated from the formula, TP-B (g/l) = −61.6 + 8.8 × Brix %, with a high coefficient of determination ($R^2 = 0.85$, $P < 0.001$).

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The formula predicting the protein % in colostrum from Brix refractometry measurements (Protein % = −2.5 + 0.7 × Brix %) had an even greater coefficient of determination ($R^2 = 0.96$, $P < 0.001$) than the TP-B formula. Therefore, Brix refractometry measurements are a good predictor for not only estimating IgG in colostrum, but also protein and lactose concentrations.

Previous studies suggest calves should receive at least 10% of body weight of 50 g/l of IgG from colostrum within a few hours of birth (McGuirk and Collins, 2004; Godden, 2008; Bartier *et al.*, 2015). As it is difficult to measure IgG on-farm, it has been suggested that calves should receive a large volume of colostrum, equal to 10 to 12% of body weight (3 to 4 litres of colostrum) at their first feed (Moran, 2002; McGuirk and Collins, 2004; Godden *et al.*, 2019). Other authors investigating the transfer of passive immunity to calves fed different colostrum volumes at different times after birth have suggested specific colostrum volumes and specific feeding times (Williams *et al.*, 2014). For example, Osaka *et al.* (2014) recommended that calves consume ⩾3 litres of colostrum with IgG concentration >40 mg/ml within 6 h after birth. However, it is difficult to follow these recommendations as there is a large variation in individual cow colostrum IgG concentrations (Marnila and Korhonen, 2011; Quigley *et al.*, 2013; Dunn *et al.*, 2017), and other variation is inevitable due to factors such as the time of collection, breed and cow management (Bartier *et al.*, 2015; Schneider *et al.*, 2020).

Establishing the colostrum quality using Brix refractometry will enable the volume of colostrum that should be fed to the neonate to avoid FPIT to be more accurately determined. For example, colostrum with a Brix of 20% would have approximately 115 g/l of IgG (Supplementary Table S1), so calves weighing ≤40 kg at birth should receive ~ 2 litres of colostrum of this quality within 4 h postpartum to avoid FPIT. If the Brix % is lower than the recommended threshold of 18 to 23 Brix%, then more colostrum should be provided at the first feed. Based on the prediction equation, a Brix measurement of 14% would be equivalent to 50 g/l of IgG, and the producer would need to feed calves weighing ≤ 40 kg at least 4 litres of the colostrum to ensure the calves received sufficient IgG (Supplementary Table S1).

Calf blood components

Blood samples were taken from the calves prior to the first feed (day 0), and then after receiving the colostrum and milk, on days 1 and 7. The blood samples were analysed for glucose, GGT, total protein and IgG. Before the first feed, all colostrum derived components were at low levels in the calf blood, in particular, GGT and IgG (11.7 IU/l and 0.3 g/l, respectively) (Table 3). These values confirmed that calves had not suckled from their dam prior to sampling (Braun *et al.*, 1982). The concentrations of all components increased after feeding (day 1), but declined by day 7 (Table 3). The exception was the IgG concentration, which was higher at day 7 than day 1.

Correlations between calf blood components

There were negligible or low correlations between the calf blood components in the samples taken before the calves received colostrum (day 0; Table 4). On day 1, the correlations between total protein measured by refractometry (TP-R) and GGT and between GGT and IgG were high ($r = 0.71$ and $r = 0.72$, respectively), and the correlation between TP-R and IgG was moderate ($r = 0.61$). The correlations between TP-R and IgG and between GGT and IgG remained constant up to day 7 ($r = 0.67$ and $r = 0.62$, respectively), but the correlations between other components were substantially lower at later time points. A higher correlation between TP-R and IgG was observed by combining all the calf data from the first week postpartum than for any of the individual days ($r = 0.81$).

The correlation between total protein measured by serum refractometer (TP-R) and IgG for calf blood samples within 7 d postpartum was similar to those reported in other studies, which ranged between from 0.75 to 0.93 (Deelen *et al.*, 2014; Hernandez *et al.*, 2016; Renaud *et al.*, 2018; Wilm *et al.*, 2018). High correlations have been observed between serum IgG and Brix refractometer measurements of serum as well ($r = 0.79$–0.93) (Deelen *et al.*, 2014; Hernandez *et al.*, 2016). These authors also reported a very high correlation between the values from these two different types of refractometers, Brix and digital serum protein ($r = 0.97$–1.0). The estimated FPIT thresholds are 8.1% to 8.5% for the Brix refractometer and 50 to 55 g/l for the serum protein refractometer (Godden *et al.*, 2019).

There was a high correlation between serum IgG and plasma GGT at day 1 ($r = 0.72$), but the correlation was lower by day 7 ($r = 0.62$) (Table 4). These correlations are similar to those reported in a study of calves less than 11 d postpartum ($r = 0.65$–0.68) (Parish *et al.*, 1997). However, much lower correlations

Table 3. Descriptive statistics of calf blood measurements

Days postpartum	Glucose (mmol/l)	GGT (U/l)	TP-R (g/l)	TP-B (g/l)	IgG (g/l)
Day 0 ($n = 35$)					
Mean ± SE	3.8 ± 0.2	11.7 ± 0.7	40.4 ± 0.6	52.5 ± 1.6	0.3 ± 0.1
Range	1.6–6.5	6.2–26.2	34.0–46.0	38.3–80.0	0–1.7
Day 1 ($n = 35$)					
Mean ± SE	6.9 ± 0.2	1305.9 ± 163.5	57.7 ± 1.4	76.6 ± 2.7	23.4 ± 2.1
Range	4.3–10.2	89.5–4034.5	38.0–74.0	50.6–107.1	7.2–58.1
Day 7 ($n = 35$)					
Mean ± SE	6.0 ± 0.1	205.7 ± 22.8	55.1 ± 1.1	63.7 ± 1.5	27.1 ± 2.4
Range	4.7–7.9	25.4–581.6	42.0–70.0	47.8–87.0	7.3–57.5

TP-R = total protein by refractometry; TP-B = total protein by Bradford assay; Day 0 = within 4 h postpartum and before receiving colostrum; Days 1 and 7 after receiving colostrum or milk twice per day.

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Table 4. Correlations between calf blood components at various times postpartum

Correlated parameters	Calf data			
	Day 0 ($n = 35$)	Day 1 ($n = 35$)	Day 7 ($n = 35$)	Days 0, 1, 7 ($n = 105$)
Glucose and TP-R	−0.16	0.07	0.09	0.57
Glucose and TP-B	−0.21	0.01	0.18	0.45
Glucose and IgG	0.05	0.02	−0.11	0.50
Glucose and GGT	−0.03	0.02	0.24	0.45
TP-R and TP-B	0.44	0.17	0.10	0.54
TP-R and IgG	0.17	0.61	0.67	0.81
TP-R and GGT	0.03	0.71	0.46	0.65
TP-B and IgG	0.46	0.50	0.05	0.51
TP-B and GGT	0.07	0.28	0.06	0.55
IgG and GGT	−0.02	0.72	0.62	0.52

TP-R = total protein by refractometry; TP-B = total protein by Bradford assay; Day 0 = within 4 h postpartum but before receiving colostrum; Days 1 and 7 after receiving colostrum or milk twice per day.

have been found in other studies of calves less than 8 d postpartum ($r = 0.398$ and $r = 0.495$) (Jezek *et al.*, 2010; Vandeputte *et al.*, 2011).

Estimating calf blood components using serum refractometry

As the correlations between the calf blood IgG and TP-R and between GGT and TP-R were high, regression models were developed to estimate the serum IgG and plasma GGT based on the serum protein refractometer measurements (Supplementary Figures S3 and S4). To predict calf blood components more accurately, all the data within 7 d postpartum were combined and a formula was generated to predict serum IgG concentration from the protein refractometer measurements, IgG (g/l) = −49.1 + 1.3 × TP-R (g/l), which had a coefficient of determination of $R^2 = 0.65$ ($P < 0.001$, Supplementary Figure S3). The correlation between serum IgG and the protein refractometer measurements was higher in the present study ($r = 0.81$) than that reported by Parish *et al.* (1997) ($r = 0.68$), most likely due to the age differences between the calves (7 d *vs.* 11 d of age, respectively). Hence, the regression model from Parish *et al.* (1997), IgG = −2,562 + total protein (g/dl) × 670, is likely to have an lower goodness of fit for predicting serum IgG than the prediction equation developed here.

Gamma-glutamyl transferase (GGT)

GGT is found in very low concentrations in the blood of newborn calves but is rapidly absorbed from the colostrum before the gut closes, and hence, is a good indicator of colostrum ingestion (Braun *et al.*, 1982; Weaver *et al.*, 2000). The level of GGT has been also proposed as an indicator of passive immunity transfer (Parish *et al.*, 1997; Wilson *et al.*, 1999; Aydogdu and Guzelbektes, 2018). Different GGT thresholds for FPIT have been suggested (Perino *et al.*, 1993; Parish *et al.*, 1997; Hogan *et al.*, 2015; Cuttance *et al.*, 2017*a*), but the threshold is yet to be firmly established (Cuttance *et al.*, 2019).

In order to estimate a FPIT threshold for GGT in the present study, a prediction equation for IgG concentration was generated based on GGT levels at day 1, GGT (U/l) = −3.9 + 55.9 × IgG (g/l) (Supplementary Figure S4). Assuming the FPIT threshold is 10 g/l IgG at day 1 postpartum, the FPIT threshold for GGT level is predicted from this equation to be approximately 555 IU/l. This level is similar to the experimental results from a study by Pekcan *et al.* (2013), who found that calves less than 2 d old with adequate passive immunity transfer had a mean of 578.25 ± 47.45 IU GGT/l. However, the prediction equation based on GGT level at day 1 postpartum only had a coefficient of determination of $R^2 = 0.52$ ($P < 0.001$, Supplementary Figure S4), and this estimate of FTP threshold for GGT is not expected to be highly accurate. In addition, the lower correlations and coefficients of determination indicate that plasma GGT activity was not prognostic of IgG concentration, particularly after day 1.

In conclusion, the results presented here validate both Brix and serum refractometry as good methods to estimate colostrum and calf blood components, respectively. Refractometry can be used to adjust the amount of colostrum fed to ensure calves receive sufficient IgG and nutrients from the colostrum or to predict IgG levels in calves and hence, the risk of FPIT. Nevertheless, other factors, such as the timing of the first feed, calf health and body weight, need to be considered to ensure sufficient transfer of passive immunity.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000340

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References

Aydogdu U and Guzelbektes H (2018) Effect of colostrum composition on passive calf immunity in primiparous and multiparous dairy cows. *Veterinarni Medicina* **63**, 1–11.

Barrington GM and Parish SM (2001) Bovine neonatal immunology. *Veterinary Clinics of North America: Food Animal Practice* **17**, 463–476.

Bartier AL, Windeyer MC and Doepel L (2015) Evaluation of on-farm tools for colostrum quality measurement. *Journal of Dairy Science* **98**, 1878–1884.

Baumrucker CR, Stark A, Wellnitz O, Dechow C and Bruckmaier RM (2014) Short communication: immunoglobulin variation in quarter-milked colostrum. *Journal of Dairy Science* **97**, 3700–3706.

Bielmann V, Gillan J, Perkins NR, Skidmore AL, Godden S and Leslie KE (2010) An evaluation of Brix refractometry instruments for measurement of colostrum quality in dairy cattle. *Journal of Dairy Science* **93**, 3713–3721.

Braun JP, Tainturier D, Laugier C, Bénard P, Thouvenot JP and Rico AG (1982) Early variations of blood plasma gamma-glutamyl transferase in newborn calves – a test of colostrum intake. *Journal of Dairy Science* **65**, 2178–2181.

Cabral RG, Chapman CE, Aragona KM, Clark E, Lunak M and Erickson PS (2016) Predicting colostrum quality from performance in the previous lactation and environmental changes. *Journal of Dairy Science* **99**, 4048–4055.

Castro N, Capote J, Bruckmaier RM and Argüello A (2011) Management effects on colostrogenesis in small ruminants: a review. *Journal of Applied Animal Research* **39**, 85–93.

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Cuttance EL, Mason WA, Denholm KS and Laven RA (2017*a*) Comparison of diagnostic tests for determining the prevalence of failure of passive transfer in New Zealand dairy calves. *New Zealand Veterinary Journal* **65**, 6–13.

Cuttance EL, Mason WA, Laven RA, McDermott J and Phyn CVC (2017*b*) Prevalence and calf-level risk factors for failure of passive transfer in dairy calves in New Zealand. *New Zealand Veterinary Journal* **65**, 297–304.

Cuttance EL, Regnerus C and Laven RA (2019) A review of diagnostic tests for diagnosing failure of transfer of passive immunity in dairy calves in New Zealand. *New Zealand Veterinary Journal* **67**, 277–286.

Deelen SM, Ollivett TL, Haines DM and Leslie KE (2014) Evaluation of a Brix refractometer to estimate serum immunoglobulin G concentration in neonatal dairy calves. *Journal of Dairy Science* **97**, 3838–3844.

Dunn A, Ashfield A, Earley B, Welsh M, Gordon A and Morrison SJ (2017) Evaluation of factors associated with immunoglobulin G, fat, protein, and lactose concentrations in bovine colostrum and colostrum management practices in grassland-based dairy systems in Northern Ireland. *Journal of Dairy Science* **100**, 2068.

Elsohaby I, McClure JT, Cameron M, Heider LC and Keefe GP (2017) Rapid assessment of bovine colostrum quality: how reliable are transmission infrared spectroscopy and digital and optical refractometers? *Journal of Dairy Science* **100**, 1427–1435.

Furman-Fratczak K, Rzasa A and Stefaniak T (2011) The influence of colostral immunoglobulin concentration in heifer calves' serum on their health and growth. *Journal of Dairy Science* **94**, 5536–5543.

Gapper LW, Copestake DE, Otter DE and Indyk HE (2007) Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: a review. *Analytical and Bioanalytical Chemistry* **389**, 93–109.

Godden S (2008) Colostrum management for dairy calves. *Veterinary Clinics of North America: Food Animal Practice* **24**, 19–39.

Godden SM, Lombard JE and Woolums AR (2019) Colostrum management for dairy calves. *Veterinary Clinics of North America: Food Animal Practice* **35**, 535–556.

Hernandez D, Nydam DV, Godden SM, Bristol LS, Kryzer A, Ranum J and Schaefer D (2016) Brix refractometry in serum as a measure of failure of passive transfer compared to measured immunoglobulin G and total protein by refractometry in serum from dairy calves. *The Veterinary Journal* **211**, 82–87.

Hogan I, Doherty M, Fagan J, Kennedy E, Conneely M, Brady P, Ryan C and Lorenz I (2015) Comparison of rapid laboratory tests for failure of passive transfer in the bovine. *Irish Veterinary Journal* **68**, 18.

Jezek J, Nemec M, Malovrh T and Klinkon M (2010) Indicators of passive immunity and health status of calves. *Acta Veterinaria-Beograd* **60**, 513–523.

Kehoe SI, Jayarao BM and Heinrichs AJ (2007) A survey of bovine colostrum composition and colostrum management practices on Pennsylvania dairy farms. *Journal of Dairy Science* **90**, 4108–4116.

Li-Chan EC and Kummer A (1997) Influence of standards and antibodies in immunochemical assays for quantitation of immunoglobulin G in bovine milk. *Journal of Dairy Science* **80**, 1038–1046.

Marnila P and Korhonen H (2011) Milk | Colostrum A2. In Fuquay JW (ed.). *Encyclopedia of Dairy Sciences (Second Edition)*. San Diego, CA, USA: Academic Press, pp. 591–597.

McGrath BA, Fox PF, McSweeney PLH and Kelly AL (2016) Composition and properties of bovine colostrum: a review. *Dairy Science & Technology* **96**, 133–158.

McGuirk SM and Collins M (2004) Managing the production, storage, and delivery of colostrum. *Veterinary Clinics of North America: Food Animal Practice* **20**, 593–603.

Moran J (2002) *Calf Rearing: A Practical Guide*, 2nd Edn, Collingwood, Australia: Landlinks Press.

Morrill KM, Robertson KE, Spring MM, Robinson AL and Tyler HD (2015) Validating a refractometer to evaluate immunoglobulin G concentration in Jersey colostrum and the effect of multiple freeze–thaw cycles on evaluating colostrum quality. *Journal of Dairy Science* **98**, 595–601.

Oliveira L, Borchardt S, Heuwieser W, Rauch E, Erhard M and Sutter F (2019) Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves. *Journal of Dairy Science* **102**, 557–566.

Osaka I, Matsui Y and Terada F (2014) Effect of the mass of immunoglobulin (Ig)G intake and age at first colostrum feeding on serum IgG concentration in Holstein calves. *Journal of Dairy Science* **97**, 6608–6612.

Parish SM, Tyler JW, Besser TE, Gay CC and Krytenberg D (1997) Prediction of serum IgG1 concentration in Holstein calves using serum gamma glutamyltransferase activity. *Journal of Veterinary Internal Medicine* **11**, 344–347.

Pekcan M, Fidanci UR, Yuceer B and Ozbeyaz C (2013) Estimation of passive immunity in newborn calves with routine clinical chemistry measurements. *Ankara Universitesi Veteriner Fakultesi Dergisi* **60**, 85–88.

Perino L, Sutherland R and Woollen N (1993) Serum gamma-glutamyltransferase activity and protein concentration at birth and after suckling in calves with adequate and inadequate passive transfer of immunoglobulin G. *American Journal of Veterinary Research* **54**, 56–59.

Quigley JD, Lago A, Chapman C, Erickson P and Polo J (2013) Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. *Journal of Dairy Science* **96**, 1148–1155.

Rayburn MC, Chigerwe M, Barry J and Kennedy E (2019) Short communication: use of a digital refractometer in assessing immunoglobulin G concentrations in colostrum and the first 5 transition milkings in an Irish dairy herd. *Journal of Dairy Science* **102**, 7459–7463.

Renaud DL, Duffield TF, LeBlanc SJ and Kelton DF (2018) Short communication: validation of methods for practically evaluating failed passive transfer of immunity in calves arriving at a veal facility. *Journal of Dairy Science* **101**, 9516–9520.

Schneider F, Failing K and Wehrend A (2020) Measurement of IgG concentration in bovine colostrum by immunoturbidimetric assay in comparison to ELISA-based assessment. *Tierarztliche Praxis. Ausgabe G, Grosstiere/nutztiere* **48**, 73–79.

Vandeputte S, Detilleux J and Rollin F (2011) Comparison of four refractometers for the investigation of the passive transfer in beef calves. *Journal of Veterinary Internal Medicine* **25**, 1465–1469.

Weaver DM, Tyler JW, VanMetre DC, Hostetler DE and Barrington GM (2000) Passive transfer of colostral immunoglobulins in calves. *Journal of Veterinary Internal Medicine* **14**, 569–577.

Williams DR, Pithua P, Garcia A, Champagne J, Haines DM and Aly SS (2014) Effect of three colostrum diets on passive transfer of immunity and preweaning health in calves on a California dairy following colostrum management training. *Veterinary Medicine International* **2014**, 698741.

Wilm J, Costa JHC, Neave HW, Weary DM and von Keyserlingk MAG (2018) Technical note: serum total protein and immunoglobulin G concentrations in neonatal dairy calves over the first 10 d of age. *Journal of Dairy Science* **101**, 6430–6436.

Wilson LK, Tyler JW, Besser TE, Parish SM and Gant R (1999) Prediction of serum IgG1 concentration in beef calves based on age and serum gamma-glutamyl-transferase activity. *Journal of Veterinary Internal Medicine* **13**, 123–125.

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Predicting colostrum and calf blood components based on refractometry

Do T Hue[1, 2], John L. Williams[1,3], Kiro Petrovski[1], and Cynthia D. K. Bottema[1*]

SUPPLEMENTARY FILE

Supplementary Methods

Enzyme-linked immunosorbent assay for immunoglobulin G (IgG) concentration

The immunoglobulin G (**IgG**) concentration in the individual cow colostrum, pooled colostrum and bulk tank milk samples was quantified by enzyme-linked immunosorbent assay (**ELISA**) in 96-well plates (Coat Nunc F96 Maxisorp plates, Thermo-Fisher Scientific) using two bovine IgG specific antibodies (Life Technologies, USA), affinity purified goat anti-bovine IgG antibody unconjugated (Novex Cat. #A18753) and affinity purified goat anti-bovine IgG antibody conjugated with horseradish peroxidase (HRP Invitrogen Cat. #18751). The pooled colostrum samples and individual cow colostrum samples from days 0 and 1 were diluted in 0.05% Tween 20-PBS solution at a ratio of $1:10^6$, while the bulk tank milk samples and the individual cow colostrum samples from days 2 and 3 were diluted at a ratio of $1:10^5$. Bovine gamma globulin (Bio-Rad Laboratories, Inc., USA) was used as the standard, with serial dilutions (0; 6.3; 12.5; 25; 37.5; 50; 75; and 100 ng/mL). 3,3',5,5'-tetramethylbenzidine (**TMB**) substrate (Ultra TMB-ELISA, Thermo Fisher Scientific) was added and the plates were incubated at room temperature in the dark for 15 minutes before the reactions were stopped with the addition of 0.1 M H_2SO_4. Antibody binding was measured as the enzymatic colour change at 450 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA). The IgG concentrations in samples were calculated based on the standard curve. Calf serum IgG concentration was determined using the same ELISA as the colostrum samples, however, calf

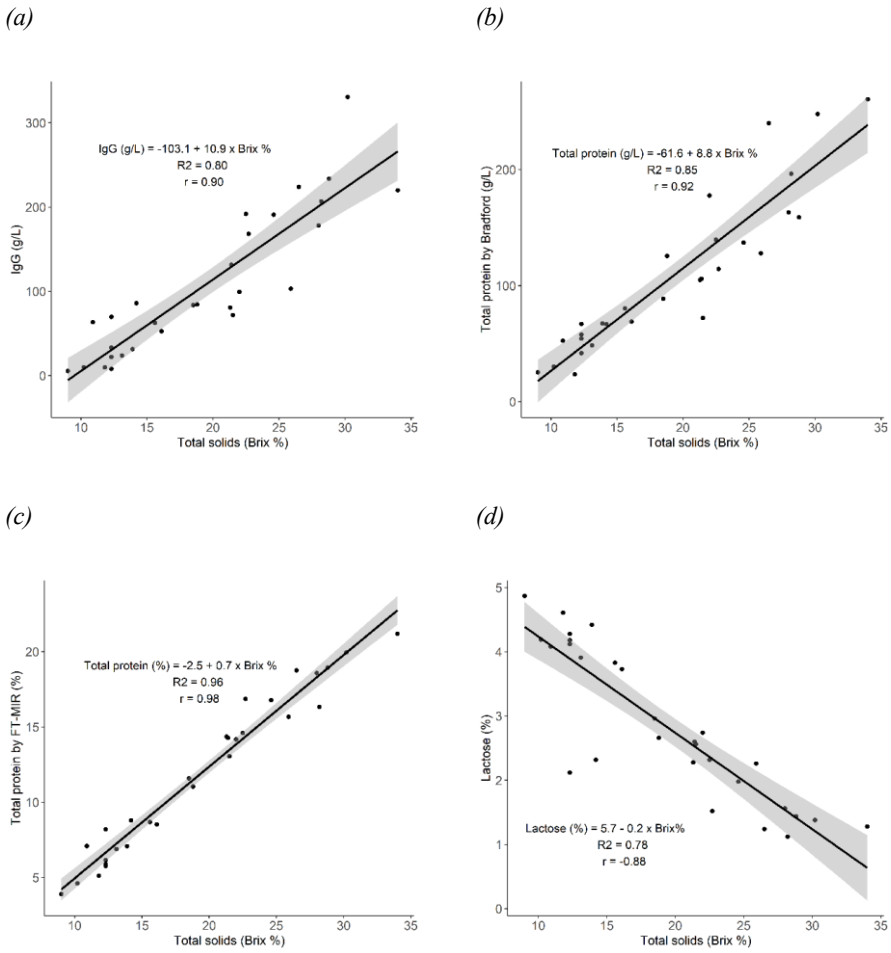
serum samples were diluted in 0.05% Tween 20-PBS solution with at a ratio of $1:10^4$ for day 0 and a ratio of $1:10^6$ for days 1 and 7.

Bradford assay for total protein concentration

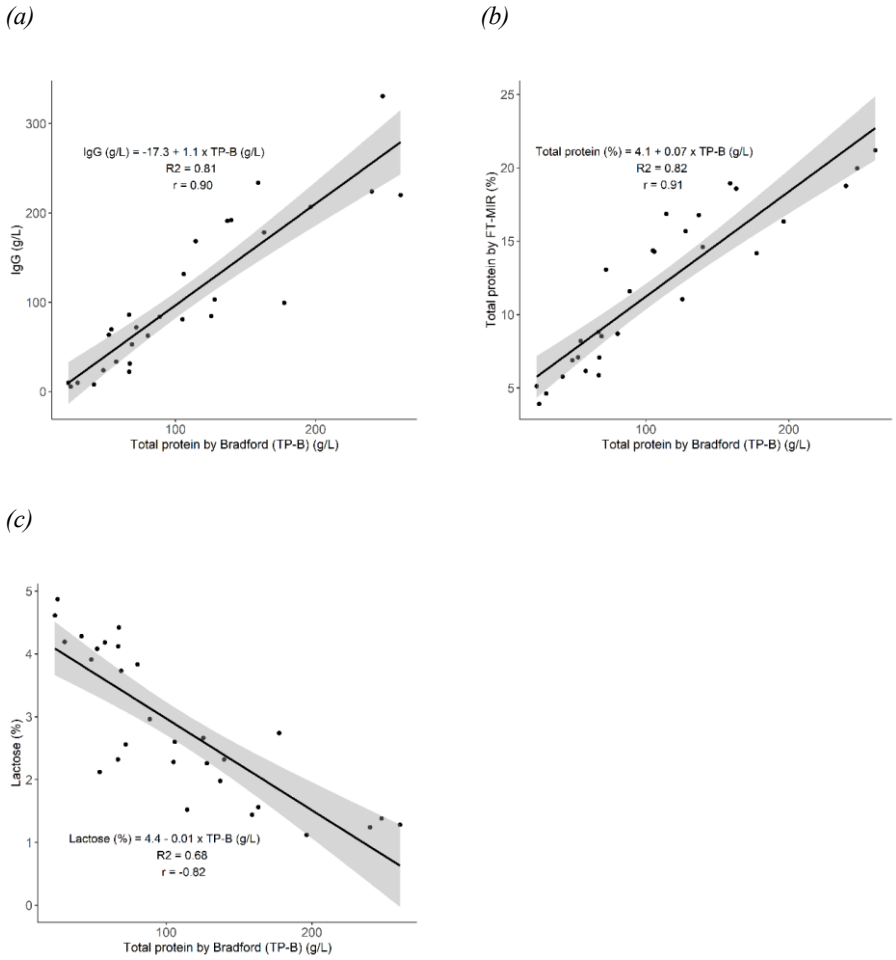
Total protein in the individual cow colostrum, pooled colostrum, and bulk tank milk was assayed in a 96 well-plate using a Quick Start Bradford Protein assay kit following the manufacturer's instructions and bovine serum albumin for the standard curves (Quick Start, Bio-Rad Laboratories, Inc., USA). The individual cow colostrum samples from days 1, 2 and 3, pooled colostrum and bulk tank milk were diluted with ultrapure water at a ratio of 1:100 and the individual cow colostrum from day 0 was diluted with a ratio of 1:200 or 1:300. The diluted samples (5 µL) were mixed with Coomassie Brilliant Blue G-250 dye (250 µL) and the colour change was measured using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA) at 595 nm (OD595). The total protein by Bradford assay (**TP-B**) in the samples was determined based on the standard curve. Calf serum total protein was measured by Bradford assay (TP-B) as described above except the serum was diluted with ultrapure water at a ratio of 1:100.

Supplementary Table S1. Estimated IgG, total protein and lactose in colostrum based on regression formulae from Brix refractometer measurements.

Brix%	**IgG (g/L)**	**Total protein (g/L)**	**Lactose (%)**
	IgG (g/L) = -103.1 + 10.9 x Brix % $R^2 = 0.80$, $P < 0.001$	TP-B (g/L) = -61.6 + 8.8 x Brix % $R^2 = 0.85$, $P < 0.001$	Lactose (%) = 5.7 – 0.2 x Brix % $R^2 = 0.78$, $P < 0.001$
12	27.7	44.0	3.3
13	38.6	52.8	3.1
14	49.5	61.6	2.9
15	60.4	70.4	2.7
16	71.3	79.2	2.5
17	82.2	88.0	2.3
18	93.1	96.8	2.1
19	104.0	105.6	1.9
20	114.9	114.4	1.7
21	125.8	123.2	1.5
22	136.7	132.0	1.3
23	147.6	140.8	1.1
24	158.5	149.6	0.9
25	169.4	158.4	0.7

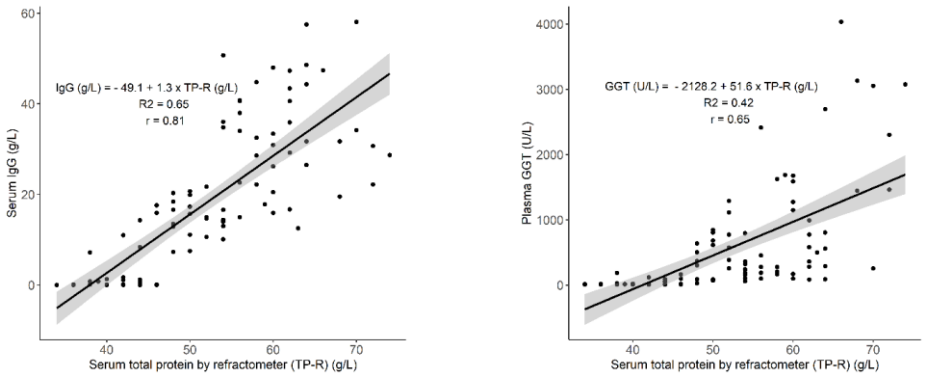


Supplementary Figure S1. Linear regression between total solids measured by Brix refractometry (Brix %) and IgG (g/L) *(a),* total protein by Bradford assay (g/L) *(b)*, protein % *(c)*, and lactose % *(d)* in individual cow colostrum day 0 and 1 postpartum and pooled colostrum samples (n = 29). R^2 = coefficient of determination, r = correlation. P-value for all panels (a, b, c, d) < 0.001.



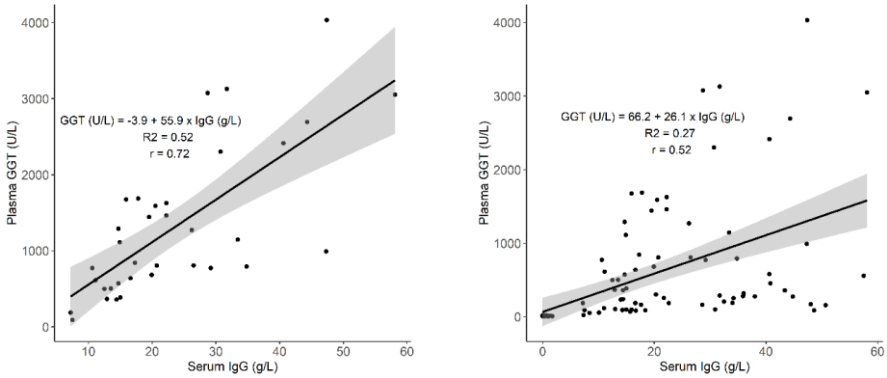
Supplementary Figure S2. Linear regression between total protein by Bradford assay (TP-B) (g/L) and IgG (g/L) *(a),* protein % by FT-MIR *(b)*, and lactose % by FT-MIR *(c)* in colostrum collected within 1 day postpartum and pooled colostrum samples (n = 29). R^2 = coefficient of determination, r = correlation. P-value for all panels (a, b, c) < 0.001.

(a) *(b)*



Supplementary Figure S3. Linear regression between serum total protein by refractometer (TP-R) and serum IgG *(a),* and plasma GGT *(b)* in calf blood measured within one week postpartum. R^2 = coefficient of determination, r = correlation. P-value for all panels (a, b) < 0.001.

(a) *(b)*



Supplementary Figure S4. Linear regression between serum IgG and plasma GGT in calf blood using data from day 1 (n=35) *(a),* and data combined from days 0, 1 and 7 (n = 105) *(b)*. R^2 = coefficient of determination, r = correlation. P-value for all panels (a, b) < 0.001.

Chapter 5

MicroRNA profile of colostrum and calf blood from Next Generation sequencing (NGS)

Chapter 5. MicroRNA profile of colostrum and calf blood from Next Generation sequencing (NGS)

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5.1. Introduction

MicroRNAs (**miRNAs**) are short non-coding RNAs, which range around 22 nucleotides in length (Bartel, 2004; Izumi et al., 2012; Sun et al., 2015). First reported by Rosalind Lee and colleagues in the worm *Caenorhabditis elegans* (Lee et al., 1993), miRNAs are found both intra- and extra-cellularly, including the body fluids of humans and animals (Chen et al., 2012; Alsaweed et al., 2015a), and milk is a rich source of miRNA (Sun et al., 2013; Alsaweed et al., 2015b). MicroRNAs play an important regulatory role in gene expression by binding to the 3'-untranslated regions (3'-UTR) of target mRNAs which results in mRNA cleavage, translational repression, or mRNA de-adenylation (Bartel, 2004; Chen et al., 2010; Wang et al., 2013). Many miRNAs have been identified and shown to be involved in various biological processes, including stem cell differentiation, immune responses, cholesterol and insulin metabolism, neurogenesis, haematopoiesis, cardiac and skeletal muscle growth, oocyte maturation and early stage embryonic development (Wienholds and Plasterk, 2005; Williams, 2007; Liu et al., 2010; Wang et al., 2013). The functions regulated by most miRNAs are unknown, but miRNAs have been predicted to regulate the expression of up to 30% of the protein coding genes by binding to the corresponding mRNA (Wienholds and Plasterk, 2005; Izumi et al., 2012).

There are several methods to identify and quantify miRNAs in samples, including qPCR, microarray, near-infrared technology, isothermal amplification and Next Generation Sequencing (NGS) (Moody et al., 2017). Next Generation Sequencing provides the most precision (Moore et al., 2016) and has several advantages over other methods (Moody et al., 2017). A key feature of NGS is the ability to sequence millions of molecules simultaneously in one lane of a sequencer to explore the expression profiles of miRNAs comprehensively and efficiently (Eminaga et al., 2013). Although Next Generation Sequencing is more expensive than other methods, it produces an unprecedented amount of data, including the identification of novel miRNA and polymorphism detection (Moody et al., 2017).

Bovine colostrum and milk contain high concentrations of miRNAs, including immune-related miRNAs (Chen et al., 2010; Hata et al., 2010; Cai et al., 2018). MicroRNAs are stored in exosomes within the colostrum and milk (Chen et al., 2010; Hata et al., 2010), and hence, are very stable even under harsh conditions, such as extreme temperatures, freeze-

thaw cycles, low pH and RNase treatment (Chen et al., 2008; Izumi et al., 2012). Therefore, miRNAs in exosomes may avoid degradation in the gastrointestinal tract (Benmoussa et al., 2016; Liao et al., 2017) and may be transferred to other cells (Hata et al., 2010; Liao et al., 2017). Milk derived miRNAs may enter the systemic circulation of newborns and exert immune and developmental functions.

To examine the possibility that miRNAs are transferred from colostrum, the miRNA profiles in the dam colostrum and pooled colostrum were determined by NGS. The microRNA profiles of whole blood samples from the three calf Groups, which received colostrum from different sources during first 3 days of their life, were also obtained and compared to establish whether the miRNA in the colostrum had been transferred to the calves.

5.2. Materials and methods

5.2.1. Materials

The experimental design and samples were as described previously (Chapter 2). However, not all samples were sequenced for miRNA. Only 12 calf blood samples from days 0 and 1, and the corresponding 4 dam colostrum day 0 samples and 4 pooled colostrum samples were chosen for miRNA-sequencing by NGS (Table 5.1, Appendix 3).

Table 5.1. Colostrum and calf blood samples for miRNA sequencing.

Samples	Group A calf		Group B calf		Group C calf		Total (n)
	n	ID calf	n	ID calf	n	ID calf	
Calf blood day 0	4	A1, A2, A4, A10	4	B1, B2, B4, B10	4	C3, C5, C8, C11	12
Calf blood day 1	4	A1, A2, A4, A10	4	B1, B2, B4, B10	4	C3, C5, C8, C11	12
Dam colostrum[a]	4	Dam 1, 2, 4 & 10					4
Pooled colostrum[b]					4	Pool 3, 5, 8 &11	4
Total	12		8		12		32

Calves (Group A, B, C) were fed colostrum from the corresponding colostrum source (e.g. dam #1 colostrum fed to calves A1 and B1, pooled colostrum #3 fed to calf C3)

[a]*Dam colostrum taken within 2 hours postpartum and fed to the corresponding pairs of Group A and B calves (i.e., colostrum from Cow #1 fed to calf A1 and B1).*

[b]*Pooled colostrum was a mix of colostrum and transition milk from other cows day 0 - 4 postpartum and fed to the corresponding Group C calves (i.e.,, pooled colostrum #3 to fed calf C3).*

5.2.2. Methods

MicroRNA levels in the colostrum and blood samples were quantified in 5 steps with the bioinformatic analysis being the last but longest step (Figure 5.1).

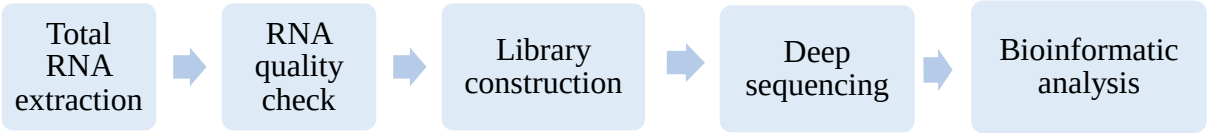


Figure 5.1. Workflow of miRNA sequencing experiment.

Step 1. Total RNA extraction

A miRNeasy Mini Kit was used to purify total RNA, including miRNAs, from the colostrum and calf blood samples. The samples were extracted by the miRNeasy Mini Kit quick-start protocol (cat. No. 217004, Qiagen), according to the manufacturer's instructions. The total RNA collected from 250 µL of colostrum or calf blood, as recommended by the protocol, was low for miRNA sequencing. To increase the total RNA yield, 500 µL of each sample was extracted in 2 duplicate tubes of 250 µL each (as detailed in Chapter 2).

Step 2. RNA quality check

The total RNA concentration was quantified with a NanoDrop ND-1000 (Supplied and Service by BIOLAB, ThermoFisher) and the OD260/280 ratio was used to determine the RNA purity, a ratio of ~2.0 is generally accepted as "pure" for RNA. The integrity of the total RNA was then assessed using a RNA ScreenTape assay (Agilent Technologies, Hewlett-Packard-StraBe 8, 76337 Waldbronn, Germany).

The samples that had a total RNA concentration of 1 µg in a maximum of 10 µL volume (1 ng per sample) with OD 260/280 range from 1.6 to 2.0 were used for the RNA library preparation. The miRNA quality and integrity were verified using an Agilent Bioanalyzer (Agilent 2100 Bioanalyzer, Version 1.2) with Small RNA Bioanalyzer chips (Small RNA Analysis 6 - 150 nucleotides) prior to library preparation (Appendix 3).

c. Library construction

The miRNA sequencing library was prepared using NEXTflex Small RNA-Seq kits v3 (Bioo Scientic Corporation, 7050 Burleson Road, Austin, Texas 78744, USA) for Illumina-compatible Next-Generation Sequencing following the manufacturer's recommendations. The NEXTflex™ Small RNA-Seq Kit v3 has been optimized and validated using total RNA (1 ng per sample). Library construction was performed using 3' and 5' adapter ligated RNA, which was reverse transcribed to generate cDNA, then the cDNA was amplified (14 cycles of PCR) (Figure 5.2). The standard protocol suggested 12-18 cycles for input amounts 2 µg-200 ng, and therefore, PCR amplification was performed with 14 cycles for both the blood and colostrum samples. Following PCR, the products were analyzed by TBE-PAGE gel electrophoresis and Agilent Bioanalyzer HS DNA Assay for small RNA.

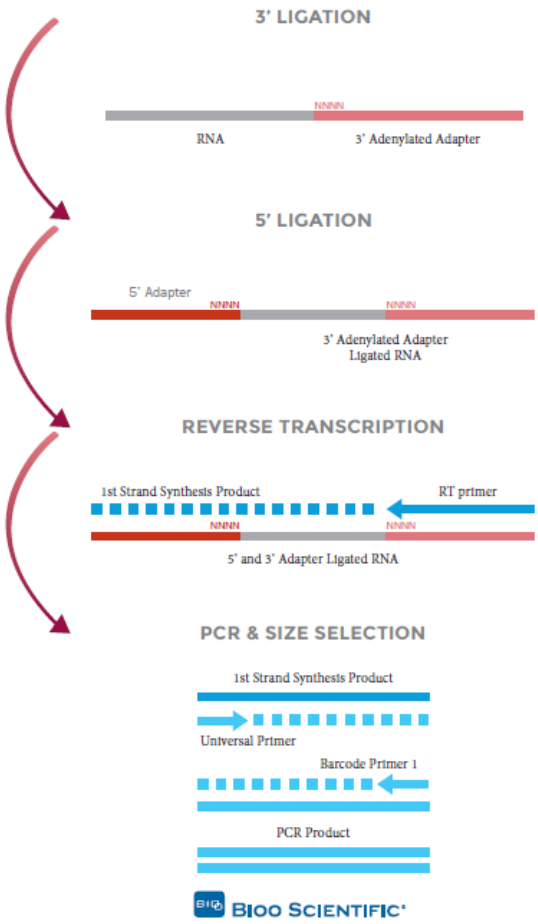


Figure 5.2. Small RNA sample preparation for miRNA sequencing library construction.

From NEXTflex™ Small RNA-Seq Kit v3 manual, Bioo Scientific Corporation, 2015.

Step 4. Deep sequencing

Following the construction of the miRNA library, sequencing was carried out on an Illumina NextSeq 500 System by the Australian Cancer Research Foundation (ACRF) Cancer Genomics Facility, Centre for Cancer Biology, SA Pathology and University of South Australia. The average read length was 75 bp and the target number of reads was 22 million per sample.

Step 5. Bioinformatic analysis

The raw reads in FASTQ file were processed using a bioinformatic pipeline that consisted of seven steps (Table 5.2).

Table 5.2. Steps for bioinformatic miRNA analysis.

Step	Content
5.1	Initial check of raw reads using FastQC report
5.2	Data filtering and trimming
5.3	Data alignment to cattle genome
5.4	Data alignment to miRNA databases
5.5	Removal of non-miRNA sequences
5.6	Differential miRNA expression analysis
5.7	Pathway analysis after target prediction

The raw read provided as FASTQ file of 4 lines for each sequence (Figure 5.3). The first line contained "@" and unique sequence identifier, the 2nd line was the nucleotide sequence of the given identifier, the 3rd line was the "+" symbol and sometime with the sequence identifier again, and the 4th line was quality score of the sequence for each position that specified the probability that the nucleotide call was wrong for each nucleotide in the read sequence. Missing nucleotides in the sequence were denoted by the character "N".

```
@NB501009:334:HLTLGBGXC:1:11101:4896:1075 1:N:0:GTAGAG
CCGTTGAAACATACACGGGAAACCTGTNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
+
AAAAAEEEEEEEEEEEEEEEAEEEEEE###E##########################################
@NB501009:334:HLTLGBGXC:1:11101:9593:1076 1:N:0:GTAGAG
CTCGTACAGTATAGATGATGTACTTGTNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
+
AAAAAEEEEEEEEEEEEEEEEEEEEEE###E##########################################
@NB501009:334:HLTLGBGXC:1:11101:3585:1076 1:N:0:GTAGAG
AATCAATAATACATGGTTGATCTTTAGNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

Figure 5.3. Example of FASTQ format for raw reads.

Step 5.1. Initial check of raw reads using FastQC report

The initial step was performed by checking raw data quality using the FastQC report. The FastQC report provided a graph and summary of raw data quality as follows:

- Basic statistics, including filename, number of sequences and sequence length,
- Per base sequence quality of each position in read,
- Per tile sequence quality on the sequencing chip,
- Per sequence quality score: plot gives the number of sequence at each mean quality score,
- Per base sequence content,
- Per sequence GC content,
- Per base N content,
- Sequence length distribution,
- Sequence duplication levels, and
- Over-represented sequences.

The report provided a quick check regarding problems to address before starting further analysis. Each sample was inspected for the number of reads sequenced, their lengths, read quality and presence of adapters. Other details reported by the FastQC software were also visually inspected.

Step 5.2. Data filtering and trimming

This step was performed to eliminate reads of low quality, remove the adapter sequences, and trim the poly-N sequences. The reads of mean quality scores ≤ 25 were removed (Huse et al., 2007) using Prinseq (version 0.20.4). The Cutadapt (version 1.9.1) was used to remove the 3′ adapter and the sequences downstream, including the barcode (Figure 5.4). Finally, the four random bases that appeared immediately 5' and 3' of the target sequence were also

removed (Figure 5.4). The trimmed reads with the length of 17–28 nucleotides were selected using Prinseq (version 0.20.4) (Figure 5.5).

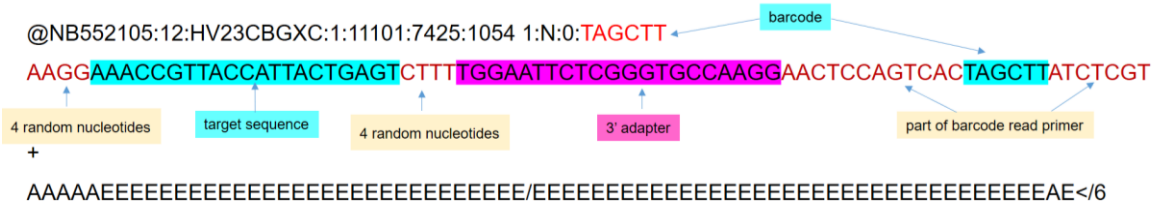


Figure 5.4. Example of raw read with adapter, target miRNA, and barcode sequence. *3' adapter sequence was TGGAATTCTCGGGTGCCAAGG and target miRNA contained 17 to 28 nucleotides.*

```
(base) [a1689729@l01 TrimmedFastq]$ head miRNA-CB12_R1_all_trim2_good.fastq
@NB501009:334:HLTLGBGXC:1:11101:22682:1084 1:N:0:CTTGTA
ATACAAGGACAATTTCTTTCTGT
+NB501009:334:HLTLGBGXC:1:11101:22682:1084 1:N:0:CTTGTA
AEEEEEEEEEEEEEEEEEEEEE
@NB501009:334:HLTLGBGXC:1:11101:23995:1086 1:N:0:CTTGTA
AGAGGTAGTAGGTTGCATAGT
+NB501009:334:HLTLGBGXC:1:11101:23995:1086 1:N:0:CTTGTA
AE/EEAAEE6EEEEEAAAEEE
@NB501009:334:HLTLGBGXC:1:11101:15828:1086 1:N:0:CTTGTA
AAACCGTTACCATTACTGAGTT
```

Figure 5.5. Example of cleaned reads (17 to 28 nucleotides) after trimming and filtering adapter and random nucleotides.

Step 5.3. Data alignment to cattle genome

BWA software (version 0.7.17) was used to align the trimmed reads to the cattle genome (a combination of the UOA_Angus_1 (GCA_003369685.2) and the X chromosome from UOA_Brahman_1 (GCF_003369695.1), (Low et al., 2019; Rosen et al., 2020) (Figure 5.6). The flag setting of **-n 1 -o 0 –e 0 –k 1** was used following the protocol from Ziemann et al. (2016) (Figure 5.7). After alignment with the cattle genome, SAMtools (version 1.10) was used to sort the aligned BAM files.

UOA_Angus_1

Organism name: Bos indicus x Bos taurus (hybrid cattle)
Infraspecific name: Breed: Angus x Brahman F1 hybrid
Sex: male
BioSample: SAMN08473802
BioProject: PRJNA432857
Submitter: University of Adelaide
Date: 2018/11/30
Assembly type: haploid (principal pseudohaplotype of diploid)
Assembly level: Chromosome
Genome representation: full
GenBank assembly accession: GCA_003369685.2 (latest)
RefSeq assembly accession: n/a
RefSeq assembly and GenBank assembly identical: n/a
WGS Project: PUFT02
Assembly method: TrioCanu v. 1.6
Expected final version: yes
Genome coverage: 136.0x
Sequencing technology: PacBio RSII; PacBio Sequel; Illumina NextSeq
Linked assembly: GCA_003369695.2 (alternate pseudohaplotype of diploid)

UOA_Brahman_1

Organism name: Bos indicus x Bos taurus (hybrid cattle)
Infraspecific name: Breed: Angus x Brahman F1 hybrid
Sex: male
BioSample: SAMN08473802
BioProject: PRJNA432857
Submitter: University of Adelaide
Date: 2018/11/30
Assembly type: alternate-pseudohaplotype
Assembly level: Chromosome
Genome representation: full
RefSeq category: representative genome
GenBank assembly accession: GCA_003369695.2 (latest)
RefSeq assembly accession: GCF_003369695.1 (latest)
RefSeq assembly and GenBank assembly identical: no (hide details)
- Only in GenBank: chromosome MT (in non-nuclear assembly-unit)
- Data displayed for RefSeq version

WGS Project: PUFS02
Assembly method: TrioCanu v. 1.6
Expected final version: yes
Genome coverage: 136.0x
Sequencing technology: PacBio Sequel; PacBio RSII; Illumina NextSeq
Linked assembly: GCA_003369685.2 (principal pseudohaplotype of diploid)

Figure 5.6. Details of cattle databases used for the bovine genome alignment.
From https://www.ncbi.nlm.nih.gov/assembly/GCA_003369685.2/, and https://www.ncbi.nlm.nih.gov/assembly/GCF_003369695.1/, for Angus and Brahman respectively, accessed November, 2020.

```
Read:       TAGCTACGCTCTACGCTATCATGCATAAAC
Seed 1 fw: TAGCTACGCT
Seed 1 rc: AGCGTAGCTA
Seed 2 fw:       CGCTCTACGC
Seed 2 rc:       GCGTAGAGCG
Seed 3 fw:             ACGCTATCAT
Seed 3 rc:             ATGATAGCGT
Seed 4 fw:                   TCATGCATAA
Seed 4 rc:                   TTATGCATGA
```

-n 1: 1% uniform base error rate if FLOAT

-o 0: no gap open

–e 0: no gap extensions

–k 1: maximum edit distance of the seed

Figure 5.7. Flag setting for alignment with cattle genome (-n 1 -o 0 –e 0 –k 1).

Step 5.4. Data alignment to miRNA databases

To identify the miRNAs, the sequences were aligned to miRNAs in two databases, miRBase and RumimiR. miRBase is a mammalian miRNA database of published conserved miRNA sequences with annotation. The last update of miRBase was Release 22.1 in October 2018

with 38589 entries. RumimiR is a ruminant miRNA database, which was last updated in January, 2020, with a total of 6560 bovine miRNAs and a total of 19847 ruminant miRNAs (RumimiR, 2020).

First, the sequences were aligned against the miRNA precursors and mature conserved mammalian miRNAs in the miRBase (http://www.mirbase.org/) using the Perl script of miRDeep2.pl. MicroRNA precursors was used for novel miRNA prediction and only mature miRNAs were studied in this project. The reads that matched miRNAs in miRBase were classified as known miRNAs (Figure 5.8). Any reads that did not match the conserved miRNAs in miRBase were designated as potentially novel miRNAs. To avoid the limitation of using just one miRNA database, the miRNAs designated as potentially novel were then aligned to the miRNA in the RumimiR database (http://rumimir.sigenae.org/). Those miRNAs that still did not align to the known miRNAs in RumimiR were classified as novel herein as they were not found in either miRNA database.

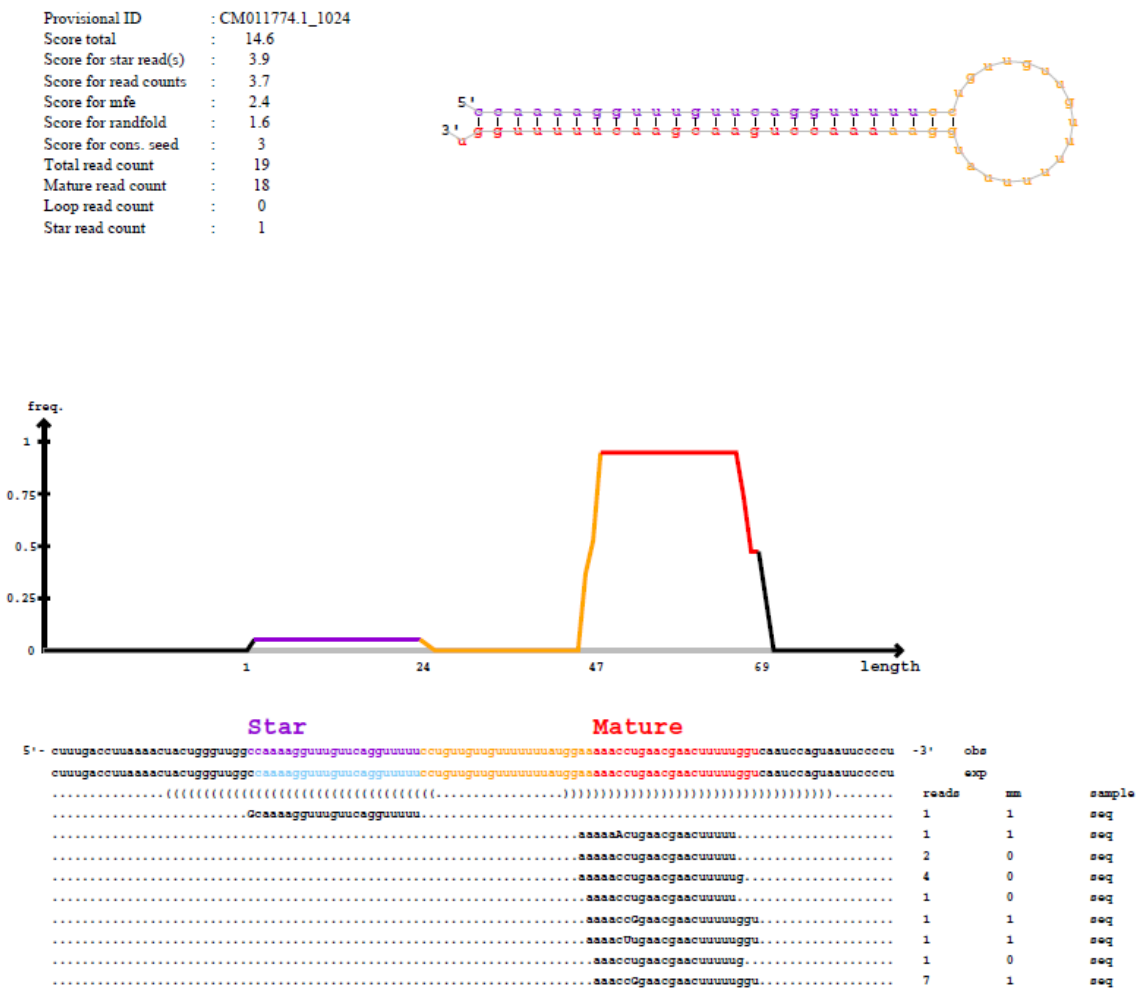


Figure 5.8. Example of sequences, mature read counts and miRNA information for each miRNA.

Step 5.5. Removal of non-miRNA sequences

According to the manual of miRDeep2, only the known and novel sequences with randfold P-values < 0.05 are true miRNA. Therefore, only these sequences were retained. To avoid biasing the analyses with rare or spurious reads, the remaining miRNAs were also filtered by retaining only those miRNAs with a minimum of five counts in at least four blood samples or two colostrum samples.

These sequences were then aligned to the ARS-UCD1.2 (GCF_002263795.1) reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_002263795.1/) for non-coding

RNA. Any RNA sequences that had a query coverage > 90 and a bitscore > 30 were classified as other types of non-coding RNAs (Table 5.3) and removed. The remaining known and novel miRNAs were used for further analysis described below.

Table 5.3. List of potential other non-coding RNAs.

Nº	Non-coding RNA	Abbreviation
1	Long non-coding RNAs	lncRNA
2	Miscellaneous RNA	misc_RNA
3	Mitochondrial ribosomal RNA	Mt_rRNA
4	Mitochondrial transfer RNA.	Mt_tRNA
5	Ribosomal RNA	rRNA
6	Small Cajal body-specific RNAs	scaRNA
7	Small nucleolar RNA	snoRNA
8	Small nuclear RNA	snRNA
9	Small RNA	sRNA

Step 5.6. Differential expression (DE) of miRNA analysis

The differential expressed miRNA analysis was performed for the mature known bovine miRNA, identified during the RumimiR database alignment, using a customized R script. The linear models (limma package) (Ritchie et al., 2015) and edgeR (version 3.11) packages (Robinson et al., 2010; Robinson and Oshlack, 2010) were mainly used to do the analysis. The trimmed mean of M (TMM) method was used for normalization of the miRNA expression of the known bovine miRNA and implemented in the R Bioconductor package edgeR as proposed by Robinson et al. (2010). This method gives the proportion of counts for a specific target across all samples. If a miRNA is present in the same proportion across all samples, it will be deemed as not being differentially expressed (Tam et al., 2015).

The linear models (limma package) were used to compare the differential expression of miRNAs for different colostrum sources (dam vs pooled colostrum), and calf blood at different time-points (day 0 vs day 1). The P-values were adjusted for multiple tests and miRNA expression differences were considered significant if the adjusted P-value (or Q-values) < 0.05 and the $|\log_2$ fold change$|$ > 1. The small sample size of four calves per Group

prevented multiple testing for miRNA differential expression comparisons between the three calf Groups for each time-point.

Step 5.7. Target prediction and pathway analysis

The prediction of the mRNAs that are targeted by known or novel miRNAs is based on comparisons between the sequences of mature miRNAs and the sequences of mRNA candidate gene targets. MicroRNA binds to the 3' UTR of their mRNA target, which triggers the repression of translation or the degradation of the mRNA depending on the complementarity between the target and the miRNA (Motameny et al., 2010). The 'seed' region, positions from 2 to 7 (or 8) from the 5'-end of the miRNA, and the 3'-UTR of the mRNA are important for miRNA–mRNA interactions (Lewis et al., 2003; Vejnar and Zdobnov, 2012) and have been found in 73% of studies (Vejnar et al., 2013) (Figure 5.9).

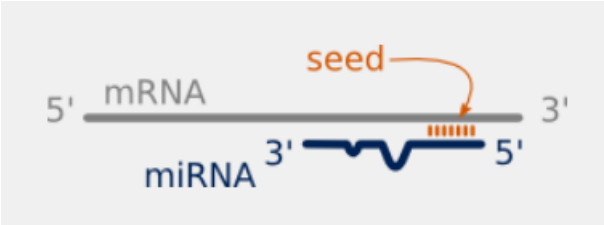


Figure 5.9. miRNA seed region.

Four main approaches can be taken to explore the interaction between a miRNA and its mRNA target based on a thermodynamic, a probabilistic, an evolutionary or a sequence based point of view (Vejnar and Zdobnov, 2012). Tools have been created using each of these four approaches to predict miRNA target sites, including miRmap, TargetScan, PITA, PicTar, miRanda, RNAhybrid, DIANA-microT, ElMMo, and PACMIT (Vejnar and Zdobnov, 2012).

In this study, the target genes of the differentially expressed (DE) miRNAs in day 0 and day 1 calf blood and the top 100 most highly expressed miRNAs in colostrum were predicted using miRmap v1.1 (https://mirmap.ezlab.org/app/) developed by Vejnar et al. (2013). The top 100 most highly expressed miRNAs in colostrum were the 100 miRNAs with the highest numbers of normalized reads. The database of miRNA target genes was downloaded from the miRmap website (https://mirmap.ezlab.org/downloads/mirmap201301e/) on December

2020, file name "mirmap201301e_bostau_targets_1to1_pt.csv.xz"). The potential target genes of the top 100 miRNAs most highly expressed across samples in colostrum and the 22 DE miRNAs in calf blood between day 0 and day 1 were identified by mapping the miRNAs with the predicted target gene database. When predicting targets, it is important to consider that one mRNA molecule might contain several binding sites for one or more miRNAs or that a single miRNA might regulate several mRNAs. Prior to pathway analysis, target genes were ranked based on the miRmap score and miRmap score with a threshold chosen based on the number of selected targets. Although the thresholds varied between the miRNAs, the miRmap scores of selected targets were all above 75.

For the pathway analysis of the top 100 most expressed miRNAs in colostrum, the 20 target genes with the highest ranking based on miRmap score > 90 were selected for each miRNA. For the pathway analysis of the 22 miRNAs in calf blood differentially expressed between day 0 and day 1, the 20 highest ranked genes were selected for each miRNA based on the miRmap scores > 84.

After the target genes were predicted, the Entrez gene ID from the cattle genome was collected for the predicted target genes, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the limma R package. Gene Ontology (http://www.geneontology.org/) is a classification system for gene functions that contains three main ontologies: cellular component, molecular function and biological process (Ozdemir, 2020). The GO term was considered enriched if the adjusted P value is < 0.05. KEGG is a database resource for understanding high-level functions and utilities of biological systems (http://www.genome.jp/kegg/). A KEGG pathways analysis was performed to test the statistical richness of the target gene candidates.

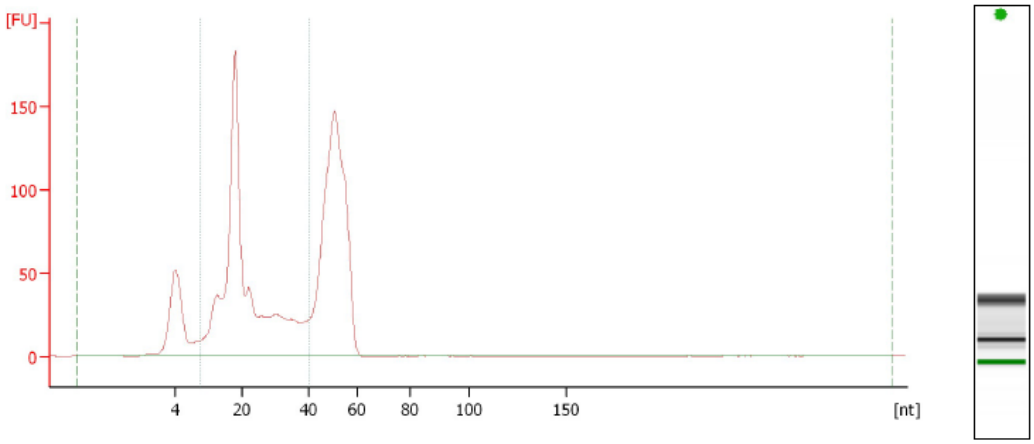
5.3. Results

5.3.1. Quality analyses

After extraction, the total RNA was analysed for quality. All samples had a ratio of A260/A280 from 1.6 to 2.0 (Appendix 3) and were acceptable for the next step of miRNA sequencing. The TapeStation results showed that all calf blood samples had high RIN values (9.3 to 9.9), whereas colostrum samples had lower RIN values (4.8 to 7.7) (Appendix 3).

The results from Bioanalyzer indicated that small RNA was present in all the blood and colostrum samples, and the miRNA concentrations were between 3,153 - 24,736 pg/µL. This accounted for 8 – 100% of small RNA based on the results from the small RNA 6 - 150 nucleotides chip (Appendix 4). MicroRNA accounted for a higher percentage of the small RNAs in the colostrum samples (59 to 67%), whereas in the blood samples, there was a large variation in the proportion of miRNA (8 to 100% of the small RNAs) (Figure 5.10 and Appendix 4).

The number of target reads was 22 million reads, but this target was only achieved for the blood samples, which had nearly three-fold more raw reads than the colostrum samples (Figure 5.11 and Table 5.4). The average raw reads of calf blood was 30 million reads (range from 20 to 42 million reads), whereas the average for the colostrum samples was 11 million reads (range from 4 to 14 million reads) (Table 5.4). The lower number of reads for the colostrum samples was presumably because the total RNA from colostrum was of poorer quality with much lower RIN values than the total RNA from the blood samples.



Overall Results for sample 2 : 24

Small RNA Concentration [pg/µl]:	4,089.1	Result Flagging Color:	
miRNA Concentration [pg/µl]:	3,153.0	Result Flagging Label:	77 % miRNA; Concentration: 3153 pg/µl
miRNA / Small RNA Ratio [%]:	77		

Region table for sample 2 : 24

Name	From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total	Color
Small RNA	0	316	33	53.1	4,089.1	100	
miRNA	10	40	22	32.8	3,153.0	77	

Figure 5.10. Example of Bioanalyzer results for a blood sample.

FU= fluorescence unit; nt = nucleotide.

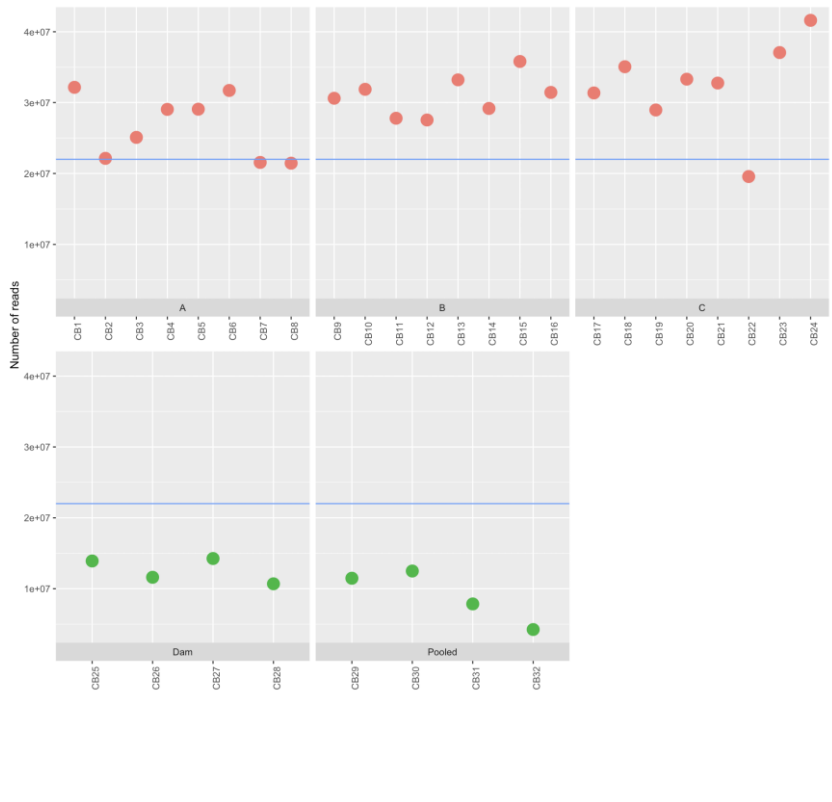


Figure 5.11. Number of raw reads for blood and colostrum samples.

Blue line = target number of reads of 22 million.

Table 5.4. Average of raw reads in different sets of samples.

	Sample	Average of raw reads	Minimum	Maximum
Sample library	Colostrum (n = 8)	10,823,712	4,238,887	14,266,062
	Calf blood (n = 24)	29,972,016	19,568,252	41,608,477
Colostrum source	Dam colostrum (n = 4)	12,625,624	10,699,779	14,266,062
	Pooled colostrum (n = 4)	9,021,801	4,238,887	12,502,948
Calf blood at time-point	Day 0 (n = 12)	30,452,056	21,558,248	37,066,316
	Day 1 (n = 12)	29,491,976	19,568,252	41,608,477
Calf blood in different groups	Group A (n = 8)	26,524,194	21,450,116	32,153,611
	Group B (n = 8)	30,929,922	27,539,044	35,811,166
	Group C (n = 8)	32,461,932	19,568,252	41,608,477

The first step of bioinformatics pipeline was to check the quality of the raw reads based on the FastQC report. As indicated in the initial FastQC file, despite concerns regarding total RNA quality for the colostrum samples, all samples provided sufficient good quality reads for further analyses.

5.3.2. Identification of miRNAs

After the reads were filtered, trimmed and clean reads mapped with the cattle genome, the sequences were aligned with miRBase database (annotated miRNA from mammals). There were 11,258 unique sequences found in total with 1,806 sequences identified as known miRNAs and 9,452 sequences predicted as novel miRNAs (Table 5.5). A unique sequence was counted only once even if the sequence appeared in many samples or appeared many times in a single sample. Some of these unique sequences with low numbers of reads may be spurious, and therefore, only sequences with minimum of 5 reads in 4 blood samples or 2 colostrum samples were retained for further analyses. This filter greatly reduced the number of sequences, as only 10% of sequences were retained (1,296 sequences).

Table 5.5. Number of unique sequences after miRNA identification steps.

Steps	Known	Novel	Total
Sequences after miRBase alignment	1,806	9,452	11,258
Sequences after filtering for minimum 5 counts in at least 4 blood or 2 colostrum samples	558	738	1,296
Sequences after removing other non-coding RNA	558	640	1,198
Sequences after RumimiR alignment of 640 novel miRNAs (from miRBase)	558 + 446	194	
Number of miRNAs after alignment with both databases	1,004	194	1,198

Unique sequences = sequences were counted only once even if they appeared in many samples or many times in one sample.

All filtered sequences were then mapped against the ARS-UCD1.2 reference for non-coding RNA to identify any sequences that were not miRNA but represented other types of small RNA (e.g., lncRNA, mtRNA, rRNA; Table 5.3). This step eliminated 77 of the novel sequences. So after removing non-coding RNAs, there were 558 known and 661 novel

sequences identified from miRBase. Lastly, a final check of the miRBase using a BLAST search determined that 21 sequences of the 661 novel miRNAs were actually known, and the final number of miRNAs identified as novel from miRBase was 640 sequences.

However, the last version of miRBase was released in October 2018 (Release 22). So in order to update the miRNA alignment, the 640 novel sequences were also mapped against the RumimiR database, which was last released in January 2020. Of the 640 miRNAs identified in the miRBase alignment as novel, 446 miRNAs were classified as known miRNAs from the RumimiR alignment. Thus, after the alignment to both databases, there were 1,004 known miRNAs and 194 novel miRNAs for total number of 1,198 unique miRNAs found in the blood and colostrum samples. Most of the miRNA sequences were 20 to 23 nucleotides long, with 22 nucleotides being most common (42% in total of miRNAs) (Figure 5.12).

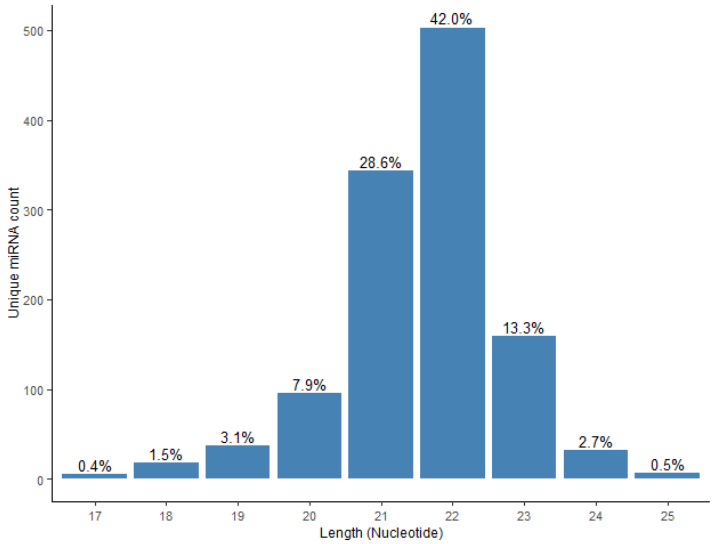


Figure 5.12. Distribution by length of miRNAs in all samples (n=32).

5.3.3. MicroRNA in colostrum and blood samples

There were four times more miRNAs in the blood samples than in the colostrum samples (1,198 vs 303, respectively). Of the 303 miRNAs found in colostrum, only one miRNA was not found in the blood. Thus, nearly all of the miRNAs detected in the colostrum were also detected in the blood (302 of 303 colostrum miRNAs) (Figure 5.13).

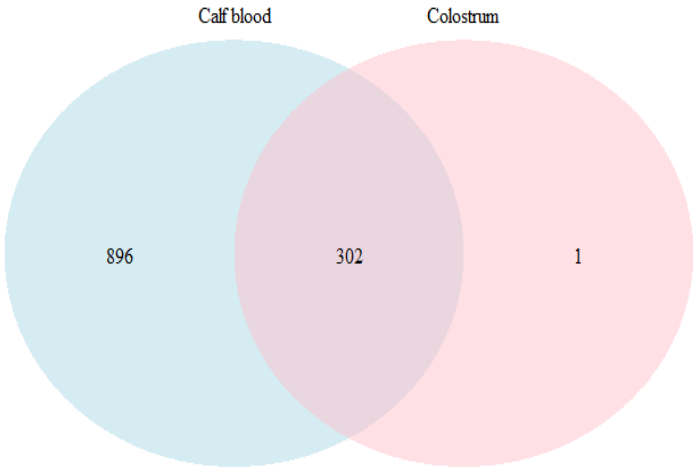


Figure 5.13. Number of miRNAs in calf blood and colostrum samples.

While 1,004 and 296 known miRNAs were found in calf blood and colostrum samples respectively, only 194 and 7 were identified as novel in calf blood and colostrum samples, respectively (Table 5.6). Consequently, there was a higher proportion of novel miRNA in the blood samples than the colostrum samples (16% vs 2%, respectively).

Table 5.6. Known and novel miRNAs in colostrum and calf blood samples.

	Known miRNA (miRBase)	Novel miRNA in miRBase, but known miRNA in RumimiR	Total known miRNA	Novel miRNA	Total miRNA
Calf blood (n = 24)	558	446	1,004	194	1,198
Colostrum (n = 8)	246	50	296	7	303
Overlapping miRNA between blood & colostrum	246	49	295	7	302
Total miRNA	558	447	1,004	194	1,198

Based on alignments with miRBase database (release 22, October 2018) and RumimiR database (updated January 2020)

5.3.4. MicroRNA expression profile in colostrum

Of the 303 miRNAs found from colostrum, 98% were known (Table 5.7). The majority of these miRNAs were found in both the dam and pooled colostrum samples (230 miRNAs,

76% of total) (Table 5.7 and Figure 5.14). This was despite the dam colostrum being collected from the individual Group A dams at day 0 (within 2 hours post-partum) and the pooled colostrum being a mix of colostrum and transition milk from many cows days 0-4 postpartum.

Table 5.7. Number of miRNAs in dam versus pooled colostrum sources.

	Known miRNA (miRBase)	Novel miRNA for miRBase, but known miRNA in RumimiR	Total known miRNA	Novel miRNA	Total miRNA
Dam colostrum (n = 4)	224	40	264	5	269
Pooled colostrum (n =4)	226	33	259	5	264
Overlapping miRNA	204	23	227	3	230
Total miRNA in colostrum (n = 8)	246	50	296	7	303

Based on alignments with miRBase database (release 22, October 2018) and RumimiR database (updated January 2020).

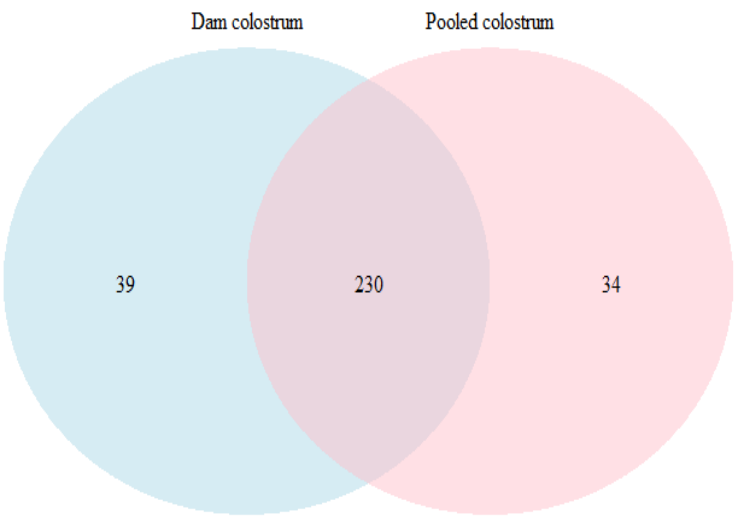


Figure 5.14. Number of miRNAs in dam and pooled colostrum samples.

Dam colostrum was from the individual Group A dams and the pooled colostrum was a mix of colostrum and transition milk from many cows 0-4 days postpartum. (n = 4 samples per colostrum source).

5.3.5. MicroRNA differential expression between colostrum sources

Only four miRNAs had significantly different read counts between the dam and pooled colostrum sources, miR-19b, miR-193b, miR-378 and miR-345-5p (Table 5.8). Of these miRNA, miR-19b and miR-378 were the more highly expressed (Table 5.9). Interestingly, four miRNAs had higher numbers of reads in the dam colostrum than in the pooled colostrum (Table 5.9). That is, their levels decreased during the lactation period.

Table 5.8. MicroRNAs differentially expressed between dam and pooled colostrum.

miRNA	log_2FC	Average Expression	t	P-Value	Adjusted P-Value
bta-miR-378	1.33	9.95	5.55	8E-05	0.02
bta-miR-19b	1.38	11.56	5.52	9E-05	0.02
bta-miR-345-5p	1.32	7.10	5.28	1E-04	0.02
bta-miR-193b	1.53	5.57	4.77	3E-04	0.04

n = 4 per colostrum source, log_2FC = log2 fold change, t = moderated t-statistic.

Table 5.9. MicroRNA read counts of the miRNA differentially expressed between dam and pooled colostrum.

Dam colostrum					Pooled colostrum				
Sample ID	miR-19b	miR-193b	miR-345-5p	miR-378	Sample ID	miR-19b	miR-193b	miR-345-5p	miR-378
CB25	83,487	1,671	3,841	30,040	CB29	11,453	272	587	3,509
CB26	50,272	467	1,599	10,239	CB30	14,158	203	1,074	5,277
CB27	40,340	785	2,049	12,602	CB31	17,449	297	597	6,320
CB28	25,477	511	1,531	11,676	CB32	9,291	80	284	2,881
Average	49,894	859	2,255	16,139	Average	13,088	213	636	4,497

5.3.6. MicroRNA profiles in calf blood

The number of miRNAs in calf blood samples was similar between the day 0 (within 4 hours after birth and before being fed) and day 1 (24 hours after birth and 2 feeds) (Table 5.10). A total of 1132 miRNAs were present in both the day 0 and day 1 calf blood samples (94%)

(Figure 5.15). The majority of miRNA overlapped between the three calf Groups at both day 0 and day 1 (63% and 65%, respectively) (Figure 5. 16).

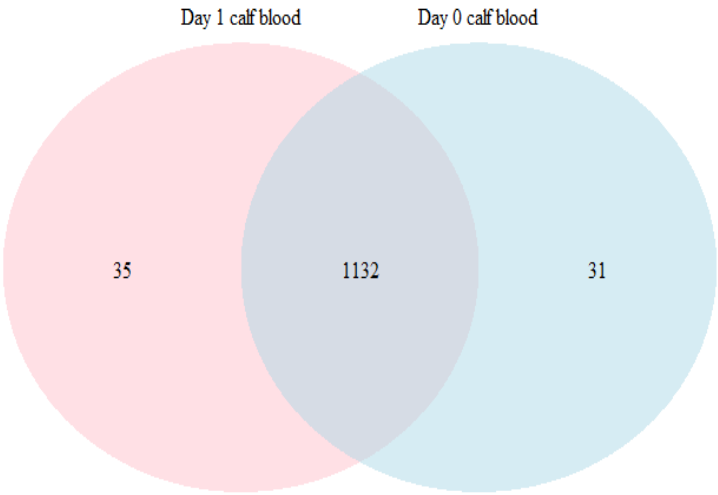


Figure 5.15. Number of miRNAs identified in blood collected from all calves at day 0 and day 1. *(n = 12 samples for each time point).*

Table 5.10. Number of known and novel miRNAs identified in blood collected from all calves at day 0 and day 1.

	Known miRNA (miRBase)	Novel miRNA for miRBase, but known miRNA in RumimiR	Total known miRNA	Novel miRNA	Total miRNA
Calf blood day 0 (n = 12)	540	437	977	186	1,163
Calf blood day 1 (n = 12)	545	430	975	192	1,167
Overlapping day 0 & day 1	527	421	948	184	1,132
Day 0 only	13	16	29	2	31
Day 1 only	18	9	27	8	35
Total miRNAs in calf blood (n = 24)	558	446	1,004	194	1,198

Based on alignments with miRBase database (release 22, October 2018) and RumimiR database (updated January 2020).

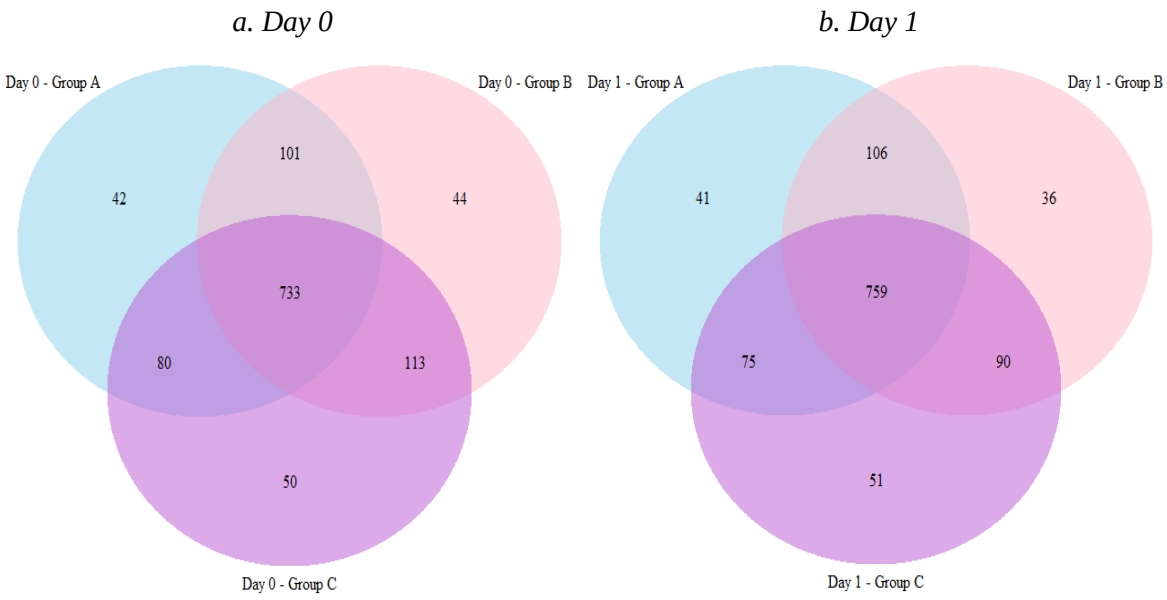


Figure 5.16. Number of miRNAs in three calf Groups identified from blood collected at day 0 and day 1. *Group A = calves received own dam colostrum, Group B = calves received colostrum from one Group A dam, Group C = calves received one bottle of pooled colostrum. n = 4 samples from each Group at each time point.*

5.3.7. Differential microRNA levels in calf blood over time

To investigate the levels of miRNA in the calf blood after they received colostrum, the calf blood samples from day 0 and day 1 were analysed, and the number of reads for 22 miRNAs differed significantly (Table 5.11). Of these 22 miRNAs, 19 miRNAs were lower in the day 1 blood samples than in the day 0 blood samples. The three miRNAs that were higher in the day 1 blood samples were let-7a-3p, miR-12042 and miR1260b (Table 5.12). These miRNAs may have been transferred from the colostrum to calf blood by day 1 or the expression of these three miRNAs by the calf may have increased after birth. Of note, let-7a-3p, miR-12042 and miR1260b were not in the list of the top 100 most highly expressed miRNA in the dam and pooled colostrum (Appendix 5.1-5.3) and they were not in the list of the 100 most highly expressed miRNAs in calf blood on day 0 and day 1 (Appendix 5.4-5.5).

Table 5.11. MicroRNAs with different levels between day 0 and day 1 calf blood.

miRNA	log_2FC	Average Expression	t	P-Value	Adjusted P-Value
bta-miR-424-5p	-1.83	1.30	-9.32	7E-10	3E-07
bta-miR-143	-1.68	6.86	-8.81	2E-09	5E-07
bta-miR-145	-1.71	1.63	-8.22	9E-09	1E-06
bta-miR-195	-1.05	2.45	-8.02	1E-08	2E-06
bta-miR-122	-2.73	2.22	-7.64	3E-08	3E-06
bta-miR-497	-1.32	1.68	-6.34	9E-07	3E-05
bta-miR-450b	-1.42	2.03	-5.89	3E-06	8E-05
bta-miR-455-5p	-2.34	-2.40	-5.54	7E-06	2E-04
bta-miR-10b	-1.46	3.82	-5.55	7E-06	2E-04
bta-miR-100	-1.13	1.37	-5.03	3E-05	5E-04
bta-let-7a-3p	1.42	3.02	4.98	3E-05	6E-04
bta-miR-503-5p	-2.11	-2.71	-4.85	5E-05	7E-04
bta-miR-125a	-1.14	2.85	-4.69	7E-05	1E-03
bta-miR-193a-3p	-2.04	-1.60	-4.31	2E-04	2E-03
bta-miR-12034	-2.42	-2.27	-3.85	7E-04	6E-03
bta-miR-455-3p	-1.55	-0.83	-3.75	9E-04	7E-03
bta-miR-1260b	1.18	3.22	3.77	8E-04	7E-03
bta-miR-196a	-1.07	-2.72	-3.24	3E-03	2E-02
bta-miR-205	-1.65	-0.11	-3.33	3E-03	2E-02
bta-miR-450a	-1.15	0.54	-3.27	3E-03	2E-02
bta-miR-12042	1.04	-2.66	2.80	9E-03	5E-02
bta-miR-1	-1.39	1.44	-3.01	6E-03	3E-02

n = 12 for each time-point; log_2FC = log2 fold change, t = moderated t-statistic)

Table 5.12. MicroRNA levels of let-7a-3p, miR-1260b and miR-12042 in calf blood at day 0 and day 1 and in the corresponding colostrum samples.

	Day 0 calf blood			Day 1 calf blood			Corresponding colostrum		
Animal ID	let-7a-3p	miR-1260b	miR-12042	let-7a-3p	miR-1260b	miR-12042	let-7a-3p	miR-1260b	miR-12042
A1	312	238	2	286	417	2	1848	4619	6
A2	97	161	1	303	1290	6	1243	1522	0
A4	83	171	2	439	756	10	2436	5062	0
A10	85	99	6	528	307	6	1039	2853	0
B1	101	111	0	4196	371	7	1848	4619	6
B2	90	114	1	343	449	8	1243	1522	0
B4	94	137	6	240	516	7	2436	5062	0
B10	101	243	7	290	262	6	1039	2853	0
C3	43	128	1	197	171	6	854	1585	0
C5	133	74	2	189	538	4	1504	1930	0
C8	39	88	0	219	97	1	962	1538	6
C11	87	64	4	272	131	5	497	550	0
Average	105	136	3	625	442	6	1412	2810	2

Units = number of reads

5.3.8. Relationship between miRNAs in day 0 colostrum and calf blood

In order to better assess whether any miRNAs were transferred from the colostrum to calf blood, the correlations between miRNA in day 0 colostrum and miRNA in day 1 calf blood were calculated (Table 5.13). There were only three miRNAs that had increased levels in calf blood by day 1 (let-7a-3p, miR-1260b, miR-12042) and might have been absorbed. All correlations between the miRNAs in day 1 calf blood and miRNAs in corresponding colostrum were low. This suggests that miRNAs in calf blood were not likely to be absorbed from the colostrum and the calves themselves are producing these miRNAs.

Table 5.13. Correlations between miRNAs in colostrum and in calf blood.

			Corresponding colostrum[a]			
		Method[b]	let-7a-3p	miR-1260b	miR-12042	miR-345-5p
Day 0	let-7a-3p	Spearman	0.45			
calf	miR-1260b	Pearson		0.49		
blood	miR-12042	Spearman			-0.54	
Day 1	let-7a-3p	Spearman	0.30			
calf	miR-1260b	Spearman		0.32		
blood	miR-12042	Spearman			-0.40	

[a] *Only the levels of those miRNA that were significantly higher at day 1 vs day 0 were analysed.*

[b] *Pearson correlation was used if both variables were parametric data. Spearman correlation was calculated if one or two variables were non-parametric data.*

n = 12 samples

5.3.9. MicroRNA target prediction and pathway analysis

In general, miRNAs are conserved between species, however, the same miRNAs may not have the same functions in different species (Van Hese et al., 2020). To understand the functions of the miRNAs in colostrum and their potential effects in the calf, the target genes for the top 100 most highly expressed miRNA in colostrum were predicted and a pathway analysis for the predominant miRNA target genes was performed. The top 100 most highly expressed miRNA were the 100 miRNAs with the highest number of normalized reads. In the addition, to understand the function of the miRNA in the calves after birth, the 22 miRNAs that had different levels between the day 0 and day 1 calf blood samples were examined.

The target genes for the 100 most highly expressed miRNAs across all colostrum samples (Appendix 5.1) were predicted by miRmap, and then Gene Ontology (GO) and KEGG analyses of the target genes were performed. The GO terms have unique identifiers and are organized hierarchically with ‘child’ terms (in the bottom of graph) being more specialized than their ‘parent’ terms. Among all the GO terms with a $P < 0.05$, there were 220 items which were found across all of the three main ontologies: biological process with 162 terms, cellular component with 33 terms, and molecular function with 25 terms (Figure 5.17 and Figure 5.18, Appendix 5.6). Many of these specific terms have the roles in the mammary

gland (e.g. ATP binding, bone development, protein and calcium iron regulation, transport, membranes), and some may have a role in development (e.g. bone and digit morphogenesis). Pathway analysis using the KEGG database only identified two pathways ($P < 0.05$) for the top 100 most highly expressed miRNAs in colostrum as being significant (autophagy and shear stress) (Table 5.14).

Table 5.14. KEGG analysis of 100 most abundant miRNAs in colostrum.

GO ID	Pathway	N	DE	P.DE	fdr
path:bta04140	Autophagy - animal	139	19	5E-05	0.02
path:bta05418	Fluid shear stress and atherosclerosis	140	18	2E-04	0.03

N = total number of genes related to the term; DE = number of genes in in the dataset for this term; P.DE = P-value; fdr= false discovery rate.

To investigate the 22 miRNAs that had significantly different levels between the day 0 and day 1 calf blood, 20 target genes for each of the 22 miRNAs were examined for their biological roles using the GO pathway analysis (Figure 5.19). There were 2 terms involved in biological processes and 10 terms involved in cellular components, particularly membranes and organelles. There were no significant pathways identified for the 22 miRNAs differentially expressed between the day 0 and day 1 calf blood from the KEGG pathway analysis.

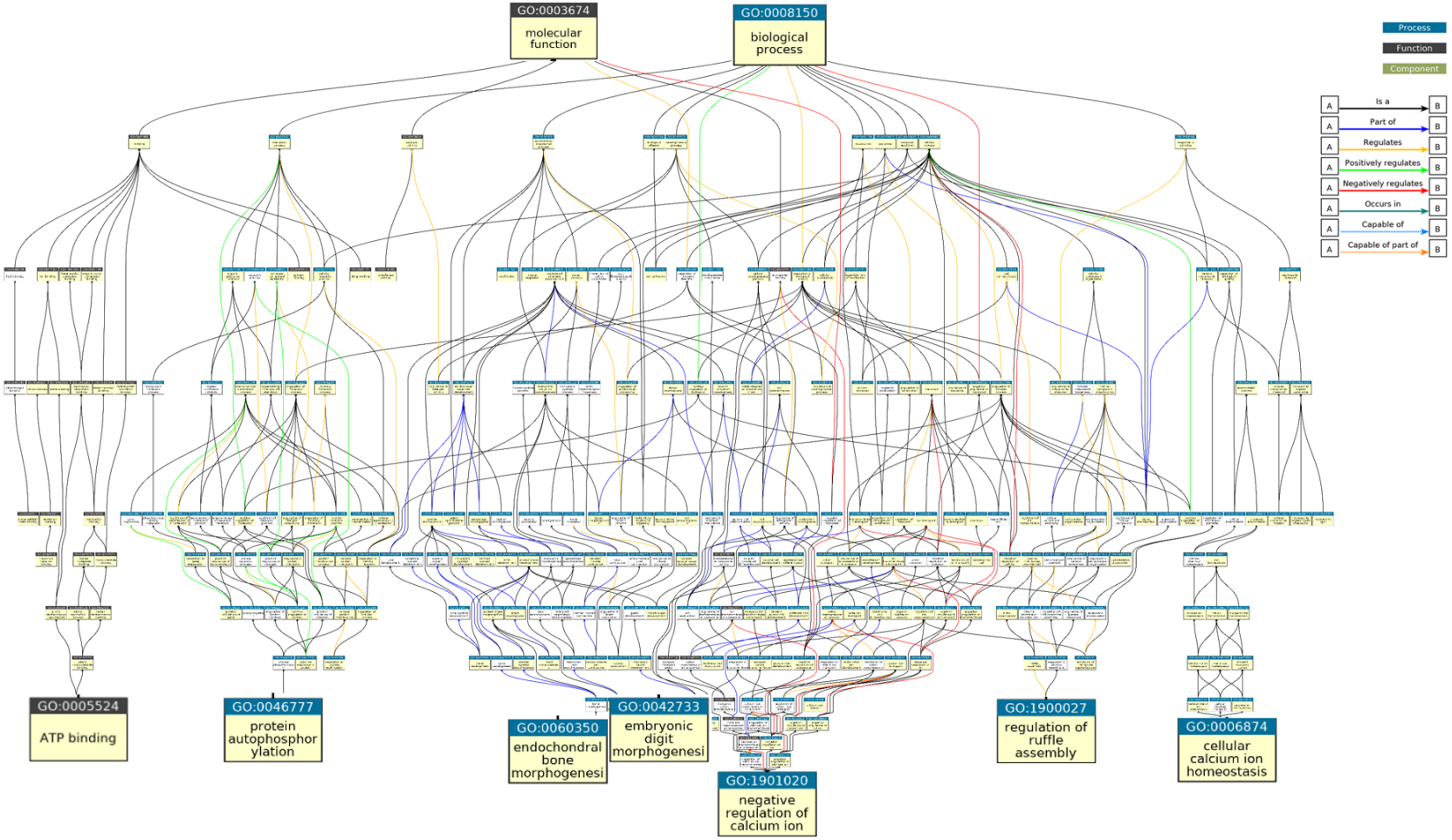


Figure 5.17. GO pathway analysis of the top 100 most highly expressed miRNAs in colostrum (Part 1).

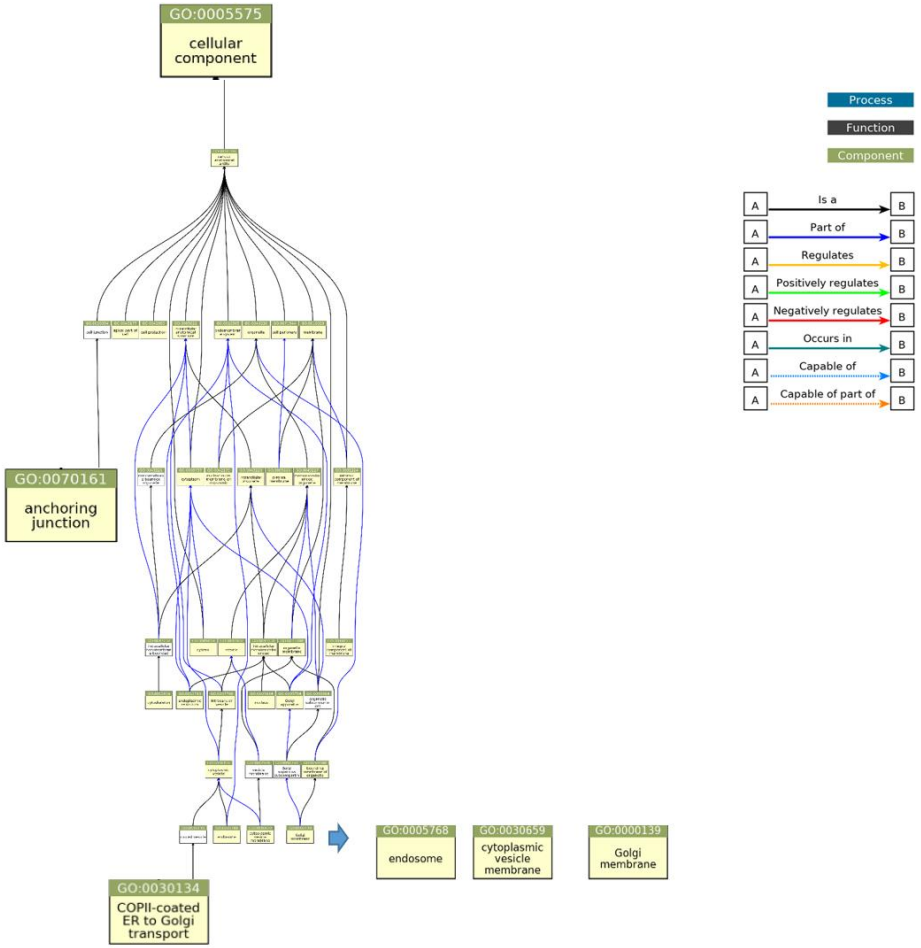


Figure 5.18. GO pathway analysis of top 100 most highly expressed miRNAs in colostrum (Part 2).

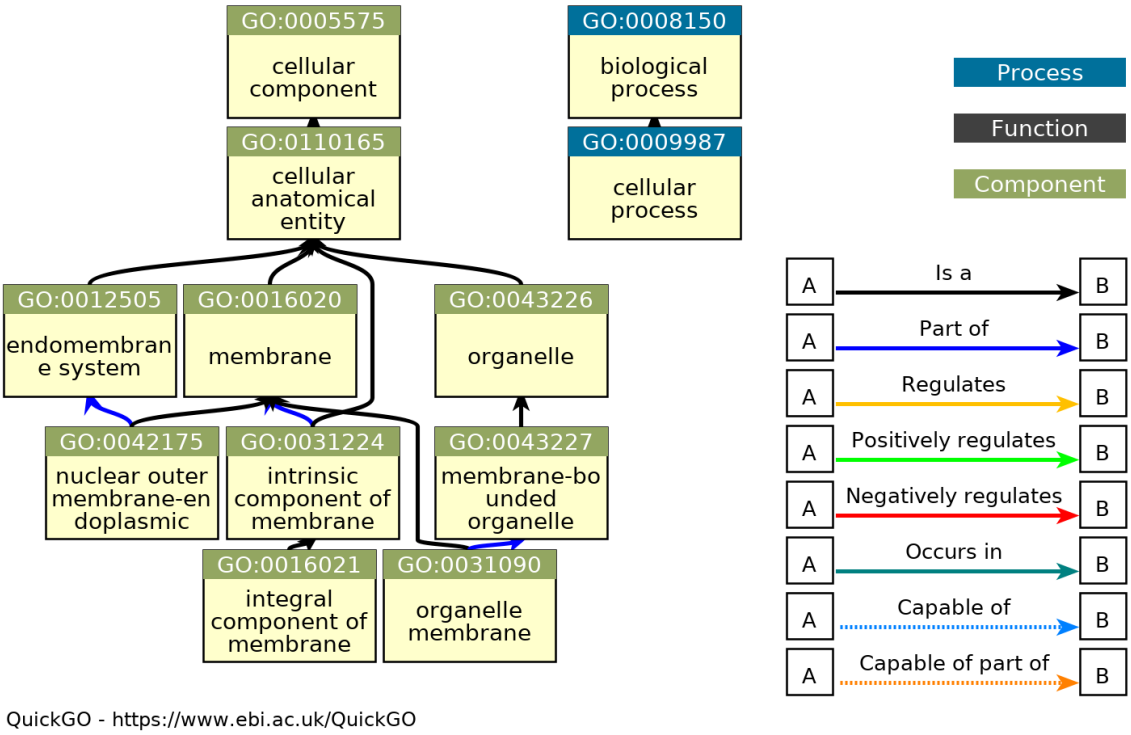


Figure 5.19. Pathway analysis of 22 miRNAs with different levels between day 0 and day 1 calf blood.

5.4. Discussion

The length of miRNAs found in the present study was between 17-25 nucleotides and 42% of the miRNAs were 22 nucleotides in length. Other studies involving milk and mammary gland miRNA reported similar size ranges (18-25 nt, Cai et al. (2018); 18-30 nt, Chen et al. (2010); 16-33 nt, Ozdemir (2020), 18-30 nt, Li et al. (2012b)) with 22 nucleotides being the most frequent length of miRNA (Li et al., 2012b; Cai et al., 2018).

The origin of bovine colostrum miRNAs is not known, and they may be derived from the cow blood, the mammary gland or both. As the miRNA in the blood of the dams was not sequenced, the origin of the miRNA found in their colostrum miRNA cannot be determined. However, in a human milk miRNA study, Alsaweed et al. (2016) found that some human milk miRNAs were more likely to be produced by the mammary gland as they were not

detected in maternal blood. Interestingly, the number of miRNAs in blood was four times higher than that in colostrum. This also suggests the origin of colostrum miRNA and blood miRNA are different, as the mammary gland secrets many fewer types of miRNAs.

5.4.1. MicroRNA profile of colostrum

The total number of miRNAs identified in the dam and pooled colostrum (303) was lower than found in previous studies (Do et al., 2017; Cai et al., 2018; Ozdemir, 2020), although the number varies depending on the method of identification and the bioinformatic analysis used (Table 5.15).

Table 5.15. MicroRNA numbers in bovine colostrum and milk in previous studies.

Sample	Known miRNAs	Novel miRNAs	Method	Reference
Milk (healthy vs mastitis)	492	980	miRNA sequencing	(Cai et al., 2018)
Raw Milk	245		Solexa sequencing	(Chen et al., 2010)
Milk	53		microarray	(Izumi et al., 2012)
Colostrum	100		microarray	(Izumi et al., 2012)
Colostrum	230		Solexa sequencing	Chen et al. (2010)
Bovine colostrum and milk	475	283	miRNA sequencing	(Do et al., 2017)
Bovine colostrum and milk	545	260	miRNA sequencing	(Ozdemir, 2020)

In this study, a total of 296 known miRNAs and 7 novel miRNAs were detected in the colostrum. The number of novel miRNAs found was much lower than previous studies, possibly because previous studies only mapped with the miRBase database (Do et al., 2017; Hou et al., 2017; Cai et al., 2018; Ozdemir, 2020) and not the recently updated RumimiR database. The number of known miRNAs was similar to a previous miRNA sequencing study of colostrum (Chen, et al. 2010), but the number was less than other studies that included both colostrum and mature milk (Do et al., 2017; Cai et al., 2018; Ozdemir, 2020). In this study, great care was taken to remove any potentially spurious data, so that only miRNAs with a minimum of five counts in at least four blood samples or two colostrum samples were retained. This step may miss low abundance sequences, such as reads detected

in only one sample, and this could also explain the differences in the number of miRNAs found in other studies (Do et al., 2017; Cai et al., 2018; Ozdemir, 2020).

The miRNA profile is dependent on the lactation period. Of the 303 miRNAs found in colostrum, 76% overlapped between the dam colostrum and the pooled colostrum. This proportion is similar to some previous studies that compared colostrum with mature milk but not all other studies. For example, 198 of the 213 miRNAs found in milk overlapped with the 230 miRNAs found in colostrum (81% of total miRNA detected) (Chen et al., 2010). On the other hand, in a microarray analysis, only 50% of the detected miRNA overlapped between milk and colostrum (Izumi et al., 2012). This will be depend on the probes selected though, which are likely to be for the most abundant miRNA.

Most of the colostrum components reduce steadily during lactation (McGrath et al., 2016; Godden et al., 2019). If miRNA had the same trend, then the miRNA levels in the dam colostrum would be expected to be higher than in pooled colostrum because the pooled colostrum in this study was a mixture of colostrum and transition milk from cows 0-4 days postpartum. However, there was only 4 miRNAs in the total of 302 detected miRNAs which were differentially expressed between dam and pooled colostrum, as the level of 298 miRNAs did not differ between dam and pooled colostrum. Hence, most of the miRNA did not appear to change immediately postpartum.

Most studies to-date have examined changes the miRNA profiles over the lactation period by focusing on differences between colostrum and mature milk rather than temporal changes in the colostrum composition over time postpartum. In a study by Do et al. (2017), the authors analysed bovine milk exosome miRNAs and found that the greatest difference in miRNAs was between day 1 colostrum and day 170 milk, where 338 miRNAs had different levels, while the levels of only 86 miRNAs were found to differ between day 1 colostrum and day 7 milk. The authors did not find any differences miRNA between day 170 and day 230 milk (Do et al., 2017). In another study comparing Holstein milk (2-4 months postpartum) with colostrum (days 1 and 2 postpartum), 120 miRNAs had increased levels in the milk and 112 miRNAs had lower levels in the milk (Ozdemir, 2020). In the Doğu Anadolu Kirmizisi cattle breed, Ozdemir (2020) found 208 miRNAs with higher levels in

milk (2-4 months postpartum) and 214 with lower levels in the milk compared to colostrum (days 1 and 2 postpartum).

Although the numbers of miRNA found in these previous studies differ, a greater concentration of miRNAs has been consistently found in colostrum compared to milk. In a study by Chen et al (2010), 108 of the 116 miRNAs had higher levels in colostrum than in milk. In the present study, only four miRNAs were found to have significantly different levels between dam and pooled colostrum. However, all four miRNAs had higher levels in colostrum compared with pooled colostrum. This low number of miRNAs that were found to be differentially expressed herein is most likely because dam colostrum was compared with pooled colostrum which will differ less than colostrum and mature milk.

The change of miRNA over the lactation period presumably reflects the transformation of the roles of these miRNAs in different biological processes and functions over time, in either the mammary gland or the calves. As these roles of the miRNAs would not be expected to alter much in the first few days postpartum, there may be little difference predicted in miRNA present. It would be of interest to expand the miRNA sequencing in this study to examine the miRNA expression in the dam colostrum at day 1, day 2, day 3 and day 7 to better define the transition of the miRNA profile.

In addition to the lactation period (Do et al., 2017; Hou et al., 2017; Ozdemir, 2020), there are other factors that may affect the miRNA profile in colostrum and milk (Van Hese et al., 2020). These include the environment (Colitti et al., 2019), nutrition, health status (Cai et al., 2018), species or breed (Ozdemir, 2020). Other factors may also affect the miRNA profile, such as parity and dam age, but these have yet to be studied (Van Hese et al., 2020). In order to understand the significance of miRNA in colostrum and milk, all of these factors should be investigated more thoroughly.

Ozdemir (2020) also found many of the immune-related miRNAs were higher in colostrum compared to milk, including bta-miR-130b, bta-miR-15b, bta-miR106a, and bta-miR-223. The higher level of immune-related miRNAs in colostrum than in milk was also found by Izumi et al. (2012) and by Sun et al (2013) in qPCR experiments. Herein, there were four

miRNAs at higher levels in the dam colostrum than the pooled colostrum (miR-19b, miR-193b, miR-345-5p, and miR-378). Most studies examining miRNA function have been in humans and unfortunately, these studies provide little clue as to the role of these miRNA in colostrum. High levels of miR-19b have been suggested as a potential biomarker for non-small cell lung cancer (Wu et al., 2014) and diabetic cardiomyopathy (Copier et al., 2017). Changes in miR-19b levels have also been associated with lymph node metastasis (Wu et al., 2014). MicroRNA-345-5p has been implicated in acute myeloid leukemia in humans as the decreased expression of miR-345-5p facilitates leukemia cell proliferation and apoptosis by targeting AKT1/2 (Ying et al., 2019). MicroRNA-378 has been proposed as a novel non-invasive biomarker in gastric cancer (Liu et al., 2012). In cattle, microRNA-193b inhibits bovine adipose cell proliferation and promotes apoptosis by targeting the Acyl-CoA synthetase short-chain family member 2 (ACSS2) (Kang et al., 2020).

Based on the pathway analysis of the top 100 most highly expressed miRNAs across all colostrum samples identified in this study, most are involved in various cellular processes, including organelles and Golgi membranes, calcium balance, cellular transport and energy, or in endothelial cell development and mammary gland function. However, there were immune functions in the pathways identified and some individual immune-related miRNAs were found amongst the colostrum miRNAs. Cai et al. (2018) observed that less than 10% of target genes of all miRNAs in Holstein milk were involved in immune system processes though.

5.4.2. MicroRNA profile of neonate calf blood

A large number of miRNAs were found in the calf blood from day 0 and day 1 (1,163 and 1,167 miRNAs, respectively), of which 94% overlapped between these two time-points (1,132 miRNAs). Yet when the levels of these miRNAs were compared between day 0 and day 1, only 22 miRNAs were found to have significantly different levels. This indicates that calves are born with a complex miRNA profile that does not change substantially after initial feeding of colostrum. Of the 22 miRNAs, three of the miRNAs had higher levels in the day 1 blood samples compared to the day 0 blood samples (let-7a-3p, miR-1260b and miR-12042). Nevertheless, the low correlations between the levels of these miRNAs in calf blood

day 1 and the corresponding colostrum suggest that these miRNAs were up-regulated after birth rather than being absorbed from colostrum.

Most studies have focused on the miRNA profiles in bovine colostrum and milk (Chen et al., 2010; Izumi et al., 2012; Hou et al., 2017; Cai et al., 2018), and only one study has considered miRNA absorption by calves (Kirchner et al., 2020). Kirchner et al. (2020) found that only three miRNAs of the top 15 most highly expressed miRNAs in colostrum exosomes were significantly increased in the calf blood after feeding colostrum (miR-200-a/b/c family). However, the authors explained the significant increase of miR-200a/b/c in the calf as differential expression instead of absorption from colostrum because of the role of the miR-200-a/b/c family in the processing of signals.

The pathway analyses of the 22 miRNAs with significantly different levels in the calf blood between day 0 and day 1 indicated that the target genes of these miRNAs are mainly involved in cell membranes. The three miRNAs (let-7a-3p, miR-1260b and miR-12042) found at higher levels in calf blood at day 1 after two feeds are also related to cellular functions. The functions of let-7a-3p and miR-1260b have been explored in humans but these studies have focused on cancer and other human diseases. A high level of miR-1260b is closely related with lymph node metastasis, venous invasion, and promotes the early-stage metastasis of colorectal cancer (Liu et al., 2016). Let-7a-3p has a role in the inhibition of neurotensin receptor 1, which plays an important part in cell proliferation and invasiveness of glioblastoma and induces intrinsic apoptosis (Dong et al., 2017). The let-7 miRNA family members though may affect IgA level in the digestive tract of the piglet (Chen et al., 2014). These miRNAs may be related to cell growth in neonates, but further studies are required to interpret their roles. Unfortunately, until the bovine target genes of these miRNAs have been identified, it is difficult to draw conclusions.

5.5. Summary and Conclusion

Both colostrum and calf blood contained a large number of miRNAs, but the number of different miRNAs in the calf blood was 4-fold higher than in colostrum. There were 303 miRNAs detected in colostrum (296 known and 7 novel miRNAs). A similar miRNA profile was found in dam and pooled colostrum with 76% of the miRNAs expressed in dam

colostrum also found in pooled colostrum. There were only four miRNAs which had significantly different levels between dam and pooled colostrum, and these four miRNAs were higher in dam colostrum. The top 100 most highly expressed miRNAs in colostrum were involved in several biological processes, but mainly contribute to cellular and mammary gland functions rather than functions that might benefit the newborn calf, such as immunity.

The total number of miRNAs found in calf blood was 1,198 miRNAs (1,004 known and 194 novel). The majority of these miRNAs (94%) were detected in the calf blood samples from both day 0 and day 1, and only 22 miRNAs had significantly different levels between the two time points. Pathway analysis of these 22 miRNAs indicated that these miRNAs were involved mostly in cell membranes and growth. Of these 22 miRNAs, three miRNAs had higher levels at day 1 after two colostrum feeds (let-7a-3p, miR-1260b and miR-12042), but these levels in the calf blood were not correlated with the levels in the corresponding colostrum. These findings suggest that miRNAs in the colostrum are not likely to be absorbed by the calves to any great extent.

Chapter 6

Quantification of immune-related miRNAs in colostrum and newborn calf blood by RT-qPCR

Chapter 6. Quantification of immune-related miRNAs in colostrum and newborn calf blood by RT-qPCR

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6.1. Introduction

Bovine colostrum provides nutrients and immunologically active factors that are essential for newborn calves (McGrath et al., 2016; Godden et al., 2019). In addition to nutrients and immune factors, such as immunoglobulins, lactoferrin and cytokines, cow milk and colostrum contains large concentrations of microRNA (miRNA) (Chen et al., 2010; Izumi et al., 2012; Cai et al., 2018). MicroRNAs regulate a wide range of biological processes, including immune function (Wienholds and Plasterk, 2005; Williams, 2007; Liu et al., 2010; Wang et al., 2013). However, whether these miRNA in the colostrum provide an immune function for neonate mammals is an open question.

In colostrum and milk, microRNAs are packaged in exosomes and are very stable (Chen et al., 2010; Hata et al., 2010). A study by Izumi et al. (2012) demonstrated that milk miRNAs are stable even under harsh conditions, including 37 ^{o}C for > 1 hour, acidic pH and in the presence of RNase. This stability suggests that colostrum miRNAs may be able to avoid degradation in the gastrointestinal tract and could be potentially absorbed in the intestine (Izumi et al., 2012).

The miRNA sequencing results in this study (Chapter 5) and elsewhere (Do et al., 2017; Kirchner et al., 2020) have confirmed that bovine colostrum, milk and calf blood contain many different types of miRNAs, including immune related miRNAs. For the past 20 years, immune-related miRNA has been a hot topic in human research. In fact, the tumour-related miRNAs and immune-related miRNAs found in the serum of cancer patients, but not in normal serum, have been used as biomarkers (Chen et al., 2008). Concentrations of circulating miRNAs in the blood of humans and other animals are high and very stable (Chen et al., 2008; Mitchell et al., 2008; Chen et al., 2012). It has not been clear though whether the miRNAs detected in neonate calf blood are produced by the calves themselves or are absorbed from the colostrum and milk.

The miRNA sequencing results (Chapter 5) suggest that miRNAs are not absorbed to any great extent by the calves. However, as the miRNA sequencing may not be sufficiently sensitive to detect the absorption of miRNA from the colostrum by the calves, this question was also investigated by RT-qPCR. Five immune-related miRNAs at various concentrations

in the colostrum were selected for further study (miR-142-5p, miR-150, miR-155, miR-181a and miR-223). These immune-related miRNAs have been reported previously to be present in both colostrum and milk (Chen et al., 2010; Sun et al., 2013). Their assessment as being "immune-related" in these studies is based on their synthesis in immune cells and their connection with various disease states (Appendix 6). Using blood samples from the 35 calves and the corresponding colostrum samples, these five immune-related miRNAs were quantified individually to determine whether they are absorbed by the neonate calf from the colostrum or produced by the calves themselves.

6.2. Materials and methods

6.2.1. Materials

The animals, experimental design and methods are as described in Chapter 2. The samples used for miRNA quantification by RT-qPCR included the dam colostrum from days 0, 1, 2 and 3 (n = 12 cows), the pooled colostrum (n = 5 samples), the bulk tank milk (n = 25 samples), and the calf blood from days 0, 1, 2, 3 and 7 (n = 35 calves).

6.2.2. Samples

Calf blood samples were collected within 4 hours of birth before feeding (day 0) and on days 1, 2, 3 and 7. Blood was drawn from the jugular vein of the calves into 6 mL BD vacutainers with EDTA, and immediately inverted gently 10 times to mix with the anticoagulant. Then 1 mL of whole blood from EDTA vacutainers was aliquoted into 3 mL of Trizol LS reagent (Catalog Numbers 10296010 and 10296028, Invitrogen, Thermo Fisher Scientific). The blood and Trizol LS reagent were mixed by pipetting, incubated at room temperature for 20 minutes, and frozen at -20°C. The samples were then transported on dry ice to The University of Adelaide, Roseworthy Campus for storage at -80°C until analysed.

Colostrum and milk samples were collected as described in Chapter 2. Colostrum and milk were thawed completely at room temperature prior to miRNA extraction, then 250 µL were added to 750 µL of Trizol LS reagent, mixed, incubated at room temperature for 20 minutes before RNA extraction.

6.2.3. RT-PCR

Five immune-related microRNAs (miR-142-5p, miR-150, miR-155, miR-181a and miR-223 Table 6.1) were quantified by RT-qPCR (Figure 6.1), using the *C. elegan* miRNA, cel-miR-39-3p, as an internal control for quantitation.

Table 6.1. Sequences of internal control (miR-39-3p) and five miRNAs.

miRBase ID	miRBase Accession Number	Sequence length	Mature miRNA Sequence	Assay ID
cel-miR-39-3p	MIMAT0000010	22	UCACCGGGUGUAAAUCAGCUUG	000200
hsa-miR-142-5p	MIMAT0000433	21	CAUAAAGUAGAAAGCACUACU	000465
bta-miR-150	MIMAT0003845	23	UCUCCCAACCCUUGUACCAGUGU	006586
hsa-miR-155	MIMAT0009241	23	UUAAUGCUAAUCGUGAUAGGGGU	002623
bta-miR-181a	MIMAT0003543	24	AACAUUCAACGCUGUCGGUGAGUU	005861
hsa-miR-223	MIMAT0000280	22	UGUCAGUUUGUCAAAUACCCCA	002295

Source: Thermo Fisher (https://www.thermofisher.com, accessed on 15/10/2018.

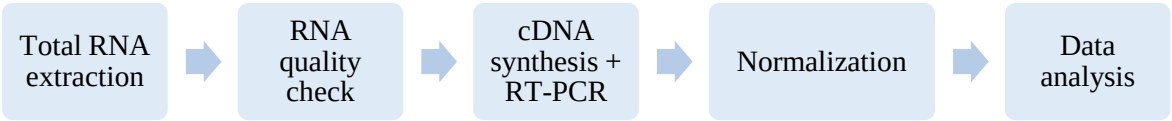

Figure 6.1. Workflow for miRNA quantification by RT-qPCR.

6.2.3.1. RNA preparation

Total RNA, including miRNA, was extracted from both colostrum and blood samples using Trizol LS (Catalog Numbers 10296010 and 10296028, Invitrogen, Thermo Fisher scientific). Cel-miR-39-3p (5 picomole/µL) (mirVana, miRNA mimic, ID: MC10956, Cat# 4464066, Lot# AS027ZX4) was used as an exogenous control by adding 1 µL to the colostrum, milk or calf blood before starting the extraction steps. All steps followed the manufacturer's protocol with some modifications as detailed in Chapter 2, Section 2.4.1.b. The concentration and quality of total RNA were assessed by a NanoDrop ND-1000 (Supplied and Service by BIOLAB, ThermoFisher). All samples that had a ratio of A260/280 in the range from 1.6 to 2.0 were used for RT-qPCR, and any sample of lower quality was re-extracted.

6.2.3.2. RT-PCR steps

The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in 5 steps on the high quality RNA samples as follows:

Step 1: Dilution of total RNA for reverse transcription (RT) reaction

Total RNA of good quality was diluted with ultra-pure water for the reverse transcription (RT) reaction. Total RNA from calf blood was diluted to approximately 2 ng/µL and the total RNA from the colostrum/milk samples was diluted to approximately 10 ng/µL.

Step 2: RT reaction

Total RNA was first transcribed into complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, product P/N 4366596 and 4366597, USA). The reagents in this kit convert total RNA into cDNA when used with specific miRNA primers supplied with the TaqMan MicroRNA assay (Table 6.1). cDNA for each sample was synthesized in duplicate. Briefly, a master mix for RT reaction was prepared and added to the reaction tubes (14 µL/tube), followed by 1 – 2 µL of diluted sample total RNA depending on the starting concentrations of the sample (Table 6.2). Each 15-µL RT reaction consisted of 14 µL (or 13 µL) master mix and 1 µL (or 2 µL) RNA sample depend on sample type.

Table 6.2. Reverse transcription reagents.

RT components	RNA from Calf blood sample	RNA from Colostrum/milk sample
Master mix		
Pooled RT primer*	6 µL	6 µL
dNTP mix (100mM total)	0.3 µL	0.3 µL
10 x RT buffer	1.5 µL	1.5 µL
Multiscribe RT enzyme (50 U/µL)	1 µL	1 µL
RNase inhibitor (20 U/ µL)	0.2 µL	0.2 µL
RNase free water	5 µL	4 µL
Total master mix/reaction	14 µL	13 µL
Diluted total RNA	1 µL of 2 ng/ µL [total RNA]	2 µL of 10 ng/ µL [total RNA]

** Pooled RT primer = 5 µL of each RT primer (miR-39-3p, miR-142-5p, miR-150, miR-155, miR-181a, miR-223) + 470 µL of RNase-fee water.*

A negative reverse transcription control (NC-RT) was included by adding 2 µL of ultra-pure water instead of diluted total RNA and was synthesized at the same time as the samples. The program for RT reaction was 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C and 5 minutes at 4°C.

To prepare the miR-39-3p for the qPCR standard curve, the miR-39-3p cDNA was transcribed following above RT steps and concentration of cDNA was calculated to 1.948 x 10^{11} copies/µL (Appendix 7).

Step 3: Dilution of cDNA for qPCR

The cDNA prepared from each of the samples was diluted at a ratio of 1:10 with RNase-free water and used as the template for the qPCR reaction. The miR-39-3p standard cDNA was diluted in serial 10-fold dilutions to create a standard curve (Table 6.3). Each dilution was aliquoted into Eppendorf tubes and stored at -80°C. Aliquots were thawed once for use to avoiding repeated multiple freeze-thaw cycles. The cDNA for the standard curve was amplified at the same time as the samples for each PCR amplification. cDNA from the standard solution (7.12 µL) was added in the PCR tube to a final volume of 15 µL, and the final concentration was determined from the standard curve.

Table 6.3. Dilutions of qPCR standards for standard curve.

Standard solution	Concentration (copies/ µL)	Standard cDNA added to PCR	Final standard concentration (copies/ µL)
Standard solution (SC1)	194,800,000,000		
SC2 (SC diluted 1: 10^2)	1,948,000,000	7.12	924,650,667
SC3 (SC diluted 1:10^3)	194,800,000	7.12	92,465,067
SC4 (SC diluted 1:10^4)	19,480,000	7.12	9,246,507
SC5 (SC diluted 1:10^5)	1,948,000	7.12	924,651
SC6 (SC diluted 1:10^6)	194,800	7.12	92,465
SC7 (SC diluted 1:10^7)	19,480	7.12	9,247

SC = Standard curve solution

Step 4: qPCR

Quantitation by real-time PCR was performed using a Corbett Robotics system (CAS Robotics 4 v4.9.1) to load the PCR materials into Gene-Disc 100 well rings, and the PCR ring was placed into a thermal cycler (Rotor-Gen 6000, Corbett Research, 2 PLEX). The reagents used for qPCR step were from TaqMan Universal Master Mix II, no uracil-N-glycoslyase (UNG) kit (Applied Biosystems) and all steps followed the protocol of manufacturer with modifications for the template amount. A negative reverse transcription control (NC-RT) was included in all RT-qPCR samples to test for contaminating DNA.

Briefly, a PCR master mix was prepared by mixing 7.5 µL of TaqMan Universal Master Mix II, no UNG 2x and 0.38 µL of TM primer 20X for each reaction. The master mix was added into each well of the ring, and the cDNA template was added to a final volume of 15 µL (Table 6.4). The cycle for PCR included 3 steps of 40 cycles as below:

Hold: 95°C for 10 minutes

Cycling: 95°C for 15 seconds, then 60°C for 60 seconds

Melting: ramp from 55 - 85°C,

The melting temperature was raised by 1 degree with each step. There was a 90 second pause for pre-melting conditioning at the first step but the pause was reduced to 5 seconds for each step thereafter. The fluorescence was measured at 470 - 510 nm, and the gain was optimized prior to the melting.

Table 6.4. PCR reagents.

Reagents	Standard solution Amount/tube (µL)	Sample Amount/tube (µL)
TagMan master mix, no UNG	7.5	7.5
TM primer (specific primer)	0.38	0.38
Ultra-pure water	0	1.12
Template	7.12 Serial dilution of cDNA of (miR39-3p)	6 Diluted (1:10) cDNA of sample
Total volume	15	15

Step 5: Normalization

To determine the original miRNA concentration, two steps of normalization were carried out using the Rotor-Gen 6000 software and equation.

First normalization: miR-39-3p was used for normalization in every PCR run. The negative control from the RT reaction and a negative control for the PCR were included with the samples in every PCR run to ensure there was no contamination at either the RT or qPCR steps. The standard curve from each run was used to assess technical variation, and if there was large variation in the standard curve, the run was repeated. A positive control, which was pooled total RNA from many samples, was included in each PCR run as a reference to compare between runs (Table 6.5).

Table 6.5. Copy number of positive control for qPCR.

miRNAs	Positive control (copies/µL)
miR-39-3p	29,126,698
miR-142-5p	298,685
miR-150	564,873
miR-155	80,857
miR-181a	29,259
miR-223	29,824,376

The first normalization step used Rotor-Gene 6000 software (Corbett, Life Science, Australia). Standard curves, which had a high R^2 (R^2 = 0.99) and efficiency in the range 0.9 – 1.1 and a good correlation between triplicates, were used for all runs. Results from the first 10 cycles of real-time amplification were removed from the analysis, and "auto Threshold" was used to obtain the baseline.

Second normalization

After obtaining the copy number from the software, the second step of normalization was to calculate the original miRNA concentration based on the cel-miR-39-3p concentration added at the RNA extraction step plus the RNA and cDNA dilutions for the RT and PCR,

respectively. The copy number of miRNAs in the sample was computed in the Excel based on RT-qPCR workflow (Figure 6.2).

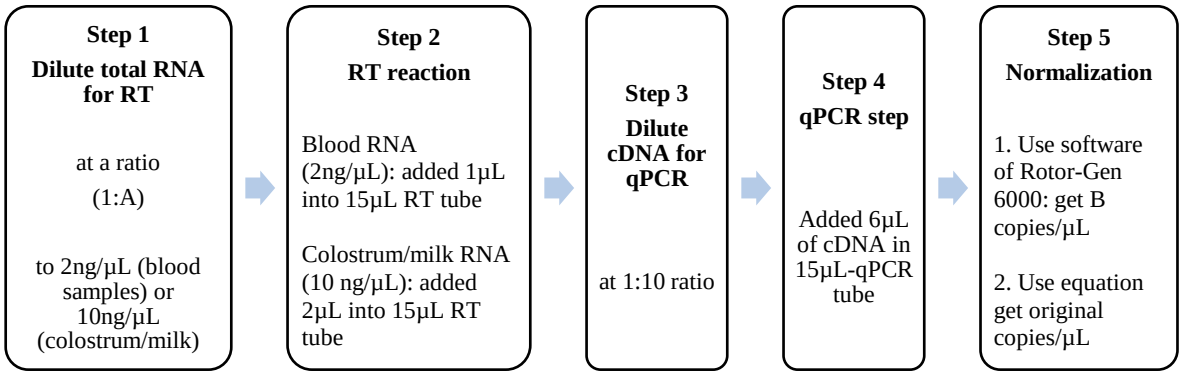

Figure 6.2. RT-qPCR workflow.

"A" in the step 1 is the dilution factor when diluting total RNA for RT reaction; "B" in the step 5 is the miRNA concentration (copies/µL) from software after first normalization.

The equations for the calf blood samples and colostrum samples were as follows:

Calf blood sample: [miRNA at step 1] = $\frac{B\ x\ 10\ x\ 15}{6}\ x\ A$

Colostrum sample: [miRNA at step 1] = $\frac{B\ x\ 10\ x\ 15}{6\ x\ 2}\ x\ A$

Where:

[miRNA at step 1]: miRNA concentration at step 1

A is dilution factor when diluting total RNA for RT reaction

B is copy number from software after normalization

As the miRNA assay was performed with separate RT and PCR assays, there were two different efficiencies for the assay. Therefore, using the concentration of cel-miR-39-3p (2.922 x 10^{12} copies) (Appendix 7) added to each sample prior the RNA isolation step, the final concentration of starting miRNA for each sample was calculated by following formula:

[Original miRNA] = $\frac{2.922\ \text{x}\ 10\text{^}12\ \text{copies}\ x\ [miRNA\ at\ step\ 1]}{[miR{-}39\ at\ step\ 1]}$

Where:

[miRNA at step 1]: miRNA concentration at step 1

[original miRNA]: original miRNA concentration in sample

6.2.3. Statistical analysis

All statistical analyses were conducted using two software packages, SAS version 9.4 (Statistical Analysis Software, Cary Inc. USA) and R (R version 3.6.3). The concentrations of five miRNAs (miR-142-5p, miR-150, miR-155, miR-181a and miR-223) in the dam colostrum, pooled colostrum and bulk tank milk were compared by a mixed linear model using the PROC MIXED procedure in SAS 9.4. The miRNA levels in dam colostrum (n = 12) over time (days 0, 1, 2 and 3) were compared by the PROC MIXED in SAS with fixed effect (time) and random effect (individual dam colostrum), which was a repeated measurement.

To compare the miRNA concentration (miR-142-5p, miR-150, miR-155, miR-181a, miR-223) in the three calf Groups (A, B and C) across time points (days 0, 1, 2, 3, and 7), the predicted means of dependent variables were compared using a mixed linear model in R by using the *asreml* package (Gilmour et al., 2015). The fixed effects were group, time point, and the interaction between the group and time point, and the random effect was the individual calves, which were repeated across time points. In addition, miRNA concentration (miR-142-5p, miR-150, miR-155, miR-181a, miR-223) in the 35 calves across time-points (days 0, 1, 2, 3 and 7) was analysed by a mixed linear model in R by using the *asreml* package (Gilmour et al., 2015) with one fixed effect (time-point) and random effect (individual calves), which were repeated across time points.

Correlations (including Pearson and Spearman correlations) between miRNAs in different samples (calf blood, colostrum or cow blood) were calculated based on the distribution of data. Normality was determined using Shapiro-Wilk test in the “nortest” package in R. A Pearson correlation was calculated if both variables had a normal distribution. The Spearman correlation was used if at least one variable was non-parametric. The Spearman correlation coefficient is based on the ranked values for each variable rather than the raw data.

6.3. Results

The samples for the quantifying the five immune-related miRNAs included the dam colostrum collected within 2 hours postpartum (day 0) and on days 1, 2 and 3. The dam colostrum was fed to their own calf (Group A) and a paired foster calf (Group B). Samples

were also taken from the colostrum pooled from several dairy cows days 0-4 postpartum and fed to the Group C calves for their first feeding (2 L). In addition, bulk tank milk was collected twice daily from the dairy cows more than 5 days postpartum and sampled. The bulk tank milk was used to feed the Group C calves from the second feeding until day 7 after birth and to feed the Group A and B calves from day 4 to 7 after birth. Blood from the three calf Groups were collected after birth and before feeding (day 0) and on days 1, 2, 3 and 7 after birth.

6.3.1. Immune-related microRNAs in colostrum and milk

MicroRNA concentration in the colostrum and bulk tank milk was determined by RT-qPCR for the five immune-related miRNAs (Figure 6.3, Appendix 8.1). Of the five miRNAs, miR-223 had the highest levels in all colostrum and milk samples. Both miR-223 and miR-155 had substantially higher concentrations than the other three miRNAs. In dam colostrum, the miR-223 and miR-155 concentration were 67.2 and 2.4 x 10^9 copies/µL at day 0 postpartum, whereas the levels of miR-142-5p, miR-150 and miR-181a were approximately 0.4 - 0.6 x 10^9 copies/µL.

Generally, the bulk tank milk had lower levels of all the miRNAs than the colostrum. There were significant differences in concentration of miR-142-5p, miR-155 and miR-181a between colostrum samples and bulk tank milk ($P < 0.05$). However, the dam colostrum only had higher concentrations of miR-155 than the pooled colostrum ($P < 0.05$). The pooled colostrum was significantly higher than bulk tank milk for the miR-181a concentration ($P < 0.05$). There were no significant differences in the miR-150 and miR-223 concentrations between dam colostrum, pooled colostrum and bulk tank milk, but the standard errors for these two miRNAs were notably very high.

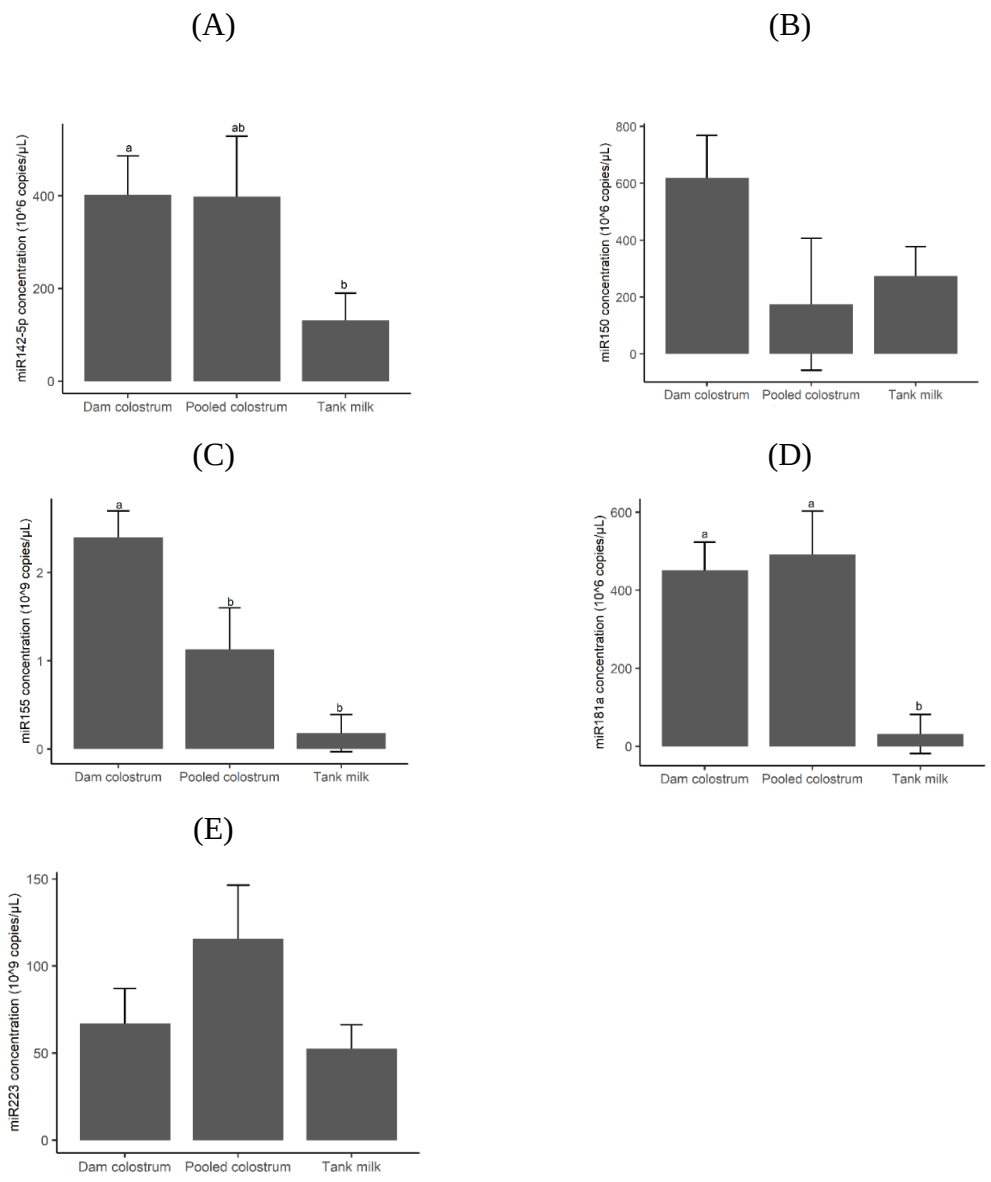

Figure 6.3. Concentration of microRNAs in day 0 dam colostrum (n = 12), pooled colostrum (n = 5), and bulk tank milk (n= 25).

(A) miR-142-5p, (B) miR-150, (C) miR-155, (D) miR-181a, and (E) miR-223. (Mean ± SE). Superscripts (a, b) indicate significant differences between least square means (P < 0.05).

6.3.2. Immune-related microRNAs dam colostrum across time-points

There was a significant difference between miRNA levels across time-points for all five miRNAs (P < 0.05). All five immune-related miRNAs in dam colostrum were highest at day 0 and then decreased 5- to 22-fold by day 3 (Figure 6.4, Appendix 8.2). All miRNAs decreased significantly by day 1 (2 to 6-fold) with the exception of miR-181a. The miR-181a concentrations in the dam colostrum at days 0 and 1 were similar but significantly

higher than the levels at days 2 and 3 (P < 0.05). MicroRNA-223 had the highest concentration and miR-155 had the second highest concentration compared to the other three miRNAs (miR-142-5p, miR-150 and miR-181a) across all time points.

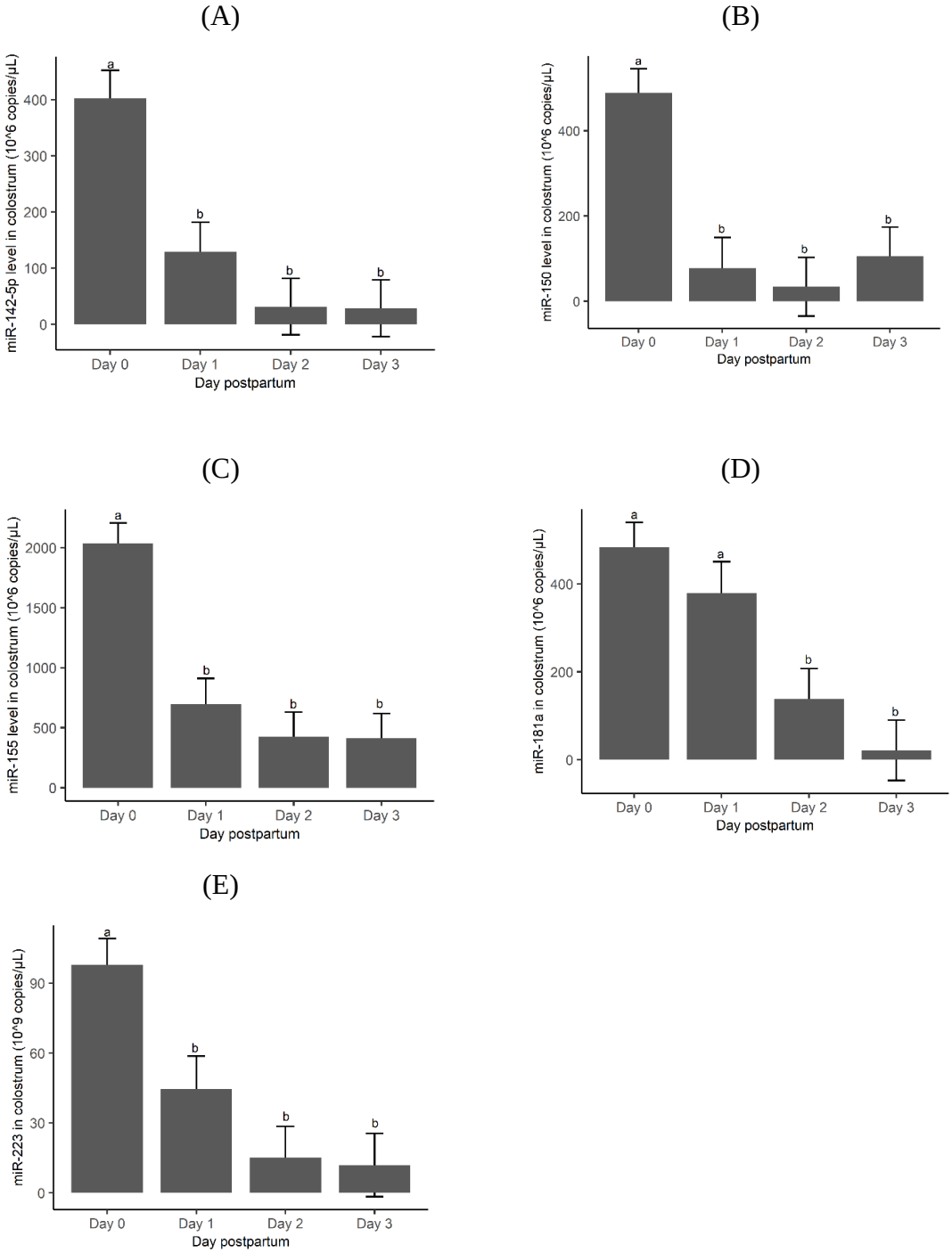

Figure 6.4. MicroRNA concentrations in dam colostrum day 0 to 3 postpartum.
(A) miR-142-5p, (B) miR-150, (C) miR-155, (D) miR-181a, and (E) miR-223. (Mean ± SE). Superscripts (a, b) indicate significant differences between means (P < 0.05). n = 12.

6.3.3. Immune-related microRNAs in the three calf groups within a week post-partum

In order to test whether any of the miRNA were transferred from colostrum to the calves, blood samples from all calves were taken at day 0, 1, 2, 3 and 7 after birth and the concentrations of the five immune-related miRNAs were measured. MicroRNA-223 had the highest concentrations of these miRNA at all time-points, followed by miR-150, miRNA-142-5p, miR-155 and miR-181a (Table 6.6). The miR-223 levels were 1000-fold greater than the miR-181a and miR-155 levels and 100-fold greater than the miR-150 and miR-142-5p levels.

Table 6.6. MicroRNA concentrations in all calves across time-points within one week after birth.

Time	miR-142-5p		miR-150		miR-155		miR-181a		miR-223	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	22.2^{z}	2.3	68.4^{x}	4.6	10.2^{y}	1.0	3.8^{y}	0.3	$5,056.8^{x}$	282.7
Day 1	18.1^{zw}	2.3	75.5^{x}	4.6	17.3^{x}	1.0	8.0^{x}	0.3	$5,255.9^{x}$	282.7
Day 2	33.9^{y}	2.3	46.9^{y}	4.6	7.2^{yz}	1.0	0.9^{zw}	0.3	$2,153.7^{y}$	282.7
Day 3*	12.9^{w}	2.3	23.5^{z}	4.6	2.7^{w}	1.0	0.2^{w}	0.3	471.6^{z}	286.8
Day 7	43.5^{x}	2.3	46.4^{y}	4.6	5.1^{zw}	1.0	1.8^{z}	0.3	$1,224.9^{xy}$	282.7

Unit: 10^9 copies/µL, n = 35.

** Day 3, Group B had 11 samples instead of 12 samples.*

$^{x, y, z, w}$ *LSM within a column with a different superscript letter are significantly different (P < 0.05).*

a. MicroRNA-142-5p

The miRNA-142-5p concentration was affected by Group, time and the interaction between Group and time (P < 0.05) (Table 6.7, Figure 6.5.A). The concentration of miR-142-5p in the calf blood was similar at days 0 and 1, suggesting that miR-142-5p was not absorbed from colostrum after two feedings. The level of miRNA-142-5p was higher at day 2 and was at the highest level by day 7 compared to other time points (P < 0.05). There were significant differences in miR-142-5p concentration (P < 0.05) between three calf Groups at day 7 with Group A calves, who were fed their own dam colostrum, having higher levels than Group B calves (fed foster cow colostrum) and Group C calves (fed pooled colostrum) (P < 0.05).

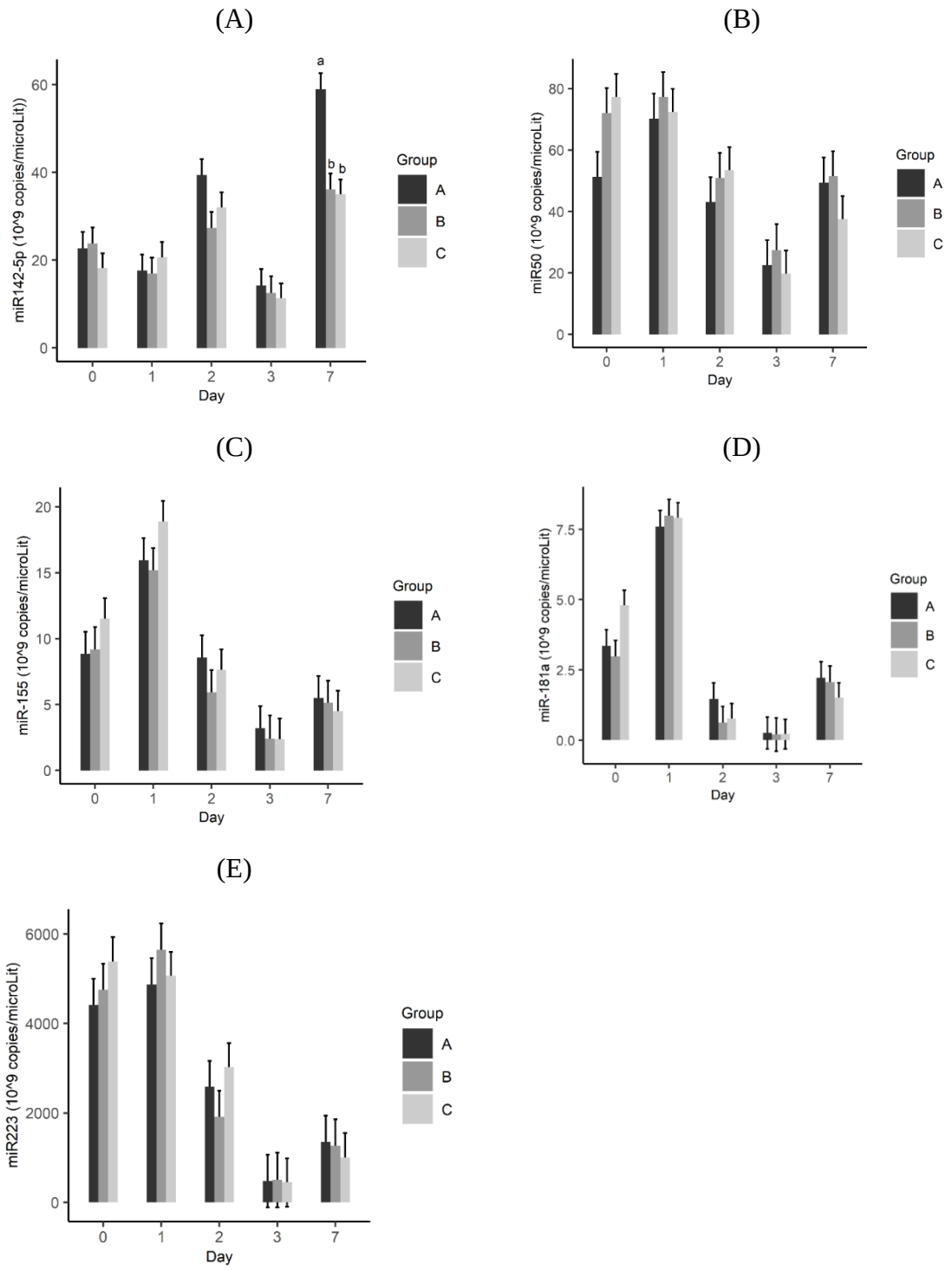

Figure 6.5. MicroRNA concentrations in three calf Groups within one week after birth. *(A) miR-142-5p, (B) miR-150, (C) miR-155, (D) miR-181a, and (E) miR-223. (Mean ± SE). Superscripts (a, b) indicate significant differences between means (P < 0.05). n = 12 for Groups A and B, n = 11 for Group C.*

Table 6.7. MicroRNA-142-5p in three calf Groups within one week after birth.

Time	Group A (n = 12)		Group B (n = 12)		Group C (n = 11)		All calves (n = 35)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	22.7	3.7	23.8	3.7	19.9	3.4	22.2^{z}	2.3
Day 1	17.6	3.7	16.9	3.7	19.9	3.4	18.1^{zw}	2.3
Day 2	39.3	3.7	27.3	3.7	35.3	3.4	33.9^{y}	2.3
Day 3*	14.3	3.7	12.5	3.7	11.9	3.4	12.9^{w}	2.3
Day 7	58.9^{a}	3.7	36.1^{b}	3.7	34.9^{b}	3.4	43.5^{x}	2.3

Units = 10^9 copies/µL.

Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed only a bottle of pooled colostrum. LSM = least square means, SE = standard error.

** Day 3, Group B had 11 samples instead of 12 samples.*

^{a,b,c} *LSM within a row of each time point with a different superscript letter are significantly different (P < 0.05).*

$^{x, y, z, w}$ *LSM within an "All calves" column with a different superscript letter are significantly different (P < 0.05).*

b. MicroRNA-150

The level of miR-150 in the calf blood gradually decreased over time (Table 6.8, Figure 6.5.B, P < 0.05). The miR-150 concentration was highest at days 0 and 1, then declined by days 2, 3 and 7 (P < 0.05). As the miR-150 level in the blood of the calves was high when the calves were born (day 0), this suggests that miR-150 may be transferred from their dam *in utero* or that the calves synthesized miRNA themselves prior to birth. This level did not increase between day 0 and day 1 (P > 0.05), and there were no differences between the calf Groups for any time point. This suggests that the miR-150 in the calf blood was not absorbed from the colostrum.

Table 6.8. MicroRNA-150 in three calf Groups within one week after birth.

Time	Group A (n = 12)		Group B (n = 12)		Group C (n = 11)		All calves (n = 35)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	51.3	7.7	72.0	7.7	83.1	8.1	68.4^{x}	4.6
Day 1	70.2	7.7	77.2	7.7	79.4	8.1	75.5^{x}	4.6
Day 2	43.0	7.7	50.8	7.7	46.9	8.1	46.9^{y}	4.6
Day 3*	22.6	7.7	27.3	7.7	20.4	8.1	23.5^{z}	4.6
Day 7	49.3	7.7	51.5	7.7	37.6	8.1	46.4^{y}	4.6

Units = 10^9 copies/µL.
Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed only a bottle of pooled colostrum. LSM = least square means, SE = standard error.
** Day 3, Group B had 11 samples instead of 12 samples.*
$^{x, y, z}$ *LSM within an "All calves" column with a different superscript letter are significantly different (P < 0.05).*

c. MicroRNA-155

The miR-155 level in the blood of all calves increased significantly from day 0 to day 1 (10 and 17 x 10^9 copies/µL, respectively; P < 0.05), then decreased by days 2, 3 and 7 with the lowest level at day 3 (Table 6.9 and Figure 6.5.C). The increased level on day 1 suggests that calves may have absorbed miR-155 from colostrum. However, if this was the case, then the Group A and B calves should have more miR-155 than Group C, as the dam colostrum had significantly more miR-155 than the pooled colostrum. There were no significant differences in miR-155 levels between the Groups though at any time, including day 1.

Table 6.9. MicroRNA-155 in three calf Groups within one week after birth.

Time	Group A (n = 12)		Group B (n = 12)		Group C (n = 11)		All calves (n = 35)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	8.8	1.7	9.2	1.7	12.8	1.8	10.2^{y}	1.0
Day 1	16.0	1.7	15.2	1.7	21.1	1.8	17.3^{x}	1.0
Day 2	8.6	1.7	5.9	1.7	7.1	1.8	7.2^{yz}	1.0
Day 3*	3.2	1.7	2.4	1.8	2.5	1.8	2.7^{w}	1.0
Day 7	5.5	1.7	5.1	1.7	4.7	1.8	5.1^{zw}	1.0

Units = 10^9 copies/µL.
Group A = calves fed own dam colostrum, Group B = calves fed foster dam colostrum, Group C = calves fed only a bottle of pooled colostrum. LSM = least square means; SE = standard errors.
** Day 3, Group B had 11 samples instead of 12 samples.*
$^{x, y, z, w}$ *LSM within an "All calves" column with a different superscript letter are significantly different (P < 0.05).*

d. MicroRNA-181a

The level of miR-181a in the calf blood was lower than the other miRNAs tested. There were no significant differences between three calf Groups at any time-points. However, like miR-150 and miR-155, the miR-181a concentration declined over time ($P < 0.05$). The highest level of miR-181a was at day 1, but decreased thereafter (Table 6.10 and Figure 6.5.D).

The miR-181a level in all calves increased from day 0 (3.8×10^9 copies/µL) to day 1 (8.0×10^9 copies/µL) after two feedings ($P < 0.05$). The concentration then declined, reaching the lowest concentration at day 3 (0.2×10^9 copies/µL). This suggests that miR-181a could have been absorbed from colostrum in the first 24 hours. There was no difference between the level of miR-181a in the dam colostrum and pooled colostrum though. Therefore, calf Group differences would not be expected and none were observed.

Table 6.10. MicroRNA-181a in three calf Groups within one week after birth.

Time	Group A (n = 12)		Group B (n = 12)		Group C (n = 11)		All calves (n = 35)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	3.4	0.6	3.0	0.6	5.1	0.5	3.8^{y}	0.3
Day 1	7.6	0.6	8.0	0.6	8.5	0.5	8.0^{x}	0.3
Day 2	1.5	0.6	0.6	0.6	0.6	0.5	0.9^{zw}	0.3
Day 3	0.3	0.6	0.2	0.6	0.2	0.5	0.2^{w}	0.3
Day 7	2.2	0.6	2.1	0.6	1.2	0.5	1.8^{z}	0.3

Units = 10^9 copies/µL.

Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed only a bottle of pooled colostrum. LSM = least square means; SE = standard errors.

** Day 3, Group B had 11 samples instead of 12 samples.*

$^{x, y, z, w}$ *LSM within an "All calves" column with a different superscript letter are significantly different ($P < 0.05$).*

e. MicroRNA-223

Of the five immune-related miRNAs examined, miR-223 had the highest concentration in colostrum, pooled colostrum, and bulk tank milk as well as in calf blood. In the calf blood, the highest levels of miR-223 in the calf blood were at day 0 and 1 (Table 6.11 and Figure

6.5.E) and there was no difference in the level between these days (50.57 x 10^{12} copies/µL, and 52.56 x 10^{12} copies/µL, respectively). The level then decreased in the calf blood with the lowest concentration at day 3 (4.72 x 10^{12} copies/µL). Before receiving colostrum, miR-223 concentration was very high in calf blood and increased slightly, though not significantly, after 2 colostrum feedings (day 1) in the Group A and B calves. This increase was not observed in the Group C calves that received 1 bottle of pooled colostrum.

Table 6.11. MicroRNA-223 in three calf Groups within one week after birth.

Time	Group A (n = 12)		Group B (n = 12)		Group C (n = 11)		All calves (n = 35)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Day 0	4,410.7	484.1	4,753.7	484.1	6092.3	505.6	5,056.8^{x}	282.7
Day 1	4,874.0	484.1	5,650.6	484.1	5241.8	505.6	5,255.9^{x}	282.7
Day 2	2,578.6	484.1	1,909.6	484.1	1956.5	505.6	2,153.7^{y}	282.7
Day 3*	473.8	484.1	496.7	505.6	441.0	505.6	471.6^{z}	286.8
Day 7	1,354.3	484.1	1,269.1	484.1	1035.5	505.6	1,224.9^{xy}	282.7

Units = 10^{9} copies/µL.

Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed only a bottle of pooled colostrum. LSM = least square means; SE = standard errors.

** Day 3, Group B had 11 samples instead of 12 samples.*

$^{x, y, z}$ *LSM within an "All calves" column with a different superscript letter are significantly different (P < 0.05).*

6.3.4. Immune-related microRNAs in cow blood

The five immune-related miRNAs were also measured in the Group A dam blood within 2 hours after parturition (Table 6.12), as the Group A calves may have received the miRNAs *in utero* from their dams. The levels of all the microRNAs were higher in the blood than the colostrum with the exception of miR-181a, which was very similar between blood and colostrum (~500 x 10^{6} copies/µL). The miR-181a level was the lowest in the blood and colostrum. MicroRNA-223 had the highest level in colostrum (67 x 10^{9} copies/µL) and the highest level in the cow blood (12,000 x 10^{9} copies/µL). The concentration of miR-150 was also much higher in the blood than the colostrum (300 x 10^{9} copies/µL vs 0.6 x 10^{9}

copies/µL, respectively). There was a 13- and 19-fold difference in concentration between blood and colostrum for miR-155 and miR-142-5p, respectively.

Table 6.12. Concentration of five immune-related miRNAs in dam blood within 2 hours postpartum.

miRNAs	n	Mean	SE	Min	Max	95% CI
miR-142-5p	12	7.8	2.9	0	35.3	1.5 - 14.1
miR-150	12	307.7	45.7	99.9	579.5	207.0 - 408.3
miR-155	12	31.3	7.2	7.10	87.2	15.5 - 47.2
miR-181a	12	0.5	0.3	0	2.8	0.1 - 1.1
miR-223	12	12302.3	2266.5	2589.4	26425.6	7313.8 - 17290.7

Unit = 10^9 copies/µL.

SE = standard error.

6.3.5. Correlations of immune-related microRNAs

a. Day 0 colostrum and cow blood miRNA correlations

The correlations between the levels of the five immune-related miRNAs in the dam colostrum and corresponding dam blood sampled within 2 hours postpartum were not high (Table 6.13). These correlations suggest that the synthesis of colostrum miRNA occurs in the mammary gland rather than being sourced from the blood. Consequently, the levels of miRNA in the blood should not use to estimate the levels of miRNA in the colostrum.

Table 6.13. Correlations between immune-related miRNAs in dam colostrum and blood within two hours post-partum.

Correlation		Day 0 colostrum				
		miR-142-5p	miR-150	miR-155	miR-181a	miR-223
Cow blood	miR-142-5p[2]	-0.39				
	miR-150[2]		-0.01			
	miR-155[2]			0.29		
	miR-181a[2]				-0.11	
	miR-223[1]					0.01

[1]*Pearson correlation if both variables were normal distribution.*

[2]*Spearman correlation if any variable was non- normal distribution. (n = 12).*

b. Cow blood and day 0 calf blood miRNA correlations

To determine whether miRNAs are transferred from the dam's blood to calf blood *in utero*, correlations between miRNA levels in the cow blood 2 hours postpartum and day 0 calf blood were calculated (Table 6.14). All correlations between immune-related miRNAs in dam blood and day 0 calf blood were low and negative, except for miR-155. The correlation between miR-155 in the dam blood and miR-155 in Group A calf blood at day 0 was 0.76 (P = 0.004). This result suggests that only miR-155 in the dam blood may be potentially transferred to calf blood during pregnancy via the uterine epithelial cells.

Table 6.14. Correlations between miRNAs in dam blood and day 0 Group A calf blood.

Correlation		Day 0 calf blood				
		miR-142-5p	miR-150	miR-155	miR-181a	miR-223
Cow blood	miR-142-5p[2]	-0.16				
	miR-150[1]		-0.30			
	miR-155[1]			0.76		
	miR-181a[2]				-0.30	
	miR-223[1]					-0.15

[1]*Pearson correlation if both variables were normal distribution.*

[2]*Spearman correlation if any variable was non- normal distribution. (n = 12).*

c. Day 0 colostrum and day 1 calf blood miRNA correlations

Correlations between the miRNA levels in the colostrum fed at day 0 and the respective calf blood at day 1 were calculated for both the dam and pooled colostrum (Table 6.15). The correlations between the miRNA levels in the day 1 calf blood and the corresponding colostrum were low and/or negative. This finding suggests that colostrum miRNAs were not absorbed into calf blood.

Table 6.15. Correlations between miRNAs in day 1 calf blood and corresponding colostrum.

Correlation		Corresponding colostrum				
		miR-142-5p	miR-150	miR-155	miR-181a	miR-223
Day 1 calf blood from Group A (n=12)	miR-142-5p[1]	-0.61				
	miR-150[2]		0.03			
	miR-155[2]			0.11		
	miR-181a[2]				-0.08	
	miR-223[2]					-0.06
Day 1 calf blood from Group B (n=12)	miR-142-5p[1]	-0.12				
	miR-150[2]		-0.03			
	miR-155[2]			-0.03		
	miR-181a[2]				-0.09	
	miR-223[1]					0.04
Day 1 calf blood from Group A & B (n=24)	miR-142-5p[2]	-0.4				
	miR-150[2]		-0.04			
	miR-155[2]			0.03		
	miR-181a[2]				-0.09	
	miR-223[2]					-0.05
Day 1 calf blood (n=35)	miR-142-5p[2]	-0.23				
	miR-150[2]		-0.01			
	miR-155[2]			-0.19		
	miR-181a[2]				-0.19	
	miR-223[2]					0.12

[1]*Pearson correlation if both variables were normal distribution.*

[2]*Spearman correlation if any variable was non- normal distribution.*

6.4. Discussion

Studies on immune-related miRNAs have been mainly in humans with only limited studies in other species (Zhou et al., 2012; Sun et al., 2013; Huang, 2015; Van Hese et al., 2020) and very little research on bovine miRNAs (Izumi et al., 2012; Sun et al., 2013; Cai et al., 2018). Human miRNA research has focused on cancer as 50% of the miRNA genes are located in fragile sites or chromosomal regions related to cancer (Faraoni et al., 2009). As a consequence, the target genes of these immune-related miRNAs have been investigated in

humans (Karaca et al., 2018) and mice being used as model species for human cancer (Blüml et al., 2011; Kramer et al., 2015).

While miRNAs have been identified in a variety of bovine tissues, in addition to colostrum and milk, little is known of their functions (Van Hese et al., 2020). Some studies have shown differential expression of cow colostrum and milk miRNAs in different environments (Colitti et al., 2019), health status (Cai et al., 2018), breeds (Ozdemir, 2020) and lactation periods (Do et al., 2017; Hou et al., 2017; Ozdemir, 2020). However, the function of the miRNAs in bovine colostrum and milk is not yet known and the miRNA concentrations needed for biological effects have not been explored (reviewed by Van Hese et al. (2020)).

In the present study, five milk-borne miRNAs, considered to be immune-related (Chen et al., 2010; Izumi et al., 2012; Sun et al., 2013) were quantified to determine if they were absorbed from the colostrum by the neonatal calves. These miRNAs are found in both B and T cells in the blood and also are present in both bovine colostrum and milk at relatively high concentrations (Chen et al., 2010; Sun et al., 2013).

6.4.1. Immune-related miRNAs in colostrum and milk

The five miRNAs quantified herein had the highest level at day 0, and then dramatically decreased over time postpartum. The studies on miRNA quantification in bovine colostrum across time postpartum are limited, with most only comparing miRNAs levels in colostrum versus mature milk (Do et al., 2017; Ozdemir, 2020).

Of the five miRNAs, miR-223 had the highest level in the colostrum and milk. The concentration of miR-223 in the individual dam colostrum, pooled colostrum and bulk tank milk were 67.2, 115.6, and 52.4 x 10^9 copies/µL, respectively, but there was no significant difference in miR-223 levels between colostrum and milk. Similar concentrations of miR-223 in colostrum and in milk were found in a study by Sun et al. (2013). MicroRNA-155 had the second highest concentration in the colostrum and milk. High concentrations of both miR-223 and miR-155 have been reported in the colostrum of goats and humans (Na et al., 2015) and in bovine mammary tissue (Wang et al., 2012).

The higher levels of miR-223 and miR-155 in both colostrum and milk compared to other miRNAs is presumably the result of higher expression levels of miR-233 and miR-155 in the mammary gland tissues during lactation (Li et al., 2012a; Wang et al., 2012; Hou et al., 2017; Van Hese et al., 2020). MicroRNA-223 and miR-155 are two of only 13 miRNAs for which expression increases in bovine mammary tissue in the lactation period compared to the dry period (Wang et al., 2012). This suggests that the miRNA profiles in colostrum and milk are related to miRNA expression in the mammary gland rather than being derived from the cow's blood.

However, the origin of miRNA in colostrum or milk is not known and could be from active secretion by epithelial cells of the mammary gland or by passive transfer from the cow's serum (Chen et al., 2010). Different colostrum components are secreted by different mechanisms. While some proteins are synthesised directly in the mammary gland, others are transferred from cow's blood, such as IgG (reviewed by Hou et al. (2017)). In particular, IgG1 is derived mainly from blood, with transport across mammary alveolar cells (Gapper et al., 2007). The low correlations between the levels of the miRNAs in the dam blood and the levels of the miRNAs in the colostrum herein suggest that these miRNAs are more likely to be produced in the mammary gland. In humans, Alsaweed et al. (2016) concluded that milk miRNAs primarily originate from the mammary epithelium and the maternal blood only has a small contribution because the authors found unique miRNAs that only appear in milk and were not detected in the maternal blood. In a study in goats, Li et al. (2012a) also concluded that milk miRNAs originate from the mammary gland cells. They observed that 13 miRNAs (let-7a, -7b, 7c, 7f, 7g, miR-21, 230a, 2103, 2107, 2143, 2148a, 2320 and 2423-5p) in the top 20 most expressed miRNAs in the mammary gland were also among the top 20 most expressed miRNAs in raw milk.

The dam colostrum had significantly higher levels of miR-142-5p, miR-155 and miR-181a than the bulk tank milk. Significant differences between colostrum and milk for these miRNAs have been reported previously (Izumi et al., 2012; Sun et al., 2013). MicroRNA-155, miR-181a, and miR-223 had higher concentrations in bovine colostrum than in mature milk based on quantitative PCR analysis (Izumi et al., 2012). MicroRNA-181a was also one of 7 miRNAs that had a higher copy number in colostrum than in milk based on Solexa

sequencing results (6.11 fold change) (Sun et al., 2013). There are only a few studies on the miRNA profile differences between colostrum and milk thus far (Do et al., 2017; Hou et al., 2017; Ozdemir, 2020). These previous studies have shown differential expression of miRNA with different numbers of miRNAs up-regulated and down-regulated in colostrum compared to milk. In general though, more miRNAs have higher levels in colostrum compared to in milk. For example, 108 miRNAs of 116 miRNAs were up-regulated in colostrum compared to mature milk in a study by Chen et al. (2010). Many immune-related miRNAs in Holstein colostrum were also higher than in milk, including miR-130b, miR-15b, miR106a, and miR-223, in a study by Ozdemir (2020).

Despite miRNAs being present in high concentrations in colostrum and milk, the functions of many miRNAs are not well delineated yet. Many miRNAs are considered to be immune-related because they are abundantly expressed in immune cells and/or play a role in lymphocyte development and cell generation (Vigorito et al., 2013; Xu et al., 2016; Shrestha et al., 2017; Yuan et al., 2018). However, some miRNAs have been shown to directly regulate cytokine levels and immune cell function (Sun et al., 2013). For example, miR-106a has been found to regulate IL-10 expression in humans, and Il-10 is known to be a key orchestrator of the immune system (Sharma et al., 2009).

Evidence of other miRNA being immune-related is based on the level of their expression being associated with health status. For instance, miR-142-5p and miR-223 levels are elevated in colostrum if the cow has mastitis (Cai et al., 2018). Naeem et al. (2012) found that three miRNAs (miR-181a, miR-16, and miR-31) were approximately 3- to 5-fold lower and miR-223 was approximately 2.5-fold higher in mammary tissues of cows with mastitis. Although these studies found the differential expression of miR-142-5p, miR-181a and miR-223 in cows with mastitis, to date, their purpose in the mammary gland has not been defined (Naeem et al., 2012; Cai et al., 2018).

The question is whether these miRNAs in colostrum and milk could be functional in the neonate. Strong evidence that microvesicles (exosomes) are able to deliver miRNAs or mRNA to cells is available (Skog et al., 2008; Zhang et al., 2010; Sun et al., 2013). Sun et al. (2013) showed that after incubation of mature milk or colostrum-derived vesicles

containing seven miRNAs, the concentration of three of the miRNAs (miR-106a, miR-181a and miR-451) increased significantly in macrophage cells, while the other miRNAs levels were unchanged. Sun et al. (2013) concluded that structure of the colostrum exosomes is important for transferring miRNAs from exosomes into cells. Exposing the macrophage cell line RAW264.7 to the miRNA containing exosomes increased in IL-1β production and decreased in IL-10, and these effects were blocked by miRNA inhibitors (Sun et al., 2013). Therefore, exosomes containing miRNAs can deliver miRNA to cells and those miRNAs can have an effect on cell function, as demonstrated in this case with an immune-related response.

6.4.2. Immune-related miRNAs in calf blood at birth

The results herein indicate that calves are born with the five immune-related miRNAs in their blood (miR142-5p, miR-150, miR-155, miR-181a and miR-223). The calves either received these miRNAs from their dams during pregnancy or these miRNAs were synthesized by calves themselves. The synepitheliochorial bovine placenta structure separates the maternal circulation and the fetal blood stream. However, the migration of fetal chorionic binucleate cells (BNC) is fundamental to the development of ruminant placenta, particularly the uterine epithelial cells (Wooding, 1992). These cells may be involved in the transfer of miRNAs between the dam and calf, and this may have been the source of the miRNA detected.

A relationship between miRNAs in fetus and their dam blood has been reported previous cattle studies (Pigati et al., 2010; Ioannidis and Donadeu, 2016), as miR-155 and miR-26a were found at high levels in the bovine embryos and also were highly expressed in their dam's blood at early pregnancy (day 16) (Ioannidis and Donadeu, 2016). In a cattle cloning study, De Bem et al. (2017) found that decreased exosomal-miRNAs in maternal blood at 21 days of gestation was correlated with the miRNA profile of the embryos.

To examine the relationship between miRNAs in fetus and maternal blood, the level of the five immune-related miRNAs was determined in the dam blood within 2 hours postpartum and in the calf blood within 4 hours postpartum. Only miR-155 was found to be correlated ($r = 0.76$, $P = 0.004$). Therefore, the transfer or absorption of maternal miRNA by the fetus

may not be a general phenomenon. The level of miR-155 was lower than miR-150 and miR-223 in the dam blood at 2 hours postpartum, so a concentration effect is not likely to be the cause. Rather, the transfer or absorption of miRNA by the fetus may be selective if it is occurring.

The release of miRNAs from cells into blood and milk can be selective. For example, malignant mammary epithelial cells produce and release miR-451 and miR-1246, but these miRNAs are retained in non-malignant mammary epithelial cells (Pigati et al., 2010). In another example, miR-106a, miR-181a and miR-451 in vesicles were shown to be transferred to milk somatic cells in *in vitro* culture but other miRNAs were not transferred (Sun et al., 2013). This suggests that the transfer of miRNAs can be selective although the mechanisms involved have not been yet delineated. Further studies examining the miRNA profiles of fetuses, newborns and their dams would provide a clearer picture of which miRNA are transferred and their likely functions.

In the case of miR-155 where there was a correlation between the levels in the calf blood and the dam blood, it was not obvious if there was a transfer from the dam blood to the fetus or from the fetus to the dam. The origin of the circulating miRNAs in the maternal blood can be from the bovine placenta (De Bem et al., 2017) and the bovine placenta is derived from the embryos (Hossain et al., 2014). The ability of bovine embryos to synthesise miRNAs and to transfer these to the blood of their dams via the placenta has been shown in a study of cloned cattle (Hossain et al., 2014). MicroRNA expression was analysed from blastocysts, from day-16 elongated embryos generated by nuclear transfer *in vivo* or artificial insemination, and from bovine placentas at day 50 and day 225 of pregnancy. A large number of miRNAs in placentas derived from nuclear transfer or *in vivo* produced embryos were deregulated compared to artificial insemination groups (Hossain et al., 2014). This suggests that miRNAs produced by embryo could be transferred to the blood of their dam via placenta. Further study of miRNA transfer during pregnancy is needed to fully understand the mechanism.

6.4.3. Immune-related miRNAs in calf blood after receiving colostrum

The miRNAs are present in all fractions of colostrum and milk (exosomes, whey and cells). Most of the miRNAs are found in exosomes, which are very stable under a range of conditions, including high temperatures, RNase digestion and low pH (Chen et al., 2008; Izumi et al., 2012). Therefore, colostrum miRNAs in exosomes are likely to be protected from digestion in gastrointestinal tracts of neonates, and the structure of exosomes may help the transfer of the miRNAs to the cells (Sun et al., 2013). However, there are only limited studies on miRNA absorption in newborns after consuming colostrum (Kirchner et al., 2020). Smythies and Smythies (2014) suggested that miRNAs packaged in exosomes in the gut can be taken up by dendritic cells and transported to mesenteric lymph nodes, but the mechanisms of this transport have not been investigated further.

The results from the present study indicate that at day 1, the level of two of the five miRNAs (miR-155 and miR-181a) increased significantly from day 0 ($P < 0.05$) and then decreased steadily after day 2. The significant increase in the miRNAs at day 1 may be explained either by miRNA absorption from colostrum or by the increased expression in the calves. Notably, there were no significant differences between the three calf Groups for miR-155 and miR-181a for any time point within 7 days after birth. This was despite the Group A and B calves receiving much larger amounts of miRNAs from the colostrum than the Group C calves, and suggests that there was an increase in expression rather than absorption from the colostrum.

The amount of miR-155 in the dam colostrum was higher than in the pooled colostrum and in the bulk tank milk ($P < 0.05$). The levels of two other miRNAs were also significant higher in dam colostrum than bulk tank milk (miR-142-5p and miR-181a; $P < 0.05$). However, there were no significant differences in any miRNA concentrations between three calf Groups after feeding colostrum at any time-points within 7 days after birth. Group A and B calves received dam colostrum twice per day for 3 days postpartum, which was far more than the Group C calves which received only 1 bottle pooled colostrum and then bulk tank milk for 3 days after birth. Therefore, the lack of differences between Groups with different colostrum feeding regimens also suggest that miRNAs were not transferred from colostrum.

Finally, the correlations between the levels of the miRNAs in all the colostrum samples and the levels in the corresponding day 1 calf blood were low. Again, these correlations suggest that the calves are synthesizing the miRNA themselves rather than absorbing the miRNA from the colostrum. The increase in miR-155 and miR-181a at day 1 is most likely to be the result of increased synthesis after birth.

Unlike the other miRNAs, only miR-142-5p concentration in calf blood increased after day 2 and reached its highest level at day 7. The level of miR-142-5p at day 7 in the Group A calves, which received their own dam colostrum, was higher than that in Group B and Group C calves ($P < 0.05$). This finding does not imply that miR-142-5p was absorbed in calves after feeding. MiR-142-5p was higher in colostrum than in bulk tank milk. If miR-142-5p absorption occurs, this level should be higher in day 1 after the calves received colostrum rather than at day 7 after receiving bulk tank milk. Interestingly, the correlation between miR-142-5p levels in the Group A calf blood at day 1 and miR-142-5p levels in their own dam colostrum at day 0 was moderate but negative ($r = -0.61$). This might suggest a relationship between the miR-142-5p in the colostrum and miR-142-5p in the calves but the results do not suggest absorption.

The conclusion that miRNAs are not widely absorbed from colostrum into neonate calf blood is similar to that of Kirchner et al. (2020), who reported that only three miRNAs of the top 15 most common miRNAs (miR-200 a/b/c family) were significantly increased in calf blood after feeding colostrum. They examined several time points after feeding (1, 3, 6 and 9-12 hours), but were unable to show an increase in any other miRNA levels in the calf blood by RT-qPCR. Kirchner et al. (2020), therefore, also concluded that there was no significant uptake of dietary miRNAs.

The five immune-related miRNAs studied here, whether synthesized or absorbed, may have a functional effect in the newborn calves in regulating their immune competence. These five miRNAs are involved in many biological processes, but most importantly, they are all key to the development of the immune system (Appendix 6), which is immature in newborn calves (Vigorito et al., 2013; Huang et al., 2015; Yuan et al., 2018). Therefore, one may postulate that these immune-related miRNA are present at high levels because they have a

role in immune system development. Unfortunately, due to the low number of calves in the present study, there was not sufficient power to show a relationship between the level of the miRNAs in the calves and their health status in the 7 days after birth. A longitudinal study, therefore, comparing newborn miRNA with long-term health effects would be of interest.

The functions of miRNAs in the calf blood have not been fully investigated, especially during the first days of their life. However, miRNAs are involved in the intestinal epithelium development (reviewed by Van Hese et al. (2020)), and the potential regulatory role of miRNAs in the development of gastrointestinal tract (GIT) neonate calves has been examined in one study (Liang et al. 2014). Liang et al. (2014) analysed mucosal miRNAs from birth to 42 days in dairy calves. They found that variation in miRNA expression in the GIT samples may reflect both changes in immune cell populations and the miRNA production within specific cell populations. In particular, miR-143 was abundant at all time-points and targeted genes involved rapid tissue development. Most of the temporally differentially expressed miRNAs found in the small intestine had putative target genes involved in the development of the mucosal immune functions including miR-146, miR-191, miR-33, miR-7, miR99/100, miR-486, miR-145, miR-196 and miR-211, etc. (Liang et al., 2014). The functions of some of these miRNAs are related to mucosal immune system development in newborn cattle (Liang et al., 2015a), which may affect the gut microbiome. The expression of miR15/16, miR-29 and miR-196 are positively correlated with the copy number of the 16S rRNA gene of *Bifidobacterium* or *Lactobacillus species* (Liang et al., 2014). MicroRNA-155 was significantly upregulated in the mid-jejunum and ileum at day 7 of age compared to 3 h postpartum, which is consistent with an increased inflammatory response during the first week of life (Liang et al., 2014). It is highly likely that an important function of many of these microRNAs relates to the regulation of microbiome development in the growing calf. This area deserves greater discussion and research.

6.5. Summary and Conclusion

Of the five immune-related miRNAs examined (miR-142-5p, miR-150, miR-155, miR-181a and miR-223), two were abundant in colostrum and bulk tank milk (miR-223 and miR-155). Dam colostrum had significantly higher levels than bulk tank milk for miR-142-5p, miR-155 and miR-181a, but only miR-155 concentration was significant different between the

dam colostrum and pooled colostrum. All five miRNAs had a similar trend in colostrum, as they were the highest level at day 0 and then decreased dramatically after day 1 ($P < 0.05$). The concentrations of miRNAs in colostrum were lower than in cow blood and calf blood (100 to 1000-fold), and miR-223 had the highest level in both calf and cow blood compared to other four immune-related miRNAs.

Calves were born with high levels of the immune-related miRNAs in their blood and there were no significant differences in miRNA levels between the three calf Groups at birth or after they were fed different colostrum (dam vs pooled colostrum). In addition, only low or negative correlations were found between the levels of the miRNAs in the colostrum and the miRNA levels in calf blood at day 1, these results imply that these miRNAs were not transferred from colostrum to the calf. The studies on miRNA absorption from colostrum and milk are extremely limited though and miRNA absorption should be further explored by quantifying more miRNAs in larger numbers of neonates using RT-qPCR.

Given the low number of calves per Group, other results should be verified as well. For instance, it is not evident why miR-142-5p increased by day 7 and whether this was a result of colostrum and milk consumption or the result of immune system development. The high correlation between miR-155 in dam blood and day 0 Group A calf blood also suggests the possible selective transfer of this miRNA during pregnancy, but again, this needs confirmation. Finally, further studies of miRNA, such as miR-155, on the effect of cow health or vaccination status on the miRNA levels in offspring is warranted. At this point, there is little to no information on the functions of miRNAs in neonates. Thus, further studies focused on the effects of miRNAs in newborn calves, particularly, on calf immune system development, are genuinely needed.

Chapter 7
General Discussion

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7.1. Introduction

A large number of studies on bovine colostrum have investigated colostrum components such as nutrients, growth factors and immune components (reviewed by (Uruakpa et al., 2002; McGrath et al., 2016; Godden et al., 2019). Many of these have focused on individual components, such as immunoglobulins or specific proteins, and only a few studies have examined miRNAs, especially immune-related miRNAs (reviewed by Van Hese et al. (2020)). The aim of this study was to examine the main components of colostrum and their absorption into the calf blood (e.g., IgG, protein, lactose and fat as well as miRNA). The questions to be addressed were whether these components are absorbed equally by calves fed different colostrum from different sources and whether there was similar transfer of the different components.

To address these questions, various components (total solids, total protein, lactose, fat, IgG and miRNAs, including immune-related miRNAs) were quantified in colostrum from different colostrum sources, specially colostrum collected within 2 hours after calving and colostrum pooled from cows up to 4 days post calving. The transfer of components from the colostrum into neonate calf blood were measured on days 1, 2, 3 and 7 after birth in three calf Groups. The Group A and Group B calves were paired and received colostrum from one of the Group A dams twice daily for 3 days after birth and then bulk tank milk until day 7. The Group C calves received only one bottle (2 L) of pooled colostrum after birth, as frequently practiced in Australia and New Zealand. The pooled colostrum was a mix of colostrum and transition milk from multiple cows days 0-4 postpartum. After the initial pooled colostrum feeding, the calves were fed bulk tank milk until day 7.

7.2. Components in colostrum and milk

Dam and pooled colostrum components

With the exception of lactose, the dam colostrum, collected within 2 hours after birth, had higher concentrations of all components than the pooled colostrum and the bulk tank milk (Chapter 3). As expected, lactose had an inverse relationship with the levels of the protein components (Chapter 4). The pooled colostrum was lower quality with less IgG, total protein and total solids than the dam colostrum because it was a mixture of colostrum and transition

milk from multiple cows day 0 to 4 postpartum. The bulk tank milk was even lower as it was from multiple cows 5 or more days postpartum (Chapter 4).

Colostrum is considered to be of good quality if the concentration of IgG is greater than 50 g/L or the total solids are higher than 18 Brix % (Morrill et al., 2015). Therefore, although the pooled colostrum had lower total solids, total protein and IgG than the dam colostrum ($P < 0.05$), the quality of pooled colostrum in this study was still classified as good based on the concentrations of the total solids (19.4 Brix %) and IgG (90.5 g/L). Consequently, it was not surprising that there were no differences in calf morbidity between colostrum types in this study (Chapter 3). To assess the effects on health resulting from differences in colostrum quality, a greater number of calves would be required. Nevertheless, there was sufficient difference in the quality of the dam and pooled colostrum to allow comparisons of colostrum component absorption.

There was a small difference in the miRNA profiles of the dam colostrum and pooled colostrum based on miRNA sequencing (Chapter 5). The miRNA profile changed little during the 4 days postpartum, with 76% of the total unique miRNAs detected overlapping between the dam colostrum collected within 2 hours postpartum and the pooled colostrum from cows 0-4 days postpartum. Of the 303 unique miRNAs detected in the colostrum, only four miRNAs had a significantly different level in the dam vs pooled colostrum. These four miRNAs (miR-19b, miR-193b, miR-345-5p and miR-378) were all at higher levels in dam colostrum compared to pooled colostrum.

Several immune-related miRNAs of specific interest (miR-142-5p, miR-150, miR-155, miR-181a and miR-223) were analysed by miRNA sequencing (Table 7.1) and by RT-qPCR (Chapter 6). The levels of these five immune-related miRNAs were not significantly different between the dam and pooled colostrum based on the miRNA sequencing data (Table 7.1), and this was confirmed for four of the miRNA by RT-qPCR. However, quantification by RT-qPCR revealed that the level of miR-155 was higher in the dam colostrum than in the pooled colostrum. The difference in the results for miR-155 may be explained by the methods and number of samples analysed. RT-qPCR is a more sensitive method and more samples were assayed by RT-qPCR (12 dam colostrum and 5 pooled

colostrum samples) than by miRNA sequencing (4 dam colostrum and 4 pooled colostrum samples).

Table 7.1. MicroRNA differentially expressed between day 0 dam colostrum and pooled colostrum.

miRNA	log_2FC	Average Expression	t	P-Value	Adjusted P-Value
bta-miR-150	0.83	5.90	2.04	0.06	0.37
bta-miR-142-5p	-0.91	9.36	-2.74	0.02	0.20
bta-miR-181a	-0.02	11.59	-0.08	0.94	0.97
bta-miR-223	-1.26	10.27	-2.95	0.01	0.16
bta-miR-155	0.01	7.77	0.04	0.97	0.98

Dam colostrum collected within 2 hours postpartum from individual cow, pooled colostrum collected from multiple cows days 0-4 postpartum.

n = 4 per colostrum source, log_2FC = log2 fold change, t = moderated t-statistic

Colostrum components across time-points

Colostrum components (particular, total solids, total protein and IgG) in the dam colostrum were highest concentrations at day 0 and decreased rapidly between day 0 to 3 postpartum (Chapter 3), which is similar to results from previous studies (Dunn et al., 2017; Godden et al., 2019). However, there is virtually no literature regarding the miRNA concentrations in bovine colostrum from 0 to 72 hours post-partum. Five immune-related miRNAs in dam colostrum were measured every 24 hours within 3 days postpartum in this study to address this gap (Chapter 6). The miRNA trend was similar to other colostrum components, with the highest level at day 0 (2 hours postpartum) and followed by a dramatic decrease after day 1. This trend has been also observed for pig colostrum miRNA (Gu et al., 2012a). Remarkably, as mentioned above, the miRNA levels in the day 0 dam colostrum and the pooled colostrum were very similar as measured by miRNA sequencing or by RT-qPCR with only a few exceptions (miR-19b, miR-193b, miR-345-5p, miR-378, and miR-155). This suggests that the miRNA profile and their expression are slower to change than the other components, which were significantly different between the dam and pooled colostrum (e.g protein %, lactose %, total solids, total protein and IgG concentrations) (Chapter 3).

Colostrum and milk components

As the levels of the colostrum components declined sharply over time postpartum, it was not surprising that the levels of the components were significantly different between the colostrum and mature milk (Chapter 3). The miRNA concentration was an exception. While most components (total solids, total protein and IgG) in the colostrum were significant higher in the dam colostrum than the bulk tank milk (Chapter 3), some miRNAs had similar levels in both (Chapter 6). Three of the five immune-related miRNAs, as measured by RT-qPCR, had higher concentrations in the dam colostrum than in the bulk tank milk (miR-142-5p, miR-155 and miR-18a), but the other two miRNAs had the same levels (miR-223 and miR-150) ($P > 0.05$). The miRNA in the bulk tank milk was not sequenced herein, so the difference in miRNA profiles between these two sources are not known though.

The miRNA profile during lactation is likely to reflect the change of biological function in the mammary gland from colostrum production to mature milk production (Do et al., 2017; Hou et al., 2017; Ozdemir, 2020. However, the miRNA can be also related to the health status of the cow. Cai et al. (2018) found that the two of miRNA quantified herein are at higher levels in milk of cows with mastitis (miR-223 and miR-142-5p), suggesting these miRNAs may be involved in the response to the infection. These differentially expressed miRNAs could be used as biomarkers for health status.

Colostrum components are produced by different mechanisms. Proteins are directly synthesised in the mammary gland or transferred from cow blood. IgG is transported from the dam blood via the mammary gland to milk, whereas IgA is synthesized by the plasma cells of mammary gland (summary by Hou et al. (2017)). Studies in human (Alsaweed et al., 2016), tammar wallaby (Modepalli et al., 2014) and goat milk (Li et al., 2012a) suggest that milk miRNA originates from the mammary gland, although the origin of miRNA in bovine colostrum has not been studied. There were low correlations between the levels of the miRNAs in the dam blood within 2 hours postpartum and the day 0 colostrum, as measured by RT-qPCR (Chapter 6). This suggests that miRNAs in colostrum are not transferred from the cow blood but are more likely to be produced in the mammary gland. The analysis of the top 100 most highly expressed miRNAs in the colostrum also suggests this may be the case. The pathway analyses determined that these abundant miRNAs were involved mostly in

mammary functions such as the development of cells, membranes and organelles, and the regulation of energy and calcium.

7.2. Immune components in newborn calves at birth

The immune components that are transferred from the dam via colostrum to newborn calves are crucial for the survival of the calves during the first weeks after birth. All the immune components present in the calf blood at birth have not yet been fully analysed. Many studies have shown that calves have very little immunoglobulin in their blood at birth (Godden et al., 2019; Van Hese et al., 2020), however, there are few studies of other components such as immune-related miRNAs.

Many components in blood of newborn calves are at very low concentrations prior to colostrum consumption (e.g. glucose, total protein, IgG and GGT) (Chapter 3). This is because the bovine placental structure prevents the transfer of proteins from the dam to foetus during pregnancy (Barrington and Parish, 2001; Castro et al., 2011). Two indicators of passive immunity transfer and colostrum ingestion (IgG and GGT) were found to be low in the newborn calves studied. The average of IgG concentration in calf blood at birth was 0.3 g/L (range of 0 to 1.7 g/L) and GGT activity was 11.7 U/L (range of 6.2 to 26.2 U/L).

In contrast, many miRNAs were detected in calf blood at day 0, before feeding (Chapter 5). This indicates that calves are born with a complex profile of miRNAs, many of which are at high levels. Whether these miRNAs are transferred from the dam to the fetus *in utero* via the blood supply or whether they are synthesized by the fetus is not known and was investigated herein. The correlations between the miRNA levels in the dam blood immediately postpartum and the calf blood at birth were low, with the exception of miR-155 ($r = 0.76$, $P = 0.004$) (Chapter 6). Hence, the transfer of miRNA between the blood of the dam and fetus may be selective. This is consistent with findings from other studies (Pigati et al., 2010; Ioannidis and Donadeu, 2016), where the authors have also suggested that if miRNA transfer occurs, then it is specific for particular miRNAs. It is also possible that miRNA can be transferred from the fetus to the dam, as there is growing evidence that the embryo or fetus communicates with the dam via miRNA that is transferred to the blood of the dam via the placenta (Hossain et al., 2014; De Bem et al., 2017).

7.3. Immune components transferred via colostrum

Comparison of calf absorption between time-points

The different colostrum components (namely total protein, IgG, GGT, and glucose) were absorbed into the calf blood after the calves received colostrum (Chapter 3). However, only total protein, measured by refractometer, and the IgG concentration were significantly different between calves fed their own dam colostrum and those fed a single bottle of pooled colostrum. There were high correlations between day 0 colostrum IgG and total solids (Brix %) with day 1 calf blood IgG (r = 0.74, r = 0.71, respectively) (Table 7.2). Slightly lower correlations were found between total protein (TP-B) in day 0 colostrum and day 1 calf serum IgG (r = 0.66). The results indicate that the amount of total protein and IgG in colostrum is directly related to their absorption by calves. Therefore, the absorption of total protein and IgG by calves can be reasonably estimated from the Brix % or IgG measured in colostrum (Chapter 4).

Table 7.2 . Relationship between components in colostrum at first feeding (day 0) and the components in the blood of the corresponding calf at day 1.

Day 0 colostrum component[1]	Day 1 calf blood component[1]	Pearson correlation (r)	P-value
Total solids	TP-R	0.69	<0.001
Total solids	TP-B	0.28	0.100
Total solids	IgG	0.74	<0.001
Total solids	GGT	0.64	<0.001
TP-B	TP-R	0.54	0.001
TP-B	TP-B	0.30	0.081
TP-B	IgG	0.66	<0.001
TP-B	GGT	0.42	0.110
IgG	TP-R	0.59	<0.001
IgG	TP-B	0.32	0.058
IgG	IgG	0.71	<0.001
IgG	GGT	0.43	0.009

[1]*Total solids = Brix refractometer; TP-R = total serum protein measured by refractometer; TP-B = total protein measured by Bradford assay; GGT = gamma-glutamyl transferase activity; IgG = immunoglobulin G. n = 35 samples.*

A similar relationship was not observed between the colostrum miRNA levels and the day 1 calf blood miRNA levels (Chapters 5 and 6). Instead, the results suggest that the bulk of miRNAs in the colostrum are not absorbed into the newborn calf. The miRNA profile in the calf blood had only minor changes between birth and 24 hours after two feeds. There were only four miRNAs out of the 1,198 total unique miRNAs that had higher levels in the calf blood on day 1 compared to day 0. The low correlations of these four miRNAs in calf blood day 1 and their levels in the corresponding colostrum suggest that miRNAs were not absorbed from the colostrum.

Absorption of five selected immune-related miRNAs was also compared in the calf blood between day 0 and day 1 by miRNA sequencing (Table 7.3) and RT-qPCR (Chapter 6). The levels of all five immune-related miRNAs were not different between day 0 and day 1 as measured by miRNA sequencing (Table 7.3). As their concentration did not increase, it would suggest that they were not absorbed. However, miRNA sequencing is not at sensitive as RT-qPCR. For sequencing data, any differential expression will not be perceived as being significant if less than 1-fold (e.g. miR-150 and miR-142-5p, Table 7.3). Therefore, the concentration of the five immune-related miRNA was also verified by RT-qPCR and two of these miRNAs (miR-155 and miR-181a) showed an increased concentration in the calf blood on day 1 ($P < 0.05$). Nevertheless, there were low correlations between these two miRNA levels in the day 1 calf blood and the corresponding colostrum, again suggesting that these five miRNAs were not absorbed into the calf blood from the colostrum.

Table 7.3. MicroRNA differential expressed in calf blood between day 0 and day 1.

miRNA	log_2FC	Average Expression	t	P-Value	Adjusted P-Value
bta-miR-150	0.50	8.71	3.12	0.00	0.03
bta-miR-142-5p	0.66	11.93	2.84	0.01	0.05
bta-miR-181a	0.12	11.16	1.62	0.12	0.28
bta-miR-223	0.10	10.71	0.91	0.37	0.57
bta-miR-155	0.09	5.70	0.76	0.46	0.65

Significance for differential expression: adjusted P-value < 0.05 and $|log_2FC| > 1$.

log_2FC = log2 fold change, t = moderated t-statistic, n = 12 samples per time point

To support the idea that miRNAs in colostrum are not absorbed into the calf blood, the miRNA levels in the three calf Groups, which received colostrum from different sources, were also compared over a longer period (Chapter 6). The Group A and Group B calves received dam colostrum for three days and the Group C calves received only one bottle of pooled colostrum and then bulk tank milk. As a consequence, the Group C calves received much less miRNA than the Group A and B calves. However, there was no significant difference in the levels of four of the five immune-related miRNAs (miR-150, miR-155, miR-181a and miR-223) between the three calf Groups at any time-points within one week postpartum. The exception was the miR-142-5p level, which differed between three calf Groups at day 7. The level of miR-142-5p increased for all 35 calves by day 7, but the miR-142-5p level at day 7 in the Group A calves was significantly higher than Group B and Group C calves. The question is whether miR-142-5p was absorbed from colostrum and/or milk. Although the calves from three Groups received different colostrum for the first three days, all the calves received bulk tank milk from day 4 to 7 after birth and the concentration of miR-142-5p in bulk tank milk was significantly lower than colostrum. This implies the increase in the miR-142-5p in the calf blood is unlikely to be caused by absorption from the colostrum or milk. The results once again suggest that the five immune-related miRNAs in colostrum and milk were not absorbed by the calves, even over a longer timeframe (one week postpartum).

Relationship between IgG and immune-related miRNAs

Immunoglobulin, particular IgG, is the main immune component in colostrum that is transferred in calf blood after colostrum consumption. Previous studies have established serum IgG as an indicator of passive immunity transfer (Buczinski et al., 2018; Godden et al., 2019). In order to examine the relationship between IgG and immune-related miRNAs, correlations between these parameters were calculated (Figure 7.1).

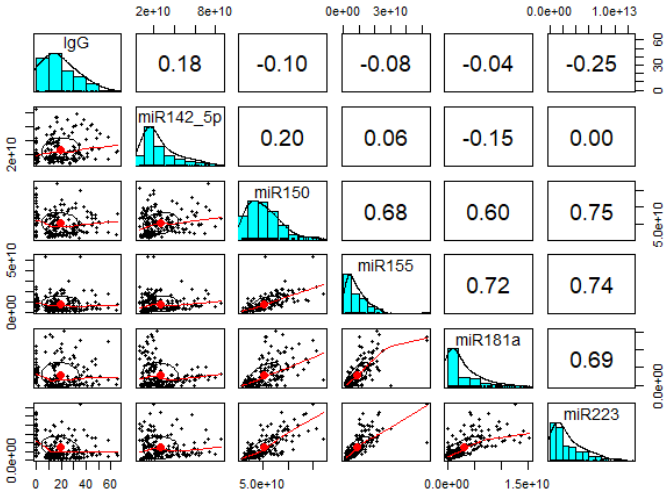

Figure 7.1. Correlations between IgG and immune-related miRNAs from day 0 to 7 after birth.

The correlations between IgG and the immune-related miRNAs in calf blood within 7 days after birth were low. The low correlations between IgG and the miRNAs confirmed that while IgG was transferred to calf blood, the miRNAs were not readily transferred from colostrum to calf blood.

Notably, with the exception of miR-142-5p, there were high correlations between all pairs of the other four miRNAs. Correlations between the pairs of the four miRNAs (miR-150, miR-155, miR-181a and miR-223) were high (ranging from 0.6 – 0.75). The highest correlation was between miR-150 and miR-223 (r = 0.75), the two miRNAs with the highest concentrations in calf blood. These four miRNAs had the same trend after birth, with high concentrations at days 0 and 1 after birth, but then decreasing steadily thereafter. This finding suggested that these four miRNAs may be downregulated in calf blood after birth.

Surprisingly, these five immune-related miRNAs were at their lowest levels at day 3, the same time point during the first week after birth when the IgG concentration was also at the lowest. Some studies have shown a relationship of immunoglobulins and immune-related miRNAs. MicroRNA-150 has been shown to significantly affect antibody production, particularly levels of immunoglobulins of various classes (Xiao et al., 2007). The immunoglobulin concentration, especially the IgA level, in miR-150 knock-out mice serum

was significantly higher than control (Xiao et al., 2007). In another mouse study, the IgG levels were significantly decreased with 80% of total anti-collagen IgG antibodies reduced in mice that were miR-155-deficient compared to wild-type mice (Blüml et al., 2011).

In summary, the protein and IgG in the colostrum were absorbed markedly by the calves within 24 hours, and the highest concentration of IgG in the calf blood was at 48 hours. Thereafter, the concentration of serum protein and IgG declined, and at the same time, their levels in the colostrum also decreased. These results are identical to most other published studies and are not surprising since gut closure begins in calves roughly 36 – 48 hours after birth (McGrath et al., 2016; Godden et al., 2019). However, this was not the case for the miRNA in the colostrum, as the results indicate that the miRNAs in colostrum were not generally absorbed into the calf blood. The only other study to consider miRNA absorption from colostrum or milk in calves also failed to detect large scale uptake of dietary miRNAs (Kirchner et al., 2020).

It is possible though that the miRNA absorption from colostrum is not being detected because of the timescale. In a study on the absorption of miRNA from cow milk in humans, miR-29b and miR-200c were shown to be absorbed into human plasma after the cow milk was consumed, as the levels increased depending on quantity of cow milk consumed (Baier et al., 2014). However, the levels only increased in a 6-hour period after the milk consumption, and then, these miRNA levels decreased to baseline by 24 hours. Interestingly, the concentration of runt-related transcription factor 2 (RUNX2), which is a target of miR-29b, increased 31% in the blood mononuclear cells after milk consumption compared with the baseline (Baier et al., 2014). This indicates that the increase of miRNA in blood may have a physiological effect although the increase in the blood was short lived.

The rapid uptake of the miRNA and quick return to baseline levels suggests that the timing for blood sample collection may be important to detect such changes. This may explain why only three of the 1,198 unique miRNAs found in calf blood increased after colostrum feeding. The absorption of colostrum miRNAs herein may not have been observed if the increase is not apparent by 24 hours as the calf blood was only collected once a day. However, Auerbach et al. (2016) did another study to validate the results from Baier et al.

(2014) in which a selection of the original samples from Baier et al. (2014) were analysed using arrays, RT-qPCR and sequencing. The results showed that there were not significant differences in miR-29b or miR-200c at any time points, and the authors concluded that the cow milk miRNAs were not absorbed into the circulation of humans. To resolve this discrepancy, experiments investigating miRNAs levels in newborn calves every hour within 24 hours after feeding could be useful.

In addition, a trial investigating potential miRNA absorption should be extended for a longer period of time. In a pig study by Gu et al. (2012a), 13 immune-related miRNAs were detected at higher levels in colostrum than in milk, and 12 of these miRNAs were also found at higher concentrations in colostrum-fed piglets rather than in mature milk-fed piglets at 4 days after birth. The authors speculated that these immune-related miRNAs in milk were being transferred into the piglets. Similarly, in a study on tammar wallaby, Modepalli et al. (2014) quantified four highly expressed milk miRNAs (miR-148, miR-22, miR-141 and miR-30a) in pouch young at different time-points of their early life (day 30, 80, 130, 150) and in weaned juveniles. The authors found significant higher levels of these miRNAs in pouch young before weaning than in the juveniles, and they concluded that miRNAs may be absorbed through the wallaby premature gut system and transferred into the newborn blood. There are several possible explanations for the apparent inconsistency between the results from piglets (Gu et al., 2012a) and tammar wallabies (Modepalli et al., 2014) versus the calves (herein and Kirchner et al. (2020)). These explanations include: 1) disparities in the specific miRNAs that were measured as these were all different between the studies and there may be selective transfer, or 2) gastrointestinal species differences between monogastrics and ruminants may allow miRNA absorption from milk-born exosomes in piglets and wallabies, but not in calves. However, at this point, calves are functionally monogastric. However, another possibility is that there is differential expression of miRNA because the neonates are more developed (e.g. pouch young vs juvenile wallabies). In the case of the piglets, which were assessed at 4 days after being fed colostrum or milk, it is well established that colostrum promotes better neonate immune and gut development than milk (Yang et al., 2015; Li et al., 2020). Therefore, these differences in the piglet miRNA profiles may reflect differential expression as a result of a more developed immune system in the colostrum-fed piglets and do not represent absorption differences. To resolve which of these

hypotheses might best explain the discrepancies between the piglet, wallaby and calf studies, a more detailed investigation of the miRNA profile of neonates over time correlated with their milk-born miRNA intake should be undertaken in several species.

The finding that many of the miRNA species are not absorbed through the gastrointestinal mechanisms may suggest that they play a role in the development of the gastrointestinal tract or microbiome of the calf. As such, the miRNA may be involved in an indirect pathway for controlling the developmental physiology of the calf and this should be considered in future experiments.

7.4. Lactocrine hypothesis

Colostrum has been shown to promote gastrointestinal tract and immune development in neonate calves better than either transition milk or bulk tank milk (Yang et al., 2015). Colostrum has been also shown to improve reproductive development in both male and female piglets in comparison to colostrum replacer (Yan et al., 2006; Rahman et al., 2014). These observations have led to the so-called “lactocrine hypothesis” (Bartol et al., 2008; Bartol et al., 2017), which is the theory that colostrum contains bioactive factors that can affect the long-term development of neonate animals. The components in colostrum responsible for this development are not known.

Colostrum contains many potential bioactive factors that may affect the neonate long-term development. Yan et al. (2006) have postulated that relaxin is the bioactive component in the piglet reproductive tract development. However, colostrum contains many other potential bioactive components, particularly growth factors and hormones (e.g. epidermal growth factor, betacellulin, insulin-like growth factor, transforming growth factor, anti-Müllerian hormone) (Baumrucker and Blum, 1993; Blum and Hammon, 2000; Tripathi and Vashishtha, 2006; Yang et al., 2015; Novo et al., 2017; Van Amburgh, 2017). Other potential maternally important bio-factors in colostrum include cells, miRNA and other proteins (Marnila and Korhonen, 2011a; Gonzalez and Santos, 2017; Van Hese et al., 2020).

Given that colostrum may have long-term effects on the neonate calf, it is vital that calves can absorb the colostrum components equally well as demonstrated in the comparison of the absorption by the Group A and Group B calves. This ensures that if the colostrum is good

quality and has high levels of IgG, then similar amounts of IgG will be absorbed. The data herein also indicate if the IgG levels are high, then the levels of total solids and total protein will be also high and lactose levels will low (Chapter 4). Lactose intolerance is a cause of bovine neonatal diarrhoea (Olchowy et al., 1993), so low lactose in colostrum is better for newborn calves. Therefore, refractometry allows the assessment of not only the quality of colostrum in terms of IgG but other nutrients as well. Presumably, any other colostrum components, such as hormones and other bioactive factors, will also have high levels if the colostrum is good quality.

However, although the results herein suggest that neonate calves will absorb nutrients from colostrum equally well, there is some evidence that maternal colostrum is superior to foster dam colostrum for neonate development (Stewart and Diekman, 1989). In a study by Stewart and Diekman (1989), they cross-fostered newborn piglets, as is common practice in the pork industry, to standardise the size of the litters. The authors observed that the cross-fostered piglets did not grow as well as those piglets that remained with their dams. This would indicate that the maternal source of the colostrum may be important.

The effects of maternal colostrum and foster dam colostrum on calves has not been examined previously. The study herein did not have a large number of calves fed dam colostrum (Group A), foster cow colostrum (Group B) or pooled colostrum (Group C) and differences in development over the course of 7 days are unlikely to have been observed. It is intriguing though that while there were no significant differences in the levels of the various blood components between Group A and Group B calves, the levels in the Group B calves were consistently in between the Group A and Group C calves. This may suggest that there is a subtle distinction between the maternal colostrum and foster cow colostrum in absorption. However, the absorption of colostrum components and their effects need to be studied in a greater number of calves over a longer timeframe to test this hypothesis. A power calculation based on the data herein indicates that greater than 35 animals per Group may be required to discern any absorption and health differences between calves receiving maternal and foster cow colostrum.

7.5. Conclusions

Calves are born with very low immune factors and protein concentration in their blood but with high levels of miRNAs, especially immune-related miRNAs. The data suggest that miRNAs are not transferred between dam and fetus *in utero* in general, but hint that there may be possible selective transfer (e.g. miR-155). Thus, further study with more immune-related miRNAs or differentially expressed miRNA between dam blood and the blood of their foetus would be interesting.

After receiving colostrum, the levels of total protein, IgG and GGT increased dramatically by day 1. The amount of colostrum protein, lactose, GGT and IgG absorbed within 48 hours by the calves depended on their levels in colostrum. There were no significant differences between the ability of calves to absorb these components from different sources as there was no significant differences between the calves that received colostrum from their dam or a foster cow. Nevertheless, there is a possibility that calves absorb the components better from their own dam, as there was consistently higher IgG and protein in the serum of calves that received colostrum of their own dam than the foster calves receiving colostrum from another cow. To explore this possibility, a much larger dataset is required.

Calves fed lower quality pooled colostrum for a shorter time had significant lower total protein and IgG concentrations than calves that received better colostrum for 3 days, however, all calves had sufficient transfer of passive immunity. Unlike the other colostrum components, most of miRNAs in colostrum did not appear to be absorbed in calves after colostrum consumption. Further studies with a greater sample size and sampling every hour within 24 hours after birth would confirm that the miRNAs are being selectively transferred and are not being globally absorbed.

In terms of industry outcomes, the Brix and serum protein refractometers were shown to be good, accurate and easy tools for use on-farm to assess colostrum quality and transfer of passive immunity, respectively. The level of IgG in colostrum was highly correlated to the amount of total solids, total protein and lactose in the colostrum. Therefore, if the level of IgG in colostrum is high, then total solids and total protein will be also high and lactose will be low, providing a good diet as well as the transfer of passive immunity for newborn calves.

It was also found that the transfer of passive immunity can be adequate if the calves are fed only a single bottle of pooled colostrum collected from cows day 0 – 4 postpartum and then bulk tank milk. However, it is necessary to ensure adequate passive immunity transfer by providing the calves with the colostrum soon after birth and by harvesting the pooled colostrum from cows on day 0 and day 1 postpartum to guarantee good quality.

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Appendix 1. Nomenclature of miRNA.

Nomenclature	Explanation
"miR" and "mir"	- "miR" refers to mature form of the miRNA - "mir" refers to the pre-miRNA and the pri-miRNA
The first three letters signify the organism	- hsa-miR-124 is a human (*Homo sapiens*) miRNA - oar-miR-124 is a sheep (*Ovis aries*) miRNA - bta-miR-155 is a cattle (*Bos taurus*) miRNA
A letter after the number in the suffix is used to differentiate among multiple members of the same family	miRNA with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case letter. Example: hsa-miR-181a: aacauucaACgcugucggugAgu hsa-miR-181b: aacauucaUUgcugucggugGgu
Additional number is given after the full name	Pre-miRNAs, pri-miRNAs and genes that lead to 100% identical mature miRNAs but that are located at different places in the genome. For example, the pre-miRNAs hsa-mir-194-1 and hsa-mir-194-2 lead to an identical mature miRNA (hsa-miR-194) but are from genes located in different genome regions
-3p or -5p suffix (or an asterisk following the name)	When two mature microRNAs originate from opposite arms of the same pre-miRNA and are found in roughly similar amounts, they are denoted with a -3p or -5p suffix. Example, miR-142-5p (from the 5' arm) and miR-142-3p (from the 3' arm) However, the mature microRNA found from one arm of the hairpin is usually much more abundant than that found from the other arm, in which case, an asterisk following the name indicates the mature species found at low levels from the opposite arm of a hairpin. For example,

	miR-56 (the predominant product) and miR-56* (from the opposite arm of the precursor) Older convention sometimes used miR-142-s and miR-142-as.
mir-1 vs *Mir*-1	The miRNAs encoding genes are also named using the same three-letter prefix according to the conventions of the species gene nomenclature. For example, the official miRNAs gene name is "*mir-1* in *Caenorhabditis elegans* and *Drosophila*, and *Mir-1* in *Rattus norvegicus*
Exceptions	*let-7* and *lin-4* because of historical reasons

Source: (Griffiths-Jones et al., 2006; miRBase, 2020)

References

Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research 34(suppl_1):D140-D144. 10.1093/nar/gkj112

miRBase. 2020. What do the miRNA names/identifiers mean? http://www.mirbase.org/help/nomenclature.shtml

Appendix 2. Protocol for bovine IgG quantification by ELISA.

a. Reagent and materials

- Nunc F96 Maxisorp plates (Thermo #442404)
- Coating buffer (Carbonate bicarbonate buffer, pH 9.6): 3.03 g Na_2CO_3 + 6 g $NaHCO_3$ + 1 L MilliQ dH_20, pH 9.6
- Goat anti-bovine IgG non-conjugated antibody (Novex #A18753)
- Goat anti-bovine IgG antibody conjugated with horseradish peroxidase (HRP) (Novex #A18751)
- Gelatine
- Phosphate Buffered Saline (PBS): 1.16 g Na_2HPO_4 + 0.1 g KCl + 0.1 g K_3PO_4 + 4 g NaCl + 500 mL MilliQ dH_20, pH 7.4.

Phosphate Buffered Saline (PBS), pH 7.4 (Sigma P3813, Lot SLBR1026V) was diluted by using a packet with a liter at 25°C and stored at room temperature.

- TWEEN 20
- IgG protein standard solution (Bovine Gama Globulin, Bio-Rad Laboratories, Inc., USA, serial dilution (0; 6.3; 12.5; 25; 37.5; 50; 75; and 100 ng/mL)).
- TMB ELISA substrate (Ultra TMB-ELISA Thermo #34028; TMB = 3,3',5,5'-tetramethylbenzidine)
- Stop reagent: 0.1 M H_2SO_4

Solutions

- 0.05% Tween 20 in PBS (0.05% PBS-Tween 20)

5 µL Tween 20 + 9995 µL PBS

- 5% gelatine in 0.05% PBS-Tween 20

5 g gelatine + 95 mL 0.05% PBS-Tween 20

Dissolve by placing in microwave and mixing well, just prior to blocking step, place in microwave for 10 secs and mix well again so gelatine is not set

- Stop reagent: 0.1 M H_2SO_4

Stock solution of **Sulfuric Acid** is calculated to be **17.822 M** based on a density of **1.84** g/mL, a formula weight of **98.08** g/mol, and a concentration of **95**% w/w. To make a **0.1 M** solution, slowly add **0.561** mL of your stock acid solution to **100** mL deionized water.

b. Plate preparation

- Step 1. Dilute the 1st antibody (Goat anti-bovine IgG non-conjugated antibody (Novex #A18753), 1 µg/mL) at a ratio 1:8000 by adding 1 µL of the 1st antibody to 8 mL of carbonate bicarbonate coating buffer. Add 50 µL of diluted antibody to each well of Coat Nunc F96 Maxisorp plates (Thermo #442404).
- Step 2. Incubate plate @ 37^0 C for 1 hr (plate was covered). *[Note: plates can be stored @ 4^0 C for up to one week.]*
- Step 3. Wash wells 3 to 5 times with 0.05% PBS-TWEEN 20 by filling each well completely using a squirt bottle. Flick out 0.05% PBS-TWEEN 20 and tap plate 3 times upside down on absorbent paper between washes. *[Note: allow the plates to dry and store @ 4^0 C for up to one week].*
- Step 4. Block non-specific binding with 5% gelatine in 0.05% PBS-TWEEN 20 by adding 280 µL /well using Multichannel-pipette.
- Step 5. Incubate plate for 1 hr @ 37^0C (plate was covered).
- Step 6. Wash wells at least 3 times with 0.05% PBS-TWEEN 20. Plates are ready for use.). *[Note: plates can be stored @ 4^0 C for up to one week.]*

c. Standard curve and sample preparation

The standard curve is made using Bovine Gamma Globulin (BGG) (Bio-Rad Laboratories, Inc., USA) serially diluted 0; 6.3; 12.5; 25; 37.5; 50; 75; and 100 ng/mL from BGG 1mg/mL and 0.05% PBS-Tween 20 as follows:

SC (1000 ng/mL) = 10 µL of BGG 1mg/mL + 10 mL of 0.05% PBS-Tween 20

SC1 (100 ng//mL) = 1 mL of SC + 9 mL of 0.05% PBS-Tween 20

SC2 (75 ng/mL) = 2 mL of SC1 + 1 mL of 0.05% PBS-Tween 20

SC3 (50 ng/mL) = 5 mL of SC1 + 5 mL of 0.05% PBS-Tween 20

SC4 (37.5 ng/mL) = 2 mL of SC3 + 1 mL of 0.05% PBS-Tween 20

SC5 (25 ng/mL) = 3 mL of SC3 + 3 mL of 0.05% PBS-Tween 20

SC6 (12.5 ng/mL) = 3 mL of SC5 + 3 mL of 0.05% PBS-Tween 20

SC7 (6.3 ng/mL) = 3 mL of SC6 + 3 mL of 0.05% PBS-Tween 20

SC8 (0 ng/mL) = 0.05% PBS-Tween 20

Standard solutions were prepared and aliquoted into Eppendorf tubes and stored at -20°C.

The samples (10 µL) were diluted in 0.05% PBS-Tween 20 solution at a ratio of 10^{-3} first (10 µL of sample + 10mL of 0.05% PBS-Tween 20, then further diluted as appropriate, as below:

Samples	Dilution factor
Calf serum day 0	10^{-4}
Calf serum day 1, 2, 3, 7	10^{-6}
Colostrum day 0, 1; Pooled colostrum	10^{-6}
Colostrum day 2, 3	10^{-5}
Bulk tank milk	10^{-4}

d. ELISA steps

1. 50 µL of standards, diluted samples were added to a pre-prepared plate.
2. The plate was incubated for 60 minutes at room temperature (25°C).
3. Wells were washed at least 3 times with 0.05% PBS-TWEEN 20
4. 50 µL/well of goat anti-bovine IgG antibody conjugated with horseradish peroxidase were added per well (HRP) (Novex #A18751)

[Note: dilute HRP-conjugated antibody 1/8000 in 0.05% PBS-TWEEN 20 before use.
1 µL of HRP + 8 mL of 0.05% PBS-Tween 20]

5. The plate was incubated for 60 minutes at room temperature (25°C).
6. Wells were washed 3 times with PBS/0.05% TWEEN 20
7. TMB substrate (Ultra TMB-ELISA Thermo #34028; TMB = 3,3',5,5'-tetramethylbenzidine) was added in plate (50 µL/well).

[Note: 5mL of TMB/plate was placed in room temperature 15 minutes before adding to place].

8. Plate was covered with foil and the reaction was allowed to proceed at room temperature until desired colour (~ 15 min).
9. 50 µL/well of 0.1 M H_2SO_4 was added to stop the reaction.
10. Absorbance was measured @ 450 nm 5 min after adding stop reagent.
11. The standard curve was plotted, and sample concentrations calculated from the standard curve.

Appendix 3. Sample details and total RNA quality for miRNA sequencing.

No	miRNA ID	ID animal-date	Sample	Total RNA concentration (ng/ul)	A260/ A280	RIN value
1	CB1	A1-d0	Blood	102.9	1.6	9.7
2	CB2	A1-d1	Blood	125	1.7	9.3
3	CB3	A2-d0	Blood	114.3	1.7	9.6
4	CB4	A2-d1	Blood	124.5	1.8	9.7
5	CB5	A4-d0	Blood	83.3	1.7	9.5
6	CB6	A4-d1	Blood	122.5	1.7	9.7
7	CB7	A10-d0	Blood	117.3	1.8	9.5
8	CB8	A10-d1	Blood	81.2	1.7	9.4
9	CB9	B1-d0	Blood	124.1	1.8	9.6
10	CB10	B1-d1	Blood	112.6	1.8	9.4
11	CB11	B2-d0	Blood	90.7	1.6	9.7
12	CB12	B2-d1	Blood	89.6	1.6	9.4
13	CB13	B4-d0	Blood	104.6	1.8	9.4
14	CB14	B4-d1	Blood	130.4	1.8	9.7
15	CB15	B10-d0	Blood	121.6	1.8	9.9
16	CB16	B10-d1	Blood	86.5	1.8	9.4
17	CB17	C3-d0	Blood	140.6	1.8	9.8
18	CB18	C3-d1	Blood	106.7	1.7	9.7
19	CB19	C5-d0	Blood	83.3	1.9	9.6
20	CB20	C5-d1	Blood	133.2	1.9	9.6
21	CB21	C8-d0	Blood	147.4	1.8	9.8
22	CB22	C8-d1	Blood	127.4	1.9	9.4
23	CB23	C11-d0	Blood	119.2	1.8	9.5
24	CB24	C11-d1	Blood	105	1.7	9.3
25	CB25	Colostrum-cow 1-d0	Colostrum	109.4	1.9	5.9
26	CB26	Colostrum-cow 2-d0	Colostrum	127.2	1.9	6.3
27	CB27	Colostrum-cow 4-d0	Colostrum	187.1	1.8	4.8
28	CB28	Colostrum-cow 10-d0	Colostrum	89.5	1.9	5.4
29	CB29	Batch15 (PC3)	Colostrum	40.6	2.0	7.5
30	CB30	Batch16 (PC5)	Colostrum	83.3	1.9	7.5
31	CB31	Batch18 (PC8)	Colostrum	97.1	1.8	7.0
32	CB32	Batch19 (PC11)	Colostrum	129.3	1.8	7.7

d0 = day 0; d1= day 1; PC = Pooled colostrum

Appendix 4. Bioanalyzer results and the raw reads.

No	miRNA ID	Sample	Small RNA Concentration [pg/µl]	miRNA Concentration [pg/µl]	% miRNA in total small RNA	Raw reads
1	CB1	Blood	25,233	4,270	17	32,153,611
2	CB2	Blood	5,833	5,713	98	22,130,399
3	CB3	Blood	38,462	4,958	13	25,088,029
4	CB4	Blood	36,032	5,331	15	29,036,315
5	CB5	Blood	24,293	4,301	18	29,062,586
6	CB6	Blood	24,020	4,508	19	31,714,248
7	CB7	Blood	26,973	4,150	15	21,558,248
8	CB8	Blood	118.9	118.9	100	21,450,116
9	CB9	Blood	6,443	3,376	52	30,607,207
10	CB10	Blood	24,199	5,576	23	31,875,301
11	CB11	Blood	3,667	3,421	93	27,783,675
12	CB12	Blood	8,221	8,178	99	27,539,044
13	CB13	Blood	13,367	13,095	98	33,215,636
14	CB14	Blood	20,737	19,436	94	29,168,282
15	CB15	Blood	134,692	13,630	10	35,811,166
16	CB16	Blood	130,133	21,585	17	31,439,064
17	CB17	Blood	241,316	20,829	9	31,364,109
18	CB18	Blood	122,809	16,481	13	35,067,285
19	CB19	Blood	107,084	11,318	11	28,951,309
20	CB20	Blood	138,914	19,722	14	33,306,930
21	CB21	Blood	157,164	12,668	8	32,762,776
22	CB22	Blood	125,431	14,883	12	19,568,252
23	CB23	Blood	8,528	3,968	47	37,066,316
24	CB24	Blood	4,089	3,153	77	41,608,477
25	CB25	Colostrum	14,886	9,986	67	13,916,975
26	CB26	Colostrum	20,709	13,415	65	11,619,678
27	CB27	Colostrum	40,879	24,736	61	14,266,062
28	CB28	Colostrum	12,996	8,250	63	10,699,779
29	CB29	Colostrum	12,334	7,561	61	11,485,288
30	CB30	Colostrum	10,787	6,362	59	12,502,948
31	CB31	Colostrum	13,333	8,682	65	7,860,082
32	CB32	Colostrum	13,919	9,270	67	4,238,887

Appendix 5. Supporting results from microRNA sequencing.

Appendix 5.1. Top 100 highly expressed miRNAs in colostrum.

No	miRNA	Average of read count	No	miRNA	Average of read count
1	bta-miR-30a-5p	1,265,238	51	bta-miR-151-3p	10,771
2	bta-miR-21-5p	387,591	52	bta-miR-223	10,473
3	bta-let-7a-5p	350,276	53	bta-miR-378	10,318
4	bta-miR-26a	212,399	54	bta-miR-339a	10,285
5	bta-let-7f	181,575	55	bta-miR-339b	10,285
6	bta-miR-2285t	139,794	56	bta-miR-15b	9,843
7	bta-miR-451	136,365	57	bta-miR-374b	9,800
8	bta-miR-148a	135,208	58	bta-miR-98	9,785
9	bta-let-7b	122,953	59	bta-miR-2284x	9,678
10	bta-let-7g	92,446	60	bta-miR-2284x	9,678
11	bta-miR-200a	79,407	61	bta-miR-2284y	9,644
12	bta-miR-30e-5p	68,103	62	bta-miR-2285av	9,644
13	bta-miR-30d	66,465	63	bta-miR-2285av	9,644
14	bta-miR-200c	55,189	64	bta-miR-2285av	9,644
15	bta-miR-26b	54,229	65	bta-miR-574	9,243
16	bta-miR-99a-5p	52,148	66	bta-miR-29a	8,645
17	bta-miR-92a	49,766	67	bta-miR-423-5p	7,338
18	bta-miR-30f	44,098	68	bta-miR-6524	7,297
19	bta-miR-30b-5p	43,148	69	bta-miR-183	7,126
20	bta-miR-27b	41,568	70	bta-miR-20a	6,842
21	bta-miR-186	40,624	71	bta-miR-429	6,738
22	bta-let-7c	36,983	72	bta-miR-140	6,719
23	bta-miR-200b	34,860	73	bta-miR-148b	6,490
24	bta-miR-19b	31,491	74	bta-miR-1246	6,260
25	bta-miR-22-3p	31,022	75	bta-miR-6119-5p	6,135
26	bta-miR-141	30,783	76	bta-miR-652	6,074
27	bta-miR-101	27,927	77	bta-miR-17-5p	5,887
28	bta-miR-181a	27,210	78	bta-miR-125b	5,766
29	bta-miR-25	26,056	79	bta-let-7e	5,608
30	bta-miR-93	25,602	80	bta-miR-32	5,357
31	bta-miR-182	24,796	81	bta-miR-142-5p	5,356
32	bta-miR-23a	22,844	82	bta-miR-15a	5,254
33	bta-let-7i	22,800	83	bta-miR-425-5p	5,244
34	bta-miR-24-3p	21,733	84	bta-miR-181b	4,661
35	bta-miR-103	20,697	85	bta-miR-152	4,388

36	bta-miR-191	20,469	86	bta-miR-96	4,329
37	bta-miR-23b-3p	19,901	87	bta-miR-486	4,068
38	bta-miR-10174-3p	19,901	88	bta-miR-532	4,042
39	bta-let-7d	19,569	89	bta-miR-3613a	4,020
40	bta-miR-16b	19,093	90	bta-miR-660	3,934
41	bta-miR-16a	17,391	91	bta-miR-146a	3,764
42	bta-miR-30c	17,161	92	bta-miR-342	3,550
43	bta-miR-27a-3p	16,404	93	bta-miR-361	3,362
44	bta-miR-320a	14,754	94	bta-miR-221	3,329
45	bta-miR-374a	14,752	95	bta-miR-125a	3,197
46	bta-miR-106b	14,260	96	bta-miR-28	3,174
47	bta-miR-151-5p	13,285	97	bta-miR-7	3,128
48	bta-miR-423-3p	13,147	98	bta-miR-146b	3,120
49	bta-miR-144	12,948	99	bta-miR-362-5p	2,854
50	bta-miR-363	12,693	100	bta-miR-2285bf	2,724

Appendix 5.2. Top 100 highly expressed miRNAs in dam colostrum.

No	miRNA	Average of read count	No	miRNA	Average of read count
1	bta-miR-30a-5p	1,445,459	51	bta-miR-151-3p	12,077
2	bta-miR-21-5p	437,968	52	bta-miR-223	8,013
3	bta-let-7a-5p	293,931	53	bta-miR-378	16,139
4	bta-miR-26a	237,576	54	bta-miR-339a	14,955
5	bta-let-7f	192,097	55	bta-miR-339b	14,955
6	bta-miR-2285t	194,405	56	bta-miR-15b	11,782
7	bta-miR-451	228,838	57	bta-miR-374b	11,834
8	bta-miR-148a	159,995	58	bta-miR-98	11,317
9	bta-let-7b	160,604	59	bta-miR-2284x	10,086
10	bta-let-7g	99,069	60	bta-miR-2284x	10,086
11	bta-miR-200a	93,680	61	bta-miR-2284y	10,041
12	bta-miR-30e-5p	77,814	62	bta-miR-2285av	10,041
13	bta-miR-30d	66,848	63	bta-miR-2285av	10,041
14	bta-miR-200c	57,367	64	bta-miR-2285av	10,041
15	bta-miR-26b	62,283	65	bta-miR-574	13,755
16	bta-miR-99a-5p	67,832	66	bta-miR-29a	9,541
17	bta-miR-92a	57,963	67	bta-miR-423-5p	7,911
18	bta-miR-30f	56,536	68	bta-miR-6524	10,262
19	bta-miR-30b-5p	59,248	69	bta-miR-183	8,267
20	bta-miR-27b	48,968	70	bta-miR-20a	9,494
21	bta-miR-186	50,851	71	bta-miR-429	6,989
22	bta-let-7c	41,096	72	bta-miR-140	9,726
23	bta-miR-200b	40,145	73	bta-miR-148b	8,632
24	bta-miR-19b	49,894	74	bta-miR-1246	8,340
25	bta-miR-22-3p	38,650	75	bta-miR-6119-5p	7,283
26	bta-miR-141	32,707	76	bta-miR-652	6,520
27	bta-miR-101	34,234	77	bta-miR-17-5p	8,025
28	bta-miR-181a	31,341	78	bta-miR-125b	7,222
29	bta-miR-25	32,700	79	bta-let-7e	6,810
30	bta-miR-93	32,772	80	bta-miR-32	5,495
31	bta-miR-182	32,817	81	bta-miR-142-5p	4,856
32	bta-miR-23a	28,757	82	bta-miR-15a	7,210
33	bta-let-7i	27,038	83	bta-miR-425-5p	6,529
34	bta-miR-24-3p	23,466	84	bta-miR-181b	5,987
35	bta-miR-103	26,255	85	bta-miR-152	5,909
36	bta-miR-191	25,846	86	bta-miR-96	6,105
37	bta-miR-23b-3p	25,572	87	bta-miR-486	6,692

38	bta-miR-10174-3p	25,572	88	bta-miR-532	5,137
39	bta-let-7d	23,734	89	bta-miR-3613a	4,459
40	bta-miR-16b	23,352	90	bta-miR-660	4,545
41	bta-miR-16a	19,332	91	bta-miR-146a	4,666
42	bta-miR-30c	21,358	92	bta-miR-342	4,711
43	bta-miR-27a-3p	17,793	93	bta-miR-361	3,725
44	bta-miR-320a	21,520	94	bta-miR-221	4,148
45	bta-miR-374a	14,697	95	bta-miR-125a	4,292
46	bta-miR-106b	19,897	96	bta-miR-28	3,507
47	bta-miR-151-5p	14,952	97	bta-miR-7	3,952
48	bta-miR-423-3p	16,824	98	bta-miR-146b	3,990
49	bta-miR-144	18,319	99	bta-miR-362-5p	3,900
50	bta-miR-363	16,576	100	bta-miR-2285bf	3,107

Appendix 5.3. Top 100 highly expressed miRNAs in pooled colostrum.

No	miRNA	Average of read count	No	miRNA	Average of read count
1	bta-miR-30a-5p	1,085,017	51	bta-miR-151-3p	9,466
2	bta-miR-21-5p	337,215	52	bta-miR-223	12,932
3	bta-let-7a-5p	406,621	53	bta-miR-378	4,497
4	bta-miR-26a	187,223	54	bta-miR-339a	5,614
5	bta-let-7f	171,052	55	bta-miR-339b	5,614
6	bta-miR-2285t	85,184	56	bta-miR-15b	7,905
7	bta-miR-451	43,892	57	bta-miR-374b	7,766
8	bta-miR-148a	110,420	58	bta-miR-98	8,253
9	bta-let-7b	85,303	59	bta-miR-2284x	9,270
10	bta-let-7g	85,824	60	bta-miR-2284x	9,270
11	bta-miR-200a	65,134	61	bta-miR-2284y	9,248
12	bta-miR-30e-5p	58,391	62	bta-miR-2285av	9,248
13	bta-miR-30d	66,083	63	bta-miR-2285av	9,248
14	bta-miR-200c	53,011	64	bta-miR-2285av	9,248
15	bta-miR-26b	46,176	65	bta-miR-574	4,731
16	bta-miR-99a-5p	36,464	66	bta-miR-29a	7,750
17	bta-miR-92a	41,569	67	bta-miR-423-5p	6,765
18	bta-miR-30f	31,660	68	bta-miR-6524	4,333
19	bta-miR-30b-5p	27,048	69	bta-miR-183	5,986
20	bta-miR-27b	34,167	70	bta-miR-20a	4,190
21	bta-miR-186	30,397	71	bta-miR-429	6,487
22	bta-let-7c	32,871	72	bta-miR-140	3,711
23	bta-miR-200b	29,575	73	bta-miR-148b	4,349
24	bta-miR-19b	13,088	74	bta-miR-1246	4,179
25	bta-miR-22-3p	23,395	75	bta-miR-6119-5p	4,986
26	bta-miR-141	28,858	76	bta-miR-652	5,627
27	bta-miR-101	21,620	77	bta-miR-17-5p	3,749
28	bta-miR-181a	23,080	78	bta-miR-125b	4,311
29	bta-miR-25	19,413	79	bta-let-7e	4,406
30	bta-miR-93	18,431	80	bta-miR-32	5,218
31	bta-miR-182	16,775	81	bta-miR-142-5p	5,856
32	bta-miR-23a	16,930	82	bta-miR-15a	3,298
33	bta-let-7i	18,562	83	bta-miR-425-5p	3,960
34	bta-miR-24-3p	20,000	84	bta-miR-181b	3,335
35	bta-miR-103	15,140	85	bta-miR-152	2,866
36	bta-miR-191	15,092	86	bta-miR-96	2,553
37	bta-miR-23b-3p	14,230	87	bta-miR-486	1,444

38	bta-miR-10174-3p	14,230	88	bta-miR-532	2,948
39	bta-let-7d	15,404	89	bta-miR-3613a	3,581
40	bta-miR-16b	14,835	90	bta-miR-660	3,324
41	bta-miR-16a	15,450	91	bta-miR-146a	2,862
42	bta-miR-30c	12,965	92	bta-miR-342	2,389
43	bta-miR-27a-3p	15,016	93	bta-miR-361	3,000
44	bta-miR-320a	7,989	94	bta-miR-221	2,511
45	bta-miR-374a	14,808	95	bta-miR-125a	2,101
46	bta-miR-106b	8,624	96	bta-miR-28	2,841
47	bta-miR-151-5p	11,617	97	bta-miR-7	2,304
48	bta-miR-423-3p	9,470	98	bta-miR-146b	2,250
49	bta-miR-144	7,577	99	bta-miR-362-5p	1,808
50	bta-miR-363	8,810	100	bta-miR-2285bf	2,341

Appendix 5.4. Top 100 highly expressed miRNAs in calf blood day 0.

No	miRNA	Average of read count	No	miRNA	Average of read count
1	bta-miR-451	13,536,419	51	bta-miR-374a	43,422
2	bta-miR-144	3,556,660	52	bta-miR-19b	40,717
3	bta-miR-16b	1,149,948	53	bta-miR-140	35,957
4	bta-miR-26a	810,998	54	bta-miR-24-3p	35,221
5	bta-miR-101	775,410	55	bta-miR-223	34,205
6	bta-miR-16a	752,518	56	bta-let-7d	33,277
7	bta-miR-30e-5p	558,957	57	bta-miR-22-3p	31,604
8	bta-miR-21-5p	544,833	58	bta-miR-185	27,466
9	bta-miR-486	423,833	59	bta-miR-410	26,002
10	bta-miR-106b	335,528	60	bta-miR-127	24,415
11	bta-miR-25	324,347	61	bta-miR-151-3p	22,956
12	bta-miR-93	321,293	62	bta-miR-194	21,800
13	bta-let-7f	305,119	63	bta-miR-505	21,401
14	bta-miR-15a	290,276	64	bta-miR-221	21,033
15	bta-miR-15b	287,208	65	bta-miR-199b	20,766
16	bta-miR-92a	283,448	66	bta-miR-342	20,527
17	bta-let-7g	234,214	67	bta-miR-19a	20,475
18	bta-miR-191	224,215	68	bta-miR-128	19,901
19	bta-let-7a-5p	207,509	69	bta-miR-2285bc	19,108
20	bta-miR-425-5p	176,864	70	bta-miR-380-3p	19,002
21	bta-let-7i	174,028	71	bta-miR-493	18,497
22	bta-miR-186	150,006	72	bta-miR-652	17,960
23	bta-miR-411a	126,416	73	bta-miR-27a-3p	17,730
24	bta-miR-339a	98,117	74	bta-miR-148a	16,876
25	bta-miR-339b	98,116	75	bta-miR-411b	16,730
26	bta-miR-494	91,643	76	bta-miR-29c	16,245
27	bta-miR-32	90,482	77	bta-miR-382	16,036
28	bta-miR-30d	83,766	78	bta-miR-154c	15,619
29	bta-miR-142-3p	83,429	79	bta-miR-23a	14,291
30	bta-miR-379	82,711	80	bta-miR-2285bf	13,756
31	bta-miR-26b	80,020	81	bta-miR-30b-5p	12,942
32	bta-miR-17-5p	73,646	82	bta-miR-301a	12,890
33	bta-miR-151-5p	70,465	83	bta-miR-23b-3p	12,608
34	bta-miR-381	69,702	84	bta-miR-10174-3p	12,608
35	bta-miR-369-3p	68,555	85	bta-miR-361	12,598
36	bta-miR-199c	67,594	86	bta-miR-484	12,311
37	bta-miR-199a-3p	67,591	87	bta-miR-363	11,809

38	bta-miR-20a	66,704	88	bta-miR-107	11,537
39	bta-miR-18a	60,906	89	bta-miR-99a-5p	11,270
40	bta-miR-487b	60,387	90	bta-miR-423-5p	10,972
41	bta-miR-142-5p	58,860	91	bta-miR-331-3p	10,962
42	bta-miR-30c	57,745	92	bta-miR-2419-5p	10,114
43	bta-miR-148b	56,347	93	bta-miR-495	9,614
44	bta-miR-6119-5p	54,086	94	bta-miR-2284aa	9,610
45	bta-miR-27b	50,614	95	bta-miR-2284aa	9,610
46	bta-miR-98	50,205	96	bta-miR-2284z	9,610
47	bta-miR-376e	49,838	97	bta-miR-1468	9,285
48	bta-miR-423-3p	45,937	98	bta-miR-222	9,111
49	bta-miR-103	44,268	99	bta-miR-376b	9,090
50	bta-miR-181a	43,879	100	bta-miR-409a	9,067

Appendix 5.5. Top 100 highly expressed miRNAs in calf blood day 1.

No	miRNA	Average of read count	No	miRNA	Average of read count
1	bta-miR-451	11,047,332	51	bta-miR-98	48,157
2	bta-miR-144	3,407,894	52	bta-miR-140	45,737
3	bta-miR-26a	941,400	53	bta-miR-19b	45,310
4	bta-miR-16b	887,662	54	bta-miR-185	45,164
5	bta-miR-101	796,228	55	bta-miR-223	43,736
6	bta-miR-16a	713,657	56	bta-miR-24-3p	41,308
7	bta-miR-486	689,025	57	bta-miR-22-3p	39,894
8	bta-miR-30e-5p	675,660	58	bta-let-7d	39,373
9	bta-miR-21-5p	484,707	59	bta-miR-151-3p	37,189
10	bta-miR-25	415,538	60	bta-miR-127	36,710
11	bta-miR-92a	388,323	61	bta-miR-2285bc	32,264
12	bta-miR-93	375,579	62	bta-miR-221	29,681
13	bta-let-7f	318,238	63	bta-miR-410	29,069
14	bta-miR-106b	292,760	64	bta-miR-342	28,948
15	bta-let-7g	290,824	65	bta-miR-505	28,675
16	bta-miR-15b	279,568	66	bta-miR-128	27,182
17	bta-miR-191	256,442	67	bta-miR-380-3p	23,718
18	bta-miR-15a	240,088	68	bta-miR-382	23,550
19	bta-let-7a-5p	226,536	69	bta-miR-199b	22,789
20	bta-let-7i	211,269	70	bta-miR-194	22,629
21	bta-miR-425-5p	206,791	71	bta-miR-148a	22,600
22	bta-miR-142-5p	197,564	72	bta-miR-493	21,775
23	bta-miR-186	197,124	73	bta-miR-652	21,651
24	bta-miR-339a	133,442	74	bta-miR-154c	20,770
25	bta-miR-339b	133,441	75	bta-miR-423-5p	20,451
26	bta-miR-494	128,733	76	bta-miR-2285bf	20,129
27	bta-miR-411a	117,465	77	bta-miR-19a	19,515
28	bta-miR-30d	114,791	78	bta-miR-363	18,826
29	bta-miR-142-3p	110,429	79	bta-miR-484	17,674
30	bta-miR-379	97,471	80	bta-miR-27a-3p	17,211
31	bta-miR-6119-5p	93,497	81	bta-miR-411b	16,976
32	bta-miR-487b	91,898	82	bta-miR-29c	16,706
33	bta-miR-151-5p	88,748	83	bta-miR-2284x	15,949
34	bta-miR-32	85,183	84	bta-miR-2284x	15,949
35	bta-miR-369-3p	81,739	85	bta-miR-2284y	15,908
36	bta-miR-381	79,836	86	bta-miR-2285av	15,908
37	bta-miR-17-5p	69,929	87	bta-miR-2285av	15,908

38	bta-miR-26b	69,689	88	bta-miR-2285av	15,908
39	bta-miR-30c	65,406	89	bta-miR-361	15,903
40	bta-miR-148b	65,080	90	bta-miR-99a-5p	15,608
41	bta-miR-376e	65,079	91	bta-miR-1468	15,300
42	bta-miR-103	64,417	92	bta-miR-107	14,833
43	bta-miR-423-3p	63,919	93	bta-miR-331-3p	14,417
44	bta-miR-181a	62,540	94	bta-miR-23b-3p	14,348
45	bta-miR-374a	60,572	95	bta-miR-10174-3p	14,348
46	bta-miR-20a	58,120	96	bta-miR-23a	13,944
47	bta-miR-199c	56,783	97	bta-miR-2419-5p	13,462
48	bta-miR-199a-3p	56,780	98	bta-miR-150	13,317
49	bta-miR-27b	52,217	99	bta-miR-487a	12,218
50	bta-miR-18a	50,746	100	bta-miR-30b-5p	11,733

Appendix 5.6. GO pathway of Top 100 most abundant miRNAs in colostrum (220 significant GO terms).

Ont = ontologies (CC = cellular component, BP = biological process, MF = molecular function). N = total number of genes related to the term; DE = number of genes in the dataset for this term; P.DE = p-value; fdr= false discovery rate.

No	GO ID	Term	Ont	N	DE	P.DE	fdr
1	GO:0110165	cellular anatomical entity	CC	4574	343	7.3E-19	1.0E-14
2	GO:0005575	cellular_component	CC	4657	346	2.1E-18	1.5E-14
3	GO:0008150	biological_process	BP	4425	325	3.0E-16	1.4E-12
4	GO:0005622	intracellular	CC	3544	269	9.4E-15	3.3E-11
5	GO:0009987	cellular process	BP	3968	292	2.1E-14	5.2E-11
6	GO:0065007	biological regulation	BP	2379	198	2.2E-14	5.2E-11
7	GO:0043229	intracellular organelle	CC	3020	235	7.0E-14	1.4E-10
8	GO:0043226	organelle	CC	3194	245	8.2E-14	1.5E-10
9	GO:0050789	regulation of biological process	BP	2191	183	2.1E-13	3.3E-10
10	GO:0016020	membrane	CC	2141	179	3.8E-13	5.4E-10
11	GO:0003674	molecular_function	MF	3794	274	2.1E-12	2.6E-09
12	GO:0005488	binding	MF	2967	225	4.3E-12	5.1E-09
13	GO:0050794	regulation of cellular process	BP	2025	167	9.0E-12	8.5E-09
14	GO:0043227	membrane-bounded organelle	CC	2816	215	8.2E-12	8.5E-09
15	GO:0005737	cytoplasm	CC	2802	214	9.0E-12	8.5E-09
16	GO:0043231	intracellular membrane-bounded organelle	CC	2477	192	4.3E-11	3.8E-08
17	GO:0051179	localization	BP	1316	118	1.2E-10	1.0E-07
18	GO:0051234	establishment of localization	BP	1065	100	3.1E-10	2.4E-07
19	GO:0016021	integral component of membrane	CC	1170	106	6.7E-10	5.0E-07
20	GO:0006810	transport	BP	1040	97	8.4E-10	6.0E-07
21	GO:0031224	intrinsic component of membrane	CC	1207	107	1.9E-09	1.3E-06
22	GO:0009653	anatomical structure morphogenesis	BP	445	53	2.6E-09	1.7E-06
23	GO:0012505	endomembrane system	CC	1004	93	2.7E-09	1.7E-06
24	GO:0043168	anion binding	MF	737	74	4.5E-09	2.6E-06
25	GO:0009887	animal organ morphogenesis	BP	145	26	9.8E-09	5.5E-06
26	GO:0032501	multicellular organismal process	BP	1326	112	1.1E-08	6.2E-06
27	GO:0043167	ion binding	MF	1629	130	2.1E-08	1.1E-05
28	GO:0051716	cellular response to stimulus	BP	1359	113	2.3E-08	1.2E-05
29	GO:0048856	anatomical structure development	BP	1028	91	3.6E-08	1.8E-05
30	GO:0032502	developmental process	BP	1123	97	4.2E-08	2.0E-05
31	GO:0048468	cell development	BP	331	41	5.7E-08	2.6E-05
32	GO:0050896	response to stimulus	BP	1764	136	7.6E-08	3.4E-05

33	GO:0006811	ion transport	BP	361	43	8.4E-08	3.6E-05
34	GO:0009888	tissue development	BP	331	40	1.6E-07	6.7E-05
35	GO:0005794	Golgi apparatus	CC	319	39	1.7E-07	6.9E-05
36	GO:0030001	metal ion transport	BP	167	26	1.9E-07	7.5E-05
37	GO:0070838	divalent metal ion transport	BP	90	18	3.4E-07	1.3E-04
38	GO:0048869	cellular developmental process	BP	717	67	3.7E-07	1.4E-04
39	GO:0072511	divalent inorganic cation transport	BP	91	18	4.0E-07	1.5E-04
40	GO:0006812	cation transport	BP	243	32	4.1E-07	1.5E-04
41	GO:0061061	muscle structure development	BP	113	20	6.1E-07	2.1E-04
42	GO:0005515	protein binding	MF	1383	109	6.2E-07	2.1E-04
43	GO:0030154	cell differentiation	BP	699	65	6.7E-07	2.1E-04
44	GO:0048731	system development	BP	788	71	6.6E-07	2.1E-04
45	GO:0098588	bounding membrane of organelle	CC	472	49	7.4E-07	2.3E-04
46	GO:0007275	multicellular organism development	BP	920	79	1.0E-06	3.2E-04
47	GO:0023052	signaling	BP	1065	88	1.2E-06	3.7E-04
48	GO:0007154	cell communication	BP	1086	89	1.5E-06	4.4E-04
49	GO:0006816	calcium ion transport	BP	80	16	1.5E-06	4.4E-04
50	GO:0008152	metabolic process	BP	2823	191	1.8E-06	5.0E-04
51	GO:0071944	cell periphery	CC	859	74	2.0E-06	5.7E-04
52	GO:0051641	cellular localization	BP	493	49	2.6E-06	7.1E-04
53	GO:0065008	regulation of biological quality	BP	759	67	2.8E-06	7.4E-04
54	GO:0019222	regulation of metabolic process	BP	1203	95	3.2E-06	8.3E-04
55	GO:0007517	muscle organ development	BP	66	14	3.2E-06	8.3E-04
56	GO:0002064	epithelial cell development	BP	42	11	4.2E-06	1.1E-03
57	GO:0031090	organelle membrane	CC	1021	83	4.7E-06	1.2E-03
58	GO:0097367	carbohydrate derivative binding	MF	591	55	4.8E-06	1.2E-03
59	GO:0042221	response to chemical	BP	787	68	4.9E-06	1.2E-03
60	GO:0005886	plasma membrane	CC	834	71	5.0E-06	1.2E-03
61	GO:0031982	vesicle	CC	510	49	6.7E-06	1.5E-03
62	GO:0048729	tissue morphogenesis	BP	100	17	7.4E-06	1.7E-03
63	GO:0071704	organic substance metabolic process	BP	2565	173	7.5E-06	1.7E-03
64	GO:0031410	cytoplasmic vesicle	CC	484	47	7.7E-06	1.7E-03
65	GO:0097708	intracellular vesicle	CC	484	47	7.7E-06	1.7E-03
66	GO:0060537	muscle tissue development	BP	71	14	8.0E-06	1.7E-03
67	GO:0030855	epithelial cell differentiation	BP	111	18	8.0E-06	1.7E-03
68	GO:0006464	cellular protein modification process	BP	847	71	8.5E-06	1.7E-03
69	GO:0036211	protein modification process	BP	847	71	8.5E-06	1.7E-03
70	GO:0044238	primary metabolic process	BP	2377	162	8.8E-06	1.8E-03

71	GO:0048518	positive regulation of biological process	BP	1105	87	9.7E-06	1.9E-03
72	GO:0060255	regulation of macromolecule metabolic process	BP	1105	87	9.7E-06	1.9E-03
73	GO:0016192	vesicle-mediated transport	BP	308	34	1.0E-05	2.0E-03
74	GO:0070887	cellular response to chemical stimulus	BP	578	53	1.1E-05	2.1E-03
75	GO:0048523	negative regulation of cellular process	BP	827	69	1.3E-05	2.5E-03
76	GO:0048519	negative regulation of biological process	BP	938	76	1.4E-05	2.5E-03
77	GO:0035637	multicellular organismal signaling	BP	18	7	1.4E-05	2.5E-03
78	GO:0046903	secretion	BP	211	26	1.6E-05	3.0E-03
79	GO:0007165	signal transduction	BP	994	79	1.8E-05	3.3E-03
80	GO:0006807	nitrogen compound metabolic process	BP	2202	150	2.1E-05	3.6E-03
81	GO:0043412	macromolecule modification	BP	901	73	2.0E-05	3.6E-03
82	GO:0051649	establishment of localization in cell	BP	360	37	2.1E-05	3.6E-03
83	GO:0045446	endothelial cell differentiation	BP	19	7	2.1E-05	3.6E-03
84	GO:0080090	regulation of primary metabolic process	BP	1032	81	2.2E-05	3.7E-03
85	GO:1901564	organonitrogen compound metabolic process	BP	1599	115	2.3E-05	3.8E-03
86	GO:0042592	homeostatic process	BP	348	36	2.3E-05	3.8E-03
87	GO:0036094	small molecule binding	MF	717	61	2.4E-05	3.9E-03
88	GO:0043170	macromolecule metabolic process	BP	2070	142	2.5E-05	4.1E-03
89	GO:0045177	apical part of cell	CC	79	14	2.9E-05	4.6E-03
90	GO:0051171	regulation of nitrogen compound metabolic process	BP	995	78	3.3E-05	5.2E-03
91	GO:0048513	animal organ development	BP	544	49	3.7E-05	5.6E-03
92	GO:0017076	purine nucleotide binding	MF	514	47	3.6E-05	5.6E-03
93	GO:0031323	regulation of cellular metabolic process	BP	1059	81	5.4E-05	8.3E-03
94	GO:0060429	epithelium development	BP	188	23	5.6E-05	8.4E-03
95	GO:0050793	regulation of developmental process	BP	435	41	5.7E-05	8.6E-03
96	GO:0010468	regulation of gene expression	BP	755	62	5.8E-05	8.6E-03
97	GO:0019538	protein metabolic process	BP	1329	97	6.1E-05	8.9E-03
98	GO:0046777	protein autophosphorylation	BP	22	7	6.2E-05	8.9E-03
99	GO:0044260	cellular macromolecule metabolic process	BP	1710	119	6.9E-05	9.8E-03
100	GO:0051246	regulation of protein metabolic process	BP	558	49	6.9E-05	9.8E-03
101	GO:0032555	purine ribonucleotide binding	MF	513	46	7.0E-05	9.8E-03

102	GO:0048747	muscle fiber development	BP	16	6	7.4E-05	1.0E-02
103	GO:0055002	striated muscle cell development	BP	30	8	7.6E-05	1.0E-02
104	GO:0032553	ribonucleotide binding	MF	515	46	7.7E-05	1.0E-02
105	GO:0014706	striated muscle tissue development	BP	66	12	8.2E-05	1.1E-02
106	GO:0003158	endothelium development	BP	23	7	8.5E-05	1.1E-02
107	GO:0016043	cellular component organization	BP	1190	88	8.6E-05	1.1E-02
108	GO:0043269	regulation of ion transport	BP	109	16	8.7E-05	1.1E-02
109	GO:0098805	whole membrane	CC	371	36	8.9E-05	1.2E-02
110	GO:0055085	transmembrane transport	BP	330	33	9.8E-05	1.2E-02
111	GO:0034220	ion transmembrane transport	BP	234	26	9.7E-05	1.2E-02
112	GO:0055001	muscle cell development	BP	31	8	9.8E-05	1.2E-02
113	GO:0035639	purine ribonucleoside triphosphate binding	MF	490	44	9.7E-05	1.2E-02
114	GO:0071310	cellular response to organic substance	BP	432	40	1.0E-04	1.3E-02
115	GO:0032413	negative regulation of ion transmembrane transporter activity	BP	11	5	1.0E-04	1.3E-02
116	GO:0061337	cardiac conduction	BP	11	5	1.0E-04	1.3E-02
117	GO:0034763	negative regulation of transmembrane transport	BP	17	6	1.1E-04	1.3E-02
118	GO:0032879	regulation of localization	BP	493	44	1.1E-04	1.3E-02
119	GO:0002070	epithelial cell maturation	BP	3	3	1.2E-04	1.4E-02
120	GO:0072189	ureter development	BP	3	3	1.2E-04	1.4E-02
121	GO:0044267	cellular protein metabolic process	BP	1186	87	1.3E-04	1.5E-02
122	GO:0000902	cell morphogenesis	BP	148	19	1.3E-04	1.5E-02
123	GO:0008144	drug binding	MF	451	41	1.3E-04	1.5E-02
124	GO:0071840	cellular component organization or biogenesis	BP	1238	90	1.3E-04	1.5E-02
125	GO:0001503	ossification	BP	80	13	1.4E-04	1.6E-02
126	GO:0010033	response to organic substance	BP	544	47	1.4E-04	1.6E-02
127	GO:0043271	negative regulation of ion transport	BP	18	6	1.6E-04	1.8E-02
128	GO:0007267	cell-cell signaling	BP	255	27	1.6E-04	1.8E-02
129	GO:0051239	regulation of multicellular organismal process	BP	547	47	1.6E-04	1.8E-02
130	GO:1904063	negative regulation of cation transmembrane transport	BP	12	5	1.7E-04	1.9E-02
131	GO:0035091	phosphatidylinositol binding	MF	42	9	1.7E-04	1.9E-02
132	GO:0051049	regulation of transport	BP	326	32	1.7E-04	1.9E-02
133	GO:0044237	cellular metabolic process	BP	2462	159	1.8E-04	1.9E-02
134	GO:0031399	regulation of protein modification process	BP	341	33	1.8E-04	1.9E-02
135	GO:0097178	ruffle assembly	BP	7	4	1.8E-04	1.9E-02
136	GO:0046872	metal ion binding	MF	1083	80	1.9E-04	2.0E-02
137	GO:0030003	cellular cation homeostasis	BP	105	15	2.0E-04	2.0E-02

138	GO:0048878	chemical homeostasis	BP	206	23	2.2E-04	2.3E-02
139	GO:0070509	calcium ion import	BP	19	6	2.2E-04	2.3E-02
140	GO:0048522	positive regulation of cellular process	BP	990	74	2.3E-04	2.3E-02
141	GO:0003824	catalytic activity	MF	1396	98	2.3E-04	2.3E-02
142	GO:0072359	circulatory system development	BP	207	23	2.4E-04	2.4E-02
143	GO:0043169	cation binding	MF	1091	80	2.4E-04	2.4E-02
144	GO:0097159	organic cyclic compound binding	MF	1245	89	2.5E-04	2.5E-02
145	GO:0034762	regulation of transmembrane transport	BP	96	14	2.5E-04	2.5E-02
146	GO:1901363	heterocyclic compound binding	MF	1229	88	2.6E-04	2.5E-02
147	GO:0034766	negative regulation of ion transmembrane transport	BP	13	5	2.7E-04	2.5E-02
148	GO:0000166	nucleotide binding	MF	606	50	2.7E-04	2.5E-02
149	GO:1901265	nucleoside phosphate binding	MF	606	50	2.7E-04	2.5E-02
150	GO:0005634	nucleus	CC	1437	100	2.7E-04	2.6E-02
151	GO:0055074	calcium ion homeostasis	BP	75	12	2.9E-04	2.7E-02
152	GO:0033993	response to lipid	BP	145	18	2.9E-04	2.7E-02
153	GO:0006873	cellular ion homeostasis	BP	109	15	3.0E-04	2.7E-02
154	GO:0045732	positive regulation of protein catabolic process	BP	36	8	3.0E-04	2.8E-02
155	GO:0000139	Golgi membrane	CC	146	18	3.1E-04	2.9E-02
156	GO:0055080	cation homeostasis	BP	122	16	3.3E-04	3.0E-02
157	GO:0010646	regulation of cell communication	BP	581	48	3.4E-04	3.0E-02
158	GO:0034330	cell junction organization	BP	99	14	3.5E-04	3.0E-02
159	GO:0031529	ruffle organization	BP	8	4	3.5E-04	3.0E-02
160	GO:0060306	regulation of membrane repolarization	BP	8	4	3.5E-04	3.0E-02
161	GO:0042733	embryonic digit morphogenesis	BP	8	4	3.5E-04	3.0E-02
162	GO:0051926	negative regulation of calcium ion transport	BP	8	4	3.5E-04	3.0E-02
163	GO:0042995	cell projection	CC	443	39	3.5E-04	3.0E-02
164	GO:0030134	COPII-coated ER to Golgi transport vesicle	CC	8	4	3.5E-04	3.0E-02
165	GO:0030554	adenyl nucleotide binding	MF	398	36	3.5E-04	3.0E-02
166	GO:0032268	regulation of cellular protein metabolic process	BP	520	44	3.6E-04	3.1E-02
167	GO:0005856	cytoskeleton	CC	520	44	3.6E-04	3.1E-02
168	GO:0023051	regulation of signaling	BP	583	48	3.7E-04	3.1E-02
169	GO:0072507	divalent inorganic cation homeostasis	BP	77	12	3.7E-04	3.1E-02
170	GO:0003008	system process	BP	311	30	3.7E-04	3.1E-02
171	GO:0046914	transition metal ion binding	MF	283	28	3.8E-04	3.2E-02
172	GO:0005783	endoplasmic reticulum	CC	255	26	3.8E-04	3.2E-02

173	GO:0120036	plasma membrane bounded cell projection organization	BP	214	23	3.9E-04	3.2E-02
174	GO:0098771	inorganic ion homeostasis	BP	124	16	4.0E-04	3.2E-02
175	GO:0032410	negative regulation of transporter activity	BP	14	5	4.0E-04	3.2E-02
176	GO:0007507	heart development	BP	78	12	4.2E-04	3.4E-02
177	GO:0010628	positive regulation of gene expression	BP	373	34	4.4E-04	3.5E-02
178	GO:0098655	cation transmembrane transport	BP	176	20	4.4E-04	3.5E-02
179	GO:0005768	endosome	CC	216	23	4.4E-04	3.5E-02
180	GO:1900027	regulation of ruffle assembly	BP	4	3	4.6E-04	3.6E-02
181	GO:0060644	mammary gland epithelial cell differentiation	BP	4	3	4.6E-04	3.6E-02
182	GO:1901020	negative regulation of calcium ion transmembrane transporter activity	BP	4	3	4.6E-04	3.6E-02
183	GO:1903170	negative regulation of calcium ion transmembrane transport	BP	4	3	4.6E-04	3.6E-02
184	GO:0071702	organic substance transport	BP	559	46	4.9E-04	3.8E-02
185	GO:0006875	cellular metal ion homeostasis	BP	91	13	5.1E-04	3.9E-02
186	GO:0098660	inorganic ion transmembrane transport	BP	178	20	5.2E-04	3.9E-02
187	GO:0007010	cytoskeleton organization	BP	246	25	5.2E-04	3.9E-02
188	GO:0006941	striated muscle contraction	BP	30	7	5.2E-04	3.9E-02
189	GO:0030879	mammary gland development	BP	30	7	5.2E-04	3.9E-02
190	GO:0048705	skeletal system morphogenesis	BP	30	7	5.2E-04	3.9E-02
191	GO:0003007	heart morphogenesis	BP	39	8	5.4E-04	3.9E-02
192	GO:0048736	appendage development	BP	22	6	5.4E-04	3.9E-02
193	GO:0060173	limb development	BP	22	6	5.4E-04	3.9E-02
194	GO:0051247	positive regulation of protein metabolic process	BP	304	29	5.5E-04	4.0E-02
195	GO:0007389	pattern specification process	BP	59	10	5.7E-04	4.1E-02
196	GO:0050790	regulation of catalytic activity	BP	439	38	5.8E-04	4.1E-02
197	GO:0007155	cell adhesion	BP	234	24	5.8E-04	4.1E-02
198	GO:0001885	endothelial cell development	BP	15	5	5.7E-04	4.1E-02
199	GO:0051656	establishment of organelle localization	BP	70	11	6.0E-04	4.2E-02
200	GO:0086003	cardiac muscle cell contraction	BP	9	4	6.1E-04	4.3E-02
201	GO:0060350	endochondral bone morphogenesis	BP	9	4	6.1E-04	4.3E-02
202	GO:0048583	regulation of response to stimulus	BP	709	55	6.2E-04	4.3E-02
203	GO:0050801	ion homeostasis	BP	142	17	6.4E-04	4.5E-02
204	GO:0070161	anchoring junction	CC	117	15	6.4E-04	4.5E-02

205	GO:0005829	cytosol	CC	662	52	6.5E-04	4.5E-02
206	GO:0034765	regulation of ion transmembrane transport	BP	82	12	6.7E-04	4.6E-02
207	GO:0042175	nuclear outer membrane-endoplasmic reticulum membrane network	CC	279	27	6.8E-04	4.6E-02
208	GO:0005524	ATP binding	MF	382	34	6.8E-04	4.6E-02
209	GO:0016740	transferase activity	MF	458	39	6.8E-04	4.6E-02
210	GO:0032559	adenyl ribonucleotide binding	MF	397	35	6.8E-04	4.6E-02
211	GO:0032940	secretion by cell	BP	182	20	6.8E-04	4.6E-02
212	GO:0007399	nervous system development	BP	323	30	6.9E-04	4.6E-02
213	GO:0055065	metal ion homeostasis	BP	106	14	7.1E-04	4.7E-02
214	GO:0022610	biological adhesion	BP	238	24	7.4E-04	4.9E-02
215	GO:0010604	positive regulation of macromolecule metabolic process	BP	618	49	7.5E-04	4.9E-02
216	GO:0014070	response to organic cyclic compound	BP	144	17	7.5E-04	4.9E-02
217	GO:0030659	cytoplasmic vesicle membrane	CC	144	17	7.5E-04	4.9E-02
218	GO:0006874	cellular calcium ion homeostasis	BP	72	11	7.6E-04	4.9E-02
219	GO:0090130	tissue migration	BP	72	11	7.6E-04	4.9E-02
220	GO:0009893	positive regulation of metabolic process	BP	667	52	7.7E-04	4.9E-02

Appendix 5.7. GO pathway of 22 miRNAs differentially expressed between calf blood day 0 and day 1.

Ont = ontologies (CC = cellular component, BP = biological process, MF = molecular function). N = total number of genes related to the term; DE = number of genes in the dataset for this term; P.DE = p-value; fdr= false discovery rate.

No	GO_ID	Term	Ont	N	DE	P.DE	fdr
1	GO:0005575	Cellular component	CC	4657	121	8.0E-08	3.7E-04
2	GO:0110165	cellular anatomical entity	CC	4574	119	1.0E-07	3.7E-04
3	GO:0016020	membrane	CC	2141	70	3.7E-08	3.7E-04
4	GO:0012505	endomembrane system	CC	1004	42	5.6E-08	3.7E-04
5	GO:0016021	integral component of membrane	CC	1170	45	2.0E-07	5.8E-04
6	GO:0031224	intrinsic component of membrane	CC	1207	45	4.9E-07	1.1E-03
7	GO:0031090	organelle membrane	CC	1021	40	6.6E-07	1.3E-03
8	GO:0043227	membrane-bounded organelle	CC	2816	79	2.4E-06	4.2E-03
9	GO:0008150	Biological process	BP	4425	110	4.5E-06	6.9E-03
10	GO:0009987	cellular process	BP	3968	101	4.9E-06	6.9E-03
11	GO:0043226	organelle	CC	3194	84	1.3E-05	1.7E-02
12	GO:0042175	nuclear outer membrane-endoplasmic reticulum membrane network	CC	279	16	2.3E-05	2.8E-02

Appendix 6. Functions of five immune-related miRNAs.

MicroRNA-142-5p

MicroRNA-142-5p is a regulator of a range of biological processes, including embryonic development, homeostasis, various diseases, cancer and immune tolerance (Shrestha et al., 2017). Although miR-142 is expressed in many tissues, recent studies suggest that it is a key regulator of cell fate in the hematopoietic system (Shrestha et al., 2017). MicroRNA-142 is an abundantly expressed in immune cells or cells of hematopoietic origin, and plays a critical role in development and homeostasis of lymphocytes (Kramer et al., 2015). The miR-142 family has 2 members (miR-142-3p and miR-142-5p) that are derived from opposite strands of a precursor (Kramer et al., 2015).

One target gene of miR-142-5p is *Suppressor Of Cytokine Signaling 1* (*SOCS1*). MicroRNA-142-5p regulates macrophage profibrogenic gene expression in chronic inflammation by silencing the *SOCS1* gene. Consequently, the level of miR -142-5p is increased in patients with liver cirrhosis and idiopathic pulmonary fibrosis (Su et al., 2015) and in patients of chronic antibody mediated rejection (Danger et al., 2013). Interestingly, miR-142-5p is found to be more highly expressed in the milk from mastitis cows compared to the milk of healthy cows, and therefore, miR-142-5p may be a potential biomarker for early detection of bacterial infection in mammary glands (Sun et al., 2015; Cai et al., 2018).

MicroRNA-150

MicroRNA-150 is considered to be a lymphopoietic-specific miRNA and studies have shown that miR-150 functions as a major regulator in determining the fate of haematopoietic stem/progenitor cells in both lymphoid and myeloid lineages (Xu et al., 2016). In addition, miR-150 acts as a tumor suppressor in malignant lymphoma (Watanabe et al., 2011). MYB, FLT3, EGR2 are important target genes of miR-150 in acute myeloid leukemia and lymphoma (Watanabe et al., 2011; Xu et al., 2016).

The top predicted target of miR-150 is the *MYB proto-oncogene transcription factor (MYB or c-Myb)* gene, which is important for lymphocyte development and required for the B1 cell generation (Xiao et al., 2007). MicroRNA-150 exerts its effects on hematopoiesis by targeting *MYB* (Huang et al., 2015). In a study on transgenic mice, over-expression of miR-

150 caused reduced *MYB* levels, while B cells that are deficient in miR-150 showed higher levels of *MYB* (Xiao et al., 2007).

MicroRNA-150 is present in high concentrations in both mature B and T cells and natural killer cells (Huang et al., 2015). However, miR-150 is also highly expressed in progenitor B cells (Fernando et al., 2012), preventing B cell differentiation from pro-B cells to pre-B cells (Sonkoly et al., 2008; Fernando et al., 2012; Liang et al., 2015)

MicroRNA-155

MiRNA-155 is a miRNA with multiple functions as it has an important role in various physiological processes, including haematopoietic lineage differentiation, immunity, and inflammation, as well as various pathological states, including cancer, cardiovascular diseases and viral infections (Faraoni et al., 2009). MicroRNA-155 is believed to target hundreds of genes (Faraoni et al., 2009). In the immune system, miR-155 can shape the transcriptome of activated myeloid and lymphoid cells to control different biological functions such as inflammation and immunological memory (Vigorito et al., 2013).

The miR-155 is involved in the normal immune function of both human B and T lymphocytes (Karaca et al., 2018). If miR-155 is deficient, the modulation of the T helper (Th)1/Th2 balance is affected and the number of germinal centre B cells is reduced (Karaca et al., 2018). In addition, miR-155 inhibits the activation of B cells present in the germinal centres and the differentiation of monocytes (Liang et al., 2015). A mouse study, (Blüml et al., 2011) concluded that miR-155 is involved in both adaptive and innate immune reactions leading to autoimmune arthritis. In mice that are miR-155 deficient, antigen-specific T cells are significantly reduced and interleukin-17 (IL-17) and IL-22 levels are decreased (Blüml et al., 2011).

MicroRNA-181a

The miR-181 family includes four members (miR181a, miR-181b, miR-181c and miR-181d), which have a few hundred identified mRNAs as their targets (Seoudi et al., 2012). MicroRNA-181a is involved in haematopoiesis and is a potential biomarker for both solid tumours and haematological tumours (Seoudi et al., 2012).

MicroRNA-181a has been implicated in the development of both B and T lymphocytes (Sonkoly et al., 2008) as miR-181a has been shown to have a role in B and T cell differentiation when ectopically expressed in hematopoietic stem/progenitor cells (Chen et al., 2004). If miR-181a is over-expressed in hematopoietic stem and progenitor cells, then the number of B cells increases (Fernando et al., 2012). MicroRNA-181a also enhances T cell receptor signalling by targeting protein and CD4+ T cell activation (Liang et al., 2015). Furthermore, miR-181a is related to T cell sensitivity and is involved in the inflammatory response of macrophages

MicroRNA-223

MicroRNA-223 functions as a key modulator of the differentiation and activation of myeloid cells, particularly neutrophils and macrophages (Yuan et al., 2018). MicroRNA-223 affects myeloid cell development and influences the maturation, proliferation and activation of granulocytes (Johnnidis et al., 2008) by enhancing granulopoiesis and inhibiting macrophage differentiation (Yuan et al., 2018). MicroRNA-223 directly targets multiple myeloid differentiation transcription factors to regulate this myeloid differentiation (Yuan et al., 2018).

MicroRNA-223 is also involved in T cell differentiation (Sonkoly et al., 2008) and is a negative regulator of neutrophil proliferation and activation (Lindsay, 2008). MicroRNA-223 is more highly expressed in milk from cows with mastitis compared to normal cows, so it may be potential biomarker for early detection of bacterial infection of the mammary gland (Sun et al., 2015; Cai et al., 2018) or sepsis in humans (Wang et al., 2010).

References of Appendix 6

Blüml, S., M. Bonelli, B. Niederreiter, A. Puchner, G. Mayr, S. Hayer, M. I. Koenders, W. B. van den Berg, J. Smolen, and K. Redlich. 2011. Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. Arthritis & Rheumatism 63(5):1281-1288.

Cai, M., H. He, X. Jia, S. Chen, J. Wang, Y. Shi, B. Liu, W. Xiao, and S. Lai. 2018. Genome-wide microRNA profiling of bovine milk-derived exosomes infected with Staphylococcus aureus. Cell Stress and Chaperones 23(4):663-672.

Chen, C.-Z., L. Li, H. F. Lodish, and D. P. Bartel. 2004. MicroRNAs modulate hematopoietic lineage differentiation. science 303(5654):83-86.

Danger, R., C. Paul, M. Giral, A. Lavault, Y. Foucher, N. Degauque, A. Pallier, M. Durand, S. Castagnet, J.-P. Duong Van Huyen, M. Delahousse, K. Renaudin, J.-P. Soulillou, and S. Brouard. 2013. Expression of miR-142-5p in Peripheral Blood Mononuclear Cells from Renal Transplant Patients with Chronic Antibody-Mediated Rejection. PLOS ONE 8(4):e60702. 10.1371/journal.pone.0060702

Faraoni, I., F. R. Antonetti, J. Cardone, and E. Bonmassar. 2009. miR-155 gene: a typical multifunctional microRNA. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1792(6):497-505.

Fernando, T. R., N. I. Rodriguez-Malave, and D. S. Rao. 2012. MicroRNAs in B cell development and malignancy. Journal of hematology & oncology 5(1):1-10.

Huang, X.-L., L. Zhang, J.-P. Li, Y.-J. Wang, Y. Duan, and J. Wang. 2015. MicroRNA-150: a potential regulator in pathogens infection and autoimmune diseases. Autoimmunity 48(8):503-510.

Johnnidis, J. B., M. H. Harris, R. T. Wheeler, S. Stehling-Sun, M. H. Lam, O. Kirak, T. R. Brummelkamp, M. D. Fleming, and F. D. Camargo. 2008. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature 451(7182):1125-1129.

Karaca, E., A. Aykut, B. Ertürk, B. Durmaz, A. Güler, B. Büke, A. Ö. Yeniel, A. M. Ergenoğlu, F. Özkınay, M. Özeren, M. Kazandı, F. Akercan, S. Sağol, C. Gündüz, and Ö. Çoğulu. 2018. MicroRNA Expression Profile in the Prenatal Amniotic Fluid Samples of Pregnant Women with Down Syndrome. Balkan Med J 35(2):163-166. 10.4274/balkanmedj.2017.0511

Kramer, N. J., W.-L. Wang, E. Y. Reyes, B. Kumar, C.-C. Chen, C. Ramakrishna, E. M. Cantin, S. L. Vonderfecht, K. D. Taganov, and N. Chau. 2015. Altered lymphopoiesis and immunodeficiency in miR-142 null mice. Blood, The Journal of the American Society of Hematology 125(24):3720-3730.

Liang, G., N. Malmuthuge, L. L. Guan, and P. Griebel. 2015. Model systems to analyze the role of miRNAs and commensal microflora in bovine mucosal immune system development. Molecular Immunology 66(1):57-67. https://doi.org/10.1016/j.molimm.2014.10.014

Seoudi, A. M., Y. A. Lashine, and A. I. Abdelaziz. 2012. MicroRNA-181a-a tale of discrepancies. Expert reviews in molecular medicine 14.

Shrestha, A., R. T. Mukhametshina, S. Taghizadeh, E. Vásquez-Pacheco, H. Cabrera-Fuentes, A. Rizvanov, B. Mari, G. Carraro, and S. Bellusci. 2017. MicroRNA-142 is a multifaceted regulator in organogenesis, homeostasis, and disease. Developmental Dynamics 246(4):285-290.

Sonkoly, E., M. Ståhle, and A. Pivarcsi. 2008. MicroRNAs and immunity: Novel players in the regulation of normal immune function and inflammation. Seminars in Cancer Biology 18(2):131-140. 10.1016/j.semcancer.2008.01.005

Su, S., Q. Zhao, C. He, D. Huang, J. Liu, F. Chen, J. Chen, J.-Y. Liao, X. Cui, and Y. Zeng. 2015. miR-142-5p and miR-130a-3p are regulated by IL-4 and IL-13 and control profibrogenic macrophage program. Nature communications 6(1):1-19.

Sun, J., K. Aswath, S. G. Schroeder, J. D. Lippolis, T. A. Reinhardt, and T. S. Sonstegard. 2015. MicroRNA expression profiles of bovine milk exosomes in response to Staphylococcus aureus infection. BMC genomics 16(1):1-10.

Vigorito, E., S. Kohlhaas, D. Lu, and R. Leyland. 2013. miR-155: an ancient regulator of the immune system. Immunological reviews 253(1):146-157.

Wang, J.-f., M.-l. Yu, G. Yu, J.-j. Bian, X.-m. Deng, X.-j. Wan, and K.-m. Zhu. 2010. Serum miR-146a and miR-223 as potential new biomarkers for sepsis. Biochemical and biophysical research communications 394(1):184-188.

Watanabe, A., H. Tagawa, J. Yamashita, K. Teshima, M. Nara, K. Iwamoto, M. Kume, Y. Kameoka, N. Takahashi, and T. Nakagawa. 2011. The role of microRNA-150 as a tumor suppressor in malignant lymphoma. Leukemia 25(8):1324-1334.

Xiao, C., D. P. Calado, G. Galler, T.-H. Thai, H. C. Patterson, J. Wang, N. Rajewsky, T. P. Bender, and K. Rajewsky. 2007. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131(1):146-159.

Xu, D.-d., P.-j. Zhou, Y. Wang, Y. Zhang, R. Zhang, L. Zhang, S.-h. Chen, W.-y. Fu, B.-b. Ruan, and H.-p. Xu. 2016. miR-150 suppresses the proliferation and tumorigenicity of leukemia stem cells by targeting the nanog signaling pathway. Frontiers in pharmacology 7:439.

Yuan, X., N. Berg, J. W. Lee, T. T. Le, V. Neudecker, N. Jing, and H. Eltzschig. 2018. MicroRNA miR-223 as regulator of innate immunity. Journal of leukocyte biology 104(3):515-524.

Appendix 7. Standard solution for RT-qPCR.

The miRNA cel-miR-39-3p is found in *Caenorhabditis elegans* and is not present in mammals. Thus, cel-miR-39-3p was used as a standard curve for qPCR and for normalization to correct for any technical variation during the RNA isolation, RT and qPCR. All steps to prepare cDNA of cel-miR-39-3p were performed as with the samples, however, instead of adding 1 µL of diluted total RNA, 1 µL of cel-miR-39-3p was added.

Cel-miR-39-3p concentration used for RT-qPCR was 5 picomole/µL, which is equivalent to 36.3 ng/ µL (using conversion from https://www.promega.com/a/apps/biomath/), and equal to 2.922 x 10^{12} copies/ µL (based on the conversion from http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr) or following formula:

$$Number\ of\ copies = \frac{X\ x\ 6.022\ x\ 10^{23}}{N\ x\ a\ x\ 1\ x\ 10^{9}}$$

Where: X = amount of amplicon (ng)

N = length of dsDNA, ssDNA or RNA amplicon

a (g/mole) = average mass of 1 bp dsDNA, or ssDNA, or RNA

(a = 660 for dsDNA, a = 330 for ssDNA, a = 340 for RNA)

One µL of 5 picomole/µL cel-miR39-3p was used as the starting material for the RT reaction, giving a cDNA concentration of cel-miR39-3p of 2.922 x 10^{12} copies in a total RT reaction volume of 15 µL or 1.948 x 10^{11} copies/ µL.

After generating cDNA of miR-39-3p, this standard cDNA solution of miR-39-3p was diluted in serial 10-fold dilutions (Table 6.3). Each dilution was stored in multiple Eppendorf tubes for long-term use while avoiding repeated multiple thawing. The cDNA for the standard curve was amplified as the same time as the samples for every PCR amplification, but the template added to the PCR was different from the samples to ensure the standard curve covered all the sample concentrations. cDNA from the standard solution (7.12 µL) was added in the PCR tube to get final volume 15 µL, and the final concentration was determined from the standard curve.

Appendix 8. Supporting results for RT-qPCR.

Appendix 8.1. Concentration of immune-related miRNAs in colostrum and milk.

miRNAs	Dam colostrum day 0 (n = 12)		Pooled colostrum (n = 5)		Bulk tank milk (n = 25)	
	LSM	SE	LSM	SE	LSM	SE
miR-142-5p	402.1[a]	84.3	398.4[ab]	130.5	131.4[b]	58.4
miR-150	618.9	149.7	174.6	231.9	274.1	103.7
miR-155	2,399.2[a]	301.5	1,125.4[b]	467.1	179.6[b]	208.9
miR-181a	451.2[a]	72.3	491.4[a]	112.0	31.7[b]	50.1
miR-223	67,190.0	19,940.0	115,600.0	30,890.0	52,430.0	13,820.0

Unit = 10^6 copies/µL.

LSM = least square means, SE = standard errors.

[a,b] LSM within a row with different superscripts differ ($P < 0.05$).

Appendix 8.2. Immune-related miRNAs in dam colostrum day 0 to 3 postpartum.

miRNAs	Day 0 (n = 12)		Day 1 (n = 11)		Day 2 (n = 12)		Day 3 (n = 12)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
miR-142-5p	402.1[a]	50.4	129.0[b]	52.7	31.3[b]	50.4	28.6[b]	50.4
miR-150	488.8[a]	56.9	78.0[b]	71.7	34.1[b]	68.7	105.2[b]	68.7
miR-155	2036.2[a]	171.2	695.3[b]	216.0	424.6[b]	206.8	412.3[b]	206.8
miR-181a	483.7[a]	56.9	379.2[a]	71.7	138.8[b]	68.7	21.6[b]	68.7
miR-223	97970.0[a]	11240.0	44470.0[b]	14180.0	15030.0[b]	13570.0	11950.0[b]	13570.0

Units = 10^6 copies/µL.

LSM = least square mean, SE = standard error.

[a,b] LSM within a row with different superscripts differ ($P < 0.05$).

Appendix 9. Evidence of poster presentations at international conferences.

Appendix 9.1. 7th International Symposium on Animal Functional Genomics and Functional Annotation of Animal Genomes Workshop 2018.

CERTIFICATE OF PARTICIPATION

Dear Hue Thi Do,

We would like to thank you for attending, presenting and participating in the 7th International Symposium on Animal Functional Genomics and Functional Annotation of Animal Genomes Workshop 2018 from November 12 -15, 2018 and the associated R short courses on November 15-16, 2018 in Adelaide, South Australia. We trust that the ISAFG 2018 Conference is memorable.

Best regards,

Cynthia Bottema
On behalf of the ISAFG Organising Committee

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Appendix 9.2. 37th International Society for Animal Genetics Conference 2019.

ISAG . 2019
37th International Society for
Animal Genetics Conference

Universitat de Lleida

CERTIFICATE OF PRESENTATION

The Scientific Committee of the

37th International Society for Animal Genetics Conference

Universitat de Lleida (Spain) 7th- 12th July, 2019

certifies that

Do HT, Williams JL, Chen T, Petrovski K, Bottema CDK

presented the poster communication

Immune-related microRNA absorption in newborn calves.

Joan Estany
Congress Co-chair

Romi Pena
Congress Co-chair

References cited

References cited

Abd El -Fattah, A. M., F. H. Abd Rabo, S. M. EL-Dieb, and H. A. El-Kashef. 2012. Changes in composition of colostrum of Egyptian buffaloes and Holstein cows. BMC Veterinary Research 8(1):19. 10.1186/1746-6148-8-19

Abuelo, A., P. Havrlant, N. Wood, and M. Hernandez-Jover. 2019. An investigation of dairy calf management practices, colostrum quality, failure of transfer of passive immunity, and occurrence of enteropathogens among Australian dairy farms. Journal of dairy science 102(9):8352-8366.

Affolter, M., L. Grass, F. Vanrobaeys, B. Casado, and M. Kussmann. 2010. Qualitative and quantitative profiling of the bovine milk fat globule membrane proteome. Journal of Proteomics 73(6):1079-1088. http://dx.doi.org/10.1016/j.jprot.2009.11.008

Alsaweed, M., P. Hartmann, D. Geddes, and F. Kakulas. 2015a. MicroRNAs in Breastmilk and the Lactating Breast: Potential Immunoprotectors and Developmental Regulators for the Infant and the Mother. International Journal of Environmental Research and Public Health 12(11):13981.

Alsaweed, M., A. R. Hepworth, C. Lefèvre, P. E. Hartmann, D. T. Geddes, and F. Hassiotou. 2015b. Human Milk MicroRNA and Total RNA Differ Depending on Milk Fractionation. Journal of Cellular Biochemistry 116(10):2397-2407. 10.1002/jcb.25207

Alsaweed, M., C. T. Lai, P. E. Hartmann, D. T. Geddes, and F. Kakulas. 2016. Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. Scientific reports 6(1):1-13.

Armengol, R. and L. Fraile. 2016. Colostrum and milk pasteurization improve health status and decrease mortality in neonatal calves receiving appropriate colostrum ingestion. Journal of Dairy Science 99(6):4718-4725. http://dx.doi.org/10.3168/jds.2015-10728

Arthington, J. D., M. B. Cattell, J. D. Quigley, G. C. McCoy, and W. L. Hurley. 2000. Passive Immunoglobin Transfer in Newborn Calves Fed Colostrum or Spray-Dried Serum Protein Alone or as a Supplement to Colostrum of Varying Quality1. Journal of Dairy Science 83(12):2834-2838. http://dx.doi.org/10.3168/jds.S0022-0302(00)75183-6

Auerbach, A., G. Vyas, A. Li, M. Halushka, and K. Witwer. 2016. Uptake of dietary milk miRNAs by adult humans: a validation study. F1000Research 5.

Baddela, V. S., V. Nayan, P. Rani, S. K. Onteru, and D. Singh. 2016. Physicochemical Biomolecular Insights into Buffalo Milk-Derived Nanovesicles. Applied Biochemistry and Biotechnology 178(3):544-557. 10.1007/s12010-015-1893-7

Baier, S. R., C. Nguyen, F. Xie, J. R. Wood, and J. Zempleni. 2014. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. The Journal of nutrition 144(10):1495-1500.

Barrington, G. M. and S. M. Parish. 2001. Bovine Neonatal Immunology. Veterinary Clinics of North America: Food Animal Practice 17(3):463-476. https://doi.org/10.1016/S0749-0720(15)30001-3

Bartel, D. P. 2004. MicroRNAs. Cell 116(2):281-297. http://dx.doi.org/10.1016/S0092-8674(04)00045-5

Bartier, A. L., M. C. Windeyer, and L. Doepel. 2015. Evaluation of on-farm tools for colostrum quality measurement. Journal of Dairy Science 98(3):1878-1884. https://doi.org/10.3168/jds.2014-8415

Baumrucker, C. R. and J. R. Blum. 1993. SECRETION OF INSULIN-LIKE GROWTH-FACTORS IN MILK AND THEIR EFFECT ON THE NEONATE. Livestock Production Science 35(1-2):49-72. 10.1016/0301-6226(93)90181-g

Baumrucker, C. R., A. Stark, O. Wellnitz, C. Dechow, and R. M. Bruckmaier. 2014. Short communication: Immunoglobulin variation in quarter-milked colostrum. Journal of Dairy Science 97(6):3700-3706. https://doi.org/10.3168/jds.2013-7107

Benmoussa, A., C. H. C. Lee, B. Laffont, P. Savard, J. Laugier, E. Boilard, C. Gilbert, I. Fliss, and P. Provost. 2016. Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions. The Journal of Nutrition 146(11):2206-2215. 10.3945/jn.116.237651

Blum, J. W. and H. Hammon. 2000. Colostrum effects on the gastrointestinal tract, and on nutritional, endocrine and metabolic parameters in neonatal calves. Livestock Production Science 66(2):151-159. 10.1016/s0301-6226(00)00222-0

Blüml, S., M. Bonelli, B. Niederreiter, A. Puchner, G. Mayr, S. Hayer, M. I. Koenders, W. B. van den Berg, J. Smolen, and K. Redlich. 2011. Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. Arthritis & Rheumatism 63(5):1281-1288.

Bourne, F. J. 1977. The mammary gland and neonatal immunity. Veterinary Science Communications 1(1):141-151. 10.1007/bf02267644

Buczinski, S., E. Gicquel, G. Fecteau, Y. Takwoingi, M. Chigerwe, and J. Vandeweerd. 2018. Systematic review and meta-analysis of diagnostic accuracy of serum refractometry and brix refractometry for the diagnosis of inadequate transfer of passive immunity in calves. Journal of veterinary internal medicine 32(1):474-483.

Buczinski, S. and J. Vandeweerd. 2016. Diagnostic accuracy of refractometry for assessing bovine colostrum quality: A systematic review and meta-analysis. Journal of dairy science 99(9):7381-7394.

Cabral, R. G., C. E. Chapman, K. M. Aragona, E. Clark, M. Lunak, and P. S. Erickson. 2016. Predicting colostrum quality from performance in the previous lactation and environmental changes. Journal of Dairy Science 99(5):4048-4055. https://doi.org/10.3168/jds.2015-9868

Cai, M., H. He, X. Jia, S. Chen, J. Wang, Y. Shi, B. Liu, W. Xiao, and S. Lai. 2018. Genome-wide microRNA profiling of bovine milk-derived exosomes infected with Staphylococcus aureus. Cell Stress and Chaperones 23(4):663-672.

Castro, N., J. Capote, R. M. Bruckmaier, and A. Argüello. 2011. Management effects on colostrogenesis in small ruminants: a review. Journal of Applied Animal Research 39(2):85-93. 10.1080/09712119.2011.581625

Chamorro, M. F., N. Cernicchiaro, and D. M. Haines. 2017. Evaluation of the effects of colostrum replacer supplementation of the milk replacer ration on the occurrence of disease, antibiotic therapy, and performance of pre-weaned dairy calves. Journal of Dairy Science 100(2):1378-1387. http://dx.doi.org/10.3168/jds.2016-11652

Chen, X., Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, and X. Guo. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell research 18(10):997-1006.

Chen, X., C. Gao, H. Li, L. Huang, Q. Sun, Y. Dong, C. Tian, S. Gao, H. Dong, D. Guan, X. Hu, S. Zhao, L. Li, L. Zhu, Q. Yan, J. Zhang, K. Zen, and C.-Y. Zhang. 2010. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. Cell Res 20(10):1128-1137. http://www.nature.com/cr/journal/v20/n10/suppinfo/cr201080s1.html

Chen, X., H. Liang, J. Zhang, K. Zen, and C.-Y. Zhang. 2012. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. Protein & Cell 3(1):28-37. 10.1007/s13238-012-2003-z

Chigerwe, M., J. Hagey, and S. Aly. 2015. Determination of neonatal serum immunoglobulin G concentrations associated with mortality during the first 4 months of life in dairy heifer calves. The Journal of Dairy Research 82(4):400-406. 10.1017/S0022029915000503

Chigerwe, M. and J. V. Hagey. 2014. Refractometer assessment of colostral and serum IgG and milk total solids concentrations in dairy cattle. BMC Veterinary Research 10(1):178. 10.1186/s12917-014-0178-7

Colitti, M., S. Sgorlon, D. Licastro, and B. Stefanon. 2019. Differential expression of miRNAs in milk exosomes of cows subjected to group relocation. Research in Veterinary Science 122:148-155. https://doi.org/10.1016/j.rvsc.2018.11.024

Conneely, M., D. P. Berry, J. P. Murphy, I. Lorenz, M. L. Doherty, and E. Kennedy. 2014. Effect of feeding colostrum at different volumes and subsequent number of transition milk feeds on the serum immunoglobulin G concentration and health status of dairy calves. Journal of Dairy Science 97(11):6991-7000. http://dx.doi.org/10.3168/jds.2013-7494

Copier, C. U., L. León, M. Fernández, D. Contador, and S. D. Calligaris. 2017. Circulating miR-19b and miR-181b are potential biomarkers for diabetic cardiomyopathy. Scientific reports 7(1):1-11.

Costa, J. F. D. R., S. M. F. Novo, C. C. Baccili, N. M. Sobreira, D. J. Hurley, and V. Gomes. 2017. Innate immune response in neonate Holstein heifer calves fed fresh or frozen colostrum. Research in Veterinary Science 115:54-60. http://dx.doi.org/10.1016/j.rvsc.2017.01.008

Cummins, C., D. Berry, R. Sayers, I. Lorenz, and E. Kennedy. 2016. Questionnaire identifying management practices surrounding calving on spring-calving dairy farms and their associations with herd size and herd expansion. animal 10(5):868-877.

Cuttance, E., W. Mason, R. Laven, K. Denholm, and D. Yang. 2018. Calf and colostrum management practices on New Zealand dairy farms and their associations with concentrations of total protein in calf serum. New Zealand veterinary journal 66(3):126-131.

Cuttance, E. L., W. A. Mason, K. S. Denholm, and R. A. Laven. 2017a. Comparison of diagnostic tests for determining the prevalence of failure of passive transfer in New Zealand dairy calves. New Zealand Veterinary Journal 65(1):6-13. 10.1080/00480169.2016.1230525

Cuttance, E. L., W. A. Mason, R. A. Laven, J. McDermott, and C. V. C. Phyn. 2017b. Prevalence and calf-level risk factors for failure of passive transfer in dairy calves in New Zealand. New Zealand Veterinary Journal 65(6):297-304. 10.1080/00480169.2017.1361876

Dawes, M. E. and J. W. Tyler. 2007. CHAPTER 43 - Principles of Colostrum Feeding. Pages 336-338 in Current Therapy in Large Animal Theriogenology (Second Edition). R. S. Youngquist and W. R. Threlfall, ed. W.B. Saunders, Saint Louis.

De Bem, T. H. C., J. C. da Silveira, R. V. Sampaio, J. R. Sangalli, M. L. F. Oliveira, R. M. Ferreira, L. A. Silva, F. Perecin, W. A. King, F. V. Meirelles, and E. S. Ramos. 2017. Low levels of exosomal-miRNAs in maternal blood are associated with early pregnancy loss in cloned cattle. Scientific reports 7(1):14319-14311. 10.1038/s41598-017-14616-1

Denholm, K., J. Hunnam, E. Cuttance, and S. McDougall. 2017. Associations between management practices and colostrum quality on New Zealand dairy farms. New Zealand Veterinary Journal 65(5):257-263.

Dilda, F., G. Gioia, L. Pisani, L. Restelli, C. Lecchi, F. Albonico, V. Bronzo, M. Mortarino, and F. Ceciliani. 2012. Escherichia coli lipopolysaccharides and Staphylococcus aureus enterotoxin B differentially modulate inflammatory microRNAs in bovine monocytes. The Veterinary Journal 192(3):514-516.

Do, D., P.-L. Dudemaine, B. Fomenky, and E. Ibeagha-Awemu. 2018. Integration of miRNA and mRNA Co-Expression Reveals Potential Regulatory Roles of miRNAs in Developmental and Immunological Processes in Calf Ileum during Early Growth. Cells 7(9):134.

Do, D. N., R. Li, P.-L. Dudemaine, and E. M. Ibeagha-Awemu. 2017. MicroRNA roles in signalling during lactation: an insight from differential expression, time course and pathway analyses of deep sequence data. Scientific Reports 7:44605. 10.1038/srep44605

https://www.nature.com/articles/srep44605#supplementary-information

Dong, Z., Q. Lei, R. Yang, S. Zhu, X.-X. Ke, L. Yang, H. Cui, and L. Yi. 2017. Inhibition of neurotensin receptor 1 induces intrinsic apoptosis via let-7a-3p/Bcl-w axis in glioblastoma. British journal of cancer 116(12):1572-1584.

Dunn, A., A. Ashfield, B. Earley, M. Welsh, A. Gordon, and S. J. Morrison. 2017. Evaluation of factors associated with immunoglobulin G, fat, protein, and lactose concentrations in bovine colostrum and colostrum management practices in grassland-based dairy systems in Northern Ireland. Journal of dairy science 100(3):2068. 10.3168/jds.2016-11724

Dzik, S., B. Miciński, I. Aitzhanova, J. Miciński, J. Pogorzelska, A. Beisenov, and I. M. Kowalski. 2017. Properties of bovine colostrum and the possibilities of use. Polish Annals of Medicine. http://dx.doi.org/10.1016/j.poamed.2017.03.004

Elsohaby, I., J. T. McClure, M. Cameron, L. C. Heider, and G. P. Keefe. 2017. Rapid assessment of bovine colostrum quality: How reliable are transmission infrared spectroscopy and digital and optical refractometers? Journal of Dairy Science 100(2):1427-1435. https://doi.org/10.3168/jds.2016-11824

Eminaga, S., D. C. Christodoulou, F. Vigneault, G. M. Church, and J. G. Seidman. 2013. Quantification of microRNA expression with next-generation sequencing. Current protocols in molecular biology 103(1):4.17. 11-14.17. 14.

Faber, S. N., N. E. Faber, T. C. McCauley, and R. L. Ax. 2005. Case Study: Effects Of Colostrum Ingestion on Lactational Performance1. The Professional Animal Scientist 21(5):420-425. http://dx.doi.org/10.15232/S1080-7446(15)31240-7

Faraoni, I., F. R. Antonetti, J. Cardone, and E. Bonmassar. 2009. miR-155 gene: a typical multifunctional microRNA. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1792(6):497-505.

Fidler, A. P., M. L. Alley, and G. W. Smith. 2011. Short communication: Serum immunoglobulin G and total protein concentrations in dairy calves fed a colostrum-replacement product. Journal of Dairy Science 94(7):3609-3612. https://doi.org/10.3168/jds.2011-4358

Floris, I., J. Kraft, and I. Altosaar. 2016. Roles of MicroRNA across Prenatal and Postnatal Periods. International Journal of Molecular Sciences 17(12):1994.

Fong, B. Y., C. S. Norris, and A. K. H. MacGibbon. 2007. Protein and lipid composition of bovine milk-fat-globule membrane. International Dairy Journal 17(4):275-288. http://dx.doi.org/10.1016/j.idairyj.2006.05.004

Franklin, S. T., D. M. Amaral-Phillips, J. A. Jackson, and A. A. Campbell. 2003. Health and Performance of Holstein Calves that Suckled or Were Hand-Fed Colostrum and Were Fed One of Three Physical Forms of Starter1. Journal of Dairy Science 86(6):2145-2153. http://dx.doi.org/10.3168/jds.S0022-0302(03)73804-1

Furman-Fratczak, K., A. Rzasa, and T. Stefaniak. 2011. The influence of colostral immunoglobulin concentration in heifer calves' serum on their health and growth. Journal of Dairy Science 94(11):5536-5543. 10.3168/jds.2010-3253

Gapper, L. W., D. E. Copestake, D. E. Otter, and H. E. Indyk. 2007. Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: a review. Analytical and bioanalytical chemistry 389(1):93-109.

Gauthier, S. F., Y. Pouliot, and J.-L. Maubois. 2006. Growth factors from bovine milk and colostrum: composition, extraction and biological activities. Lait 86(2):99-125.

Gelsinger, S. L. and A. J. Heinrichs. 2017. Comparison of immune responses in calves fed heat-treated or unheated colostrum. Journal of Dairy Science 100(5):4090-4101. https://doi.org/10.3168/jds.2016-12010

Gilmour, A., B. Gogel, B. Cullis, S. Welham, and R. Thompson. 2015. ASReml user guide release 4.1 structural specification. Hemel hempstead: VSN international ltd.

Godden, S. 2008. Colostrum Management for Dairy Calves. Veterinary Clinics of North America: Food Animal Practice 24(1):19-39. http://dx.doi.org/10.1016/j.cvfa.2007.10.005

Godden, S. M., J. E. Lombard, and A. R. Woolums. 2019. Colostrum Management for Dairy Calves. Veterinary Clinics of North America: Food Animal Practice 35(3):535-556. https://doi.org/10.1016/j.cvfa.2019.07.005

Gonzalez, D. D. and M. J. D. Santos. 2017. Bovine colostral cells-the often forgotten component of colostrum. Javma-Journal of the American Veterinary Medical Association 250(9):998-1005. 10.2460/javma.250.9.998

Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research 34(suppl_1):D140-D144. 10.1093/nar/gkj112

Gu, Y., M. Li, T. Wang, Y. Liang, Z. Zhong, X. Wang, Q. Zhou, L. Chen, Q. Lang, Z. He, X. Chen, J. Gong, X. Gao, X. Li, and X. Lv. 2012a. Lactation-Related MicroRNA Expression Profiles of Porcine Breast Milk Exosomes. Plos One 7(8). 10.1371/journal.pone.0043691

Gu, Y., M. Li, T. Wang, Y. Liang, Z. Zhong, X. Wang, Q. Zhou, L. Chen, Q. Lang, Z. He, X. Chen, J. Gong, X. Gao, X. Li, and X. Lv. 2012b. Lactation-Related MicroRNA Expression Profiles of Porcine Breast Milk Exosomes. PLOS ONE 7(8):e43691. 10.1371/journal.pone.0043691

Hata, T., K. Murakami, H. Nakatani, Y. Yamamoto, T. Matsuda, and N. Aoki. 2010. Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. Biochemical and Biophysical Research Communications 396(2):528-533. http://dx.doi.org/10.1016/j.bbrc.2010.04.135

Hernandez-Castellano, A. M. Almeida, N. Castro, and A. Arguello. 2014. The Colostrum Proteome, Ruminant Nutrition and Immunity: A Review. Current Protein and Peptide Science 15(1):64-74.

Hernandez, D., D. V. Nydam, S. M. Godden, L. S. Bristol, A. Kryzer, J. Ranum, and D. Schaefer. 2016. Brix refractometry in serum as a measure of failure of passive transfer compared to measured immunoglobulin G and total protein by refractometry in serum from dairy calves. The Veterinary Journal 211:82-87. https://doi.org/10.1016/j.tvjl.2015.11.004

Hogan, I., M. Doherty, J. Fagan, E. Kennedy, M. Conneely, P. Brady, C. Ryan, and I. Lorenz. 2015. Comparison of rapid laboratory tests for failure of passive transfer in the bovine. Irish Veterinary Journal 68(1):18. 10.1186/s13620-015-0047-0

Hossain, M. M., D. Tesfaye, D. Salilew-Wondim, E. Held, M. J. Pröll, F. Rings, G. Kirfel, C. Looft, E. Tholen, and J. Uddin. 2014. Massive deregulation of miRNAs from nuclear reprogramming errors during trophoblast differentiation for placentogenesis in cloned pregnancy. BMC genomics 15(1):1-15.

Hou, J., X. An, Y. Song, B. Cao, H. Yang, Z. Zhang, W. Shen, and Y. Li. 2017. Detection and comparison of microRNAs in the caprine mammary gland tissues of colostrum and common milk stages. BMC genetics 18(1):1-8.

Huang, X.-L., L. Zhang, J.-P. Li, Y.-J. Wang, Y. Duan, and J. Wang. 2015. MicroRNA-150: a potential regulator in pathogens infection and autoimmune diseases. Autoimmunity 48(8):503-510.

Huang, Y. 2015. Expressional analysis of immune-related miRNAs in breast milk. Genetics and Molecular Research 14(3):11371-11376.

Hurley, W. L. and P. K. Theil. 2011. Perspectives on Immunoglobulins in Colostrum and Milk. Nutrients 3(4):442.

Huse, S. M., J. A. Huber, H. G. Morrison, M. L. Sogin, and D. M. Welch. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome biology 8(7):R143.

Inabu, Y., J. Pyo, S. Pletts, L. L. Guan, M. A. Steele, and T. Sugino. 2019. Effect of extended colostrum feeding on plasma glucagon-like peptide-1 concentration in newborn calves. Journal of Dairy Science 102(5):4619-4627. https://doi.org/10.3168/jds.2018-15616

Ioannidis, J. and F. X. Donadeu. 2016. Circulating miRNA signatures of early pregnancy in cattle. BMC genomics 17(1):184.

Izumi, H., N. Kosaka, T. Shimizu, K. Sekine, T. Ochiya, and M. Takase. 2012. Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions. Journal of Dairy Science 95(9):4831-4841. http://dx.doi.org/10.3168/jds.2012-5489

Izumi, H., N. Kosaka, T. Shimizu, K. Sekine, T. Ochiya, and M. Takase. 2014. Time-Dependent Expression Profiles of microRNAs and mRNAs in Rat Milk Whey. PLOS ONE 9(2):e88843. 10.1371/journal.pone.0088843

Izumi, H., M. Tsuda, Y. Sato, N. Kosaka, T. Ochiya, H. Iwamoto, K. Namba, and Y. Takeda. 2015. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. Journal of Dairy Science 98(5):2920-2933. http://dx.doi.org/10.3168/jds.2014-9076

Jaster, E. H. 2005. Evaluation of Quality, Quantity, and Timing of Colostrum Feeding on Immunoglobulin G1 Absorption in Jersey Calves. Journal of Dairy Science 88(1):296-302. 10.3168/jds.S0022-0302(05)72687-4

Jones, C. M., R. E. James, J. D. Quigley Iii, and M. L. McGilliard. 2004. Influence of Pooled Colostrum or Colostrum Replacement on IgG and Evaluation of Animal Plasma in Milk Replacer. Journal of Dairy Science 87(6):1806-1814. http://dx.doi.org/10.3168/jds.S0022-0302(04)73337-8

Kang, Z., S. Zhang, E. Jiang, F. Wan, X. Lan, and M. Liu. 2020. Mir-193b Regulates the Differentiation, Proliferation, and Apoptosis of Bovine Adipose Cells by Targeting the ACSS2/AKT Axis. Animals 10(8):1265.

Karaca, E., A. Aykut, B. Ertürk, B. Durmaz, A. Güler, B. Büke, A. Ö. Yeniel, A. M. Ergenoğlu, F. Özkınay, M. Özeren, M. Kazandı, F. Akercan, S. Sağol, C. Gündüz, and Ö. Çoğulu. 2018. MicroRNA Expression Profile in the Prenatal Amniotic Fluid Samples of Pregnant Women with Down Syndrome. Balkan Med J 35(2):163-166. 10.4274/balkanmedj.2017.0511

Kehoe, S., A. Heinrichs, M. Moody, C. Jones, and M. Long. 2011. Comparison of immunoglobulin G concentrations in primiparous and multiparous bovine colostrum1. Professional Animal Scientist 27(3):176-180. 10.15232/S1080-7446(15)30471-X

Kirchner, B., D. Buschmann, V. Paul, and M. W. Pfaffl. 2020. Postprandial transfer of colostral extracellular vesicles and their protein and miRNA cargo in neonatal calves. PLOS ONE 15(2):e0229606. 10.1371/journal.pone.0229606

Kramer, N. J., W.-L. Wang, E. Y. Reyes, B. Kumar, C.-C. Chen, C. Ramakrishna, E. M. Cantin, S. L. Vonderfecht, K. D. Taganov, and N. Chau. 2015. Altered lymphopoiesis and immunodeficiency in miR-142 null mice. Blood, The Journal of the American Society of Hematology 125(24):3720-3730.

Krohn, C. C., J. Foldager, and L. Mogensen. 1999. Long-term Effect of Colostrum Feeding Methods on Behaviour in Female Dairy Calves. Acta Agriculturae Scandinavica, Section A - Animal Science 49(1):57-64. 10.1080/090647099421540

Lago, A., M. Socha, A. Geiger, D. Cook, N. Silva-del-Río, C. Blanc, R. Quesnell, and C. Leonardi. 2018. Efficacy of colostrum replacer versus maternal colostrum on immunological status, health, and growth of preweaned dairy calves. Journal of Dairy Science 101(2):1344-1354. https://doi.org/10.3168/jds.2017-13032

Langel, S. N., W. A. Wark, S. N. Garst, R. E. James, M. L. McGilliard, C. S. Petersson-Wolfe, and I. Kanevsky-Mullarky. 2016. Effect of feeding whole compared with cell-free colostrum on calf immune status: Vaccination response. Journal of Dairy Science 99(5):3979-3994. https://doi.org/10.3168/jds.2015-9892

Langer, P. 2009. Differences in the Composition of Colostrum and Milk in Eutherians Reflect Differences in Immunoglobulin Transfer. Journal of Mammalogy 90(2):332-339. 10.1644/08-MAMM-A-071.1

Lee, R. C. and V. Ambros. 2001. An Extensive Class of Small RNAs in Caenorhabditis elegans. Science 294(5543):862-864. 10.1126/science.1065329

Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5):843-854. http://dx.doi.org/10.1016/0092-8674(93)90529-Y

Lewis, B. P., I. h. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge. 2003. Prediction of Mammalian MicroRNA Targets. Cell 115(7):787-798. https://doi.org/10.1016/S0092-8674(03)01018-3

Li, Y., X. Pan, D. N. Nguyen, S. Ren, A. Moodley, and P. T. Sangild. 2020. Bovine colostrum before or after formula feeding improves systemic immune protection and gut function in newborn preterm pigs. Frontiers in immunology 10:3062.

Li, Z., X. Lan, W. Guo, J. Sun, Y. Huang, J. Wang, T. Huang, C. Lei, X. Fang, and H. Chen. 2012a. Comparative transcriptome profiling of dairy goat microRNAs from dry period and peak lactation mammary gland tissues. Plos one 7(12):e52388.

Li, Z., H. Liu, X. Jin, L. Lo, and J. Liu. 2012b. Expression profiles of microRNAs from lactating and non-lactating bovine mammary glands and identification of miRNA related to lactation. BMC genomics 13(1):731.

Liang, G., N. Malmuthuge, and P. Griebel. 2015a. Model systems to analyze the role of miRNAs and commensal microflora in bovine mucosal immune system development. Molecular immunology 66(1):57-67.

Liang, G., N. Malmuthuge, L. L. Guan, and P. Griebel. 2015b. Model systems to analyze the role of miRNAs and commensal microflora in bovine mucosal immune system development. Molecular Immunology 66(1):57-67. https://doi.org/10.1016/j.molimm.2014.10.014

Liang, G., N. Malmuthuge, T. B. McFadden, H. Bao, P. J. Griebel, and P. Stothard. 2014. Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PloS one 9(3):e92592.

Liao, Y., X. Du, J. Li, and B. Lönnerdal. 2017. Human milk exosomes and their microRNAs survive digestion in vitro and are taken up by human intestinal cells. Molecular nutrition & food research 61(11):1700082.

Liu, D.-R., Q.-L. Guan, M.-T. Gao, L. Jiang, and H.-X. Kang. 2016. miR-1260b is a potential prognostic biomarker in colorectal cancer. Medical science monitor: international medical journal of experimental and clinical research 22:2417.

Liu, H., L. Zhu, B. Liu, L. Yang, X. Meng, W. Zhang, Y. Ma, and H. Xiao. 2012. Genome-wide microRNA profiles identify miR-378 as a serum biomarker for early detection of gastric cancer. Cancer Letters 316(2):196-203. https://doi.org/10.1016/j.canlet.2011.10.034

Liu, H. C., J. A. Hicks, N. Trakooljul, and S. H. Zhao. 2010. Current knowledge of microRNA characterization in agricultural animals. Animal Genetics 41(3):225-231. 10.1111/j.1365-2052.2009.01995.x

Low, W. Y., R. Tearle, C. Liu, S. Koren, A. Rhie, D. M. Bickhart, B. D. Rosen, Z. N. Kroneberg, S. B. Kingan, and E. Tseng. 2019. Haplotype-resolved cattle genomes provide insights into structural variation and adaptation. BioRxiv:720797.

Malmuthuge, N., Y. Chen, G. Liang, L. A. Goonewardene, and L. L. Guan. 2015. Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. Journal of Dairy Science 98(11):8044-8053. http://dx.doi.org/10.3168/jds.2015-9607

Marnila, P. and H. Korhonen. 2011a. Milk | Colostrum. Pages 591-597 in Encyclopedia of Dairy Sciences (Second Edition). J. W. Fuquay, ed. Academic Press, San Diego.

Marnila, P. and H. Korhonen. 2011b. Milk | Colostrum A2 - Fuquay, John W. Pages 591-597 in Encyclopedia of Dairy Sciences (Second Edition). Academic Press, San Diego.

Mather, I. H. 2000. A Review and Proposed Nomenclature for Major Proteins of the Milk-Fat Globule Membrane1,2. Journal of Dairy Science 83(2):203-247. http://dx.doi.org/10.3168/jds.S0022-0302(00)74870-3

Matukumalli, L. K. S., Tad S. ; Van Tassell, Curtis P. ; Gasbarre, Louis C. ; Capuco, Anthony V. ; Coutinho, Luiz L. ; Matukumalli, Lakshmi K. ; Smith, Timothy P. L. ; Sonstegard, Tad S. 2006. Discovery and profiling of bovine microRNAs from immune-related and embryonic tissues.

McGrath, B. A., P. F. Fox, P. L. H. McSweeney, and A. L. Kelly. 2016. Composition and properties of bovine colostrum: a review. Dairy Science & Technology 96(2):133-158. 10.1007/s13594-015-0258-x

McGuirk, S. M. and M. Collins. 2004. Managing the production, storage, and delivery of colostrum. Veterinary Clinics of North America: Food Animal Practice 20(3):593-603. 10.1016/j.cvfa.2004.06.005

miRBase. 2020. What do the miRNA names/identifiers mean? http://www.mirbase.org/help/nomenclature.shtml

Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, and A. Allen. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences 105(30):10513-10518.

Modepalli, V., A. Kumar, L. A. Hinds, J. A. Sharp, K. R. Nicholas, and C. Lefevre. 2014. Differential temporal expression of milk miRNA during the lactation cycle of the marsupial tammar wallaby (Macropus eugenii). BMC genomics 15(1):1-18.

Moody, L., H. He, Y.-X. Pan, and H. Chen. 2017. Methods and novel technology for microRNA quantification in colorectal cancer screening. Clinical epigenetics 9:119-119. 10.1186/s13148-017-0420-9

Moore, A. C., J. S. Winkjer, and T.-T. Tseng. 2016. Bioinformatics Resources for MicroRNA Discovery. Biomark Insights 10(Suppl 4):53-58. 10.4137/BMI.S29513

Moran, J. 2002. Calf rearing : a practical guide / by John Moran.

Moran, J. 2012. Rearing young stock on tropical dairy farms in Asia. CSIRO Publishing, Collingwood, Vic.

Morrill, K. M., K. E. Robertson, M. M. Spring, A. L. Robinson, and H. D. Tyler. 2015. Validating a refractometer to evaluate immunoglobulin G concentration in Jersey colostrum and the effect of multiple freeze–thaw cycles on evaluating colostrum quality. Journal of Dairy Science 98(1):595-601. https://doi.org/10.3168/jds.2014-8730

Motameny, S., S. Wolters, P. Nürnberg, and B. Schumacher. 2010. Next generation sequencing of miRNAs–strategies, resources and methods. Genes 1(1):70-84.

Na, R., G. Sun, X. Sun, Q. XY, L. Chen, and Y. Huang. 2015. Expressional analysis of immune-related miRNAs in breast milk. Genetics and Molecular Research 14(3):11371-11376.

Naeem, A., K. Zhong, S. Moisá, J. Drackley, K. Moyes, and J. Loor. 2012. Bioinformatics analysis of microRNA and putative target genes in bovine mammary tissue infected with Streptococcus uberis. Journal of dairy science 95(11):6397-6408.

Novo, S. M. F., J. F. D. Costa, C. C. Baccili, N. M. Sobreira, B. T. Silva, P. L. de Oliveira, D. J. Hurley, and V. Gomes. 2017. Effect of maternal cells transferred with colostrum on the health of neonate calves. Research in Veterinary Science 112:97-104. 10.1016/j.rvsc.2017.01.025

Oh, S., M. R. Park, S. J. Son, and Y. Kim. 2015. Comparison of total RNA isolation methods for analysis of immune-related microRNAs in market milks. Korean Journal for Food Science of Animal Resources 35(4):459.

Olchowy, T. W., R. D. Linnabary, F. M. Andrews, and R. C. Longshore. 1993. Lactose intolerance in a calf. Journal of veterinary internal medicine 7(1):12-15.

Oliveira, L. d. C. C., S. Borchardt, W. Heuwieser, E. Rauch, M. Erhard, and F. Sutter. 2019. Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves. Journal of dairy science 102(1):557-566.

Osaka, I., Y. Matsui, and F. Terada. 2014. Effect of the mass of immunoglobulin (Ig)G intake and age at first colostrum feeding on serum IgG concentration in Holstein calves. Journal of Dairy Science 97(10):6608-6612. https://doi.org/10.3168/jds.2013-7571

Oyeniyi, O. O. and A. G. Hunter. 1978. Colostral Constituents including Immunoglobulins in the First Three Milkings Postpartum¹. Journal of Dairy Science 61(1):44-48. 10.3168/jds.S0022-0302(78)83549-8

Ozdemir, S. 2020. Identification and comparison of exosomal microRNAs in the milk and colostrum of two different cow breeds. Gene 743:11. 10.1016/j.gene.2020.144609

Pakkanen, R. and J. Aalto. 1997. Growth factors and antimicrobial factors of bovine colostrum. International Dairy Journal 7(5):285-297. http://dx.doi.org/10.1016/S0958-6946(97)00022-8

Parish, S. M., J. W. Tyler, T. E. Besser, C. C. Gay, and D. Krytenberg. 1997. Prediction of Serum IgG1 Concentration in Holstein Calves Using Serum Gamma Glutamyltransferase Activity. Journal of Veterinary Internal Medicine 11(6):344-347. 10.1111/j.1939-1676.1997.tb00478.x

Perino, L., R. Sutherland, and N. Woollen. 1993. Serum gamma-glutamyltransferase activity and protein concentration at birth and after suckling in calves with adequate and

inadequate passive transfer of immunoglobulin G. American Journal of Veterinary Research 54(1):56-59.

Phipps, A., D. Beggs, A. Murray, P. Mansell, and M. Pyman. 2018. A survey of northern Victorian dairy farmers to investigate dairy calf management: colostrum feeding and management. Australian veterinary journal 96(4):101-106.

Pigati, L., S. C. Yaddanapudi, R. Iyengar, D.-J. Kim, S. A. Hearn, D. Danforth, M. L. Hastings, and D. M. Duelli. 2010. Selective release of microRNA species from normal and malignant mammary epithelial cells. PloS one 5(10):e13515.

Priestley, D., J. H. Bittar, L. Ibarbia, C. A. Risco, and K. N. Galvão. 2013. Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. Journal of Dairy Science 96(5):3247-3256. http://dx.doi.org/10.3168/jds.2012-6339

Puppel, K., M. Gołębiewski, G. Grodkowski, J. Slósarz, M. Kunowska-Slósarz, P. Solarczyk, M. Łukasiewicz, M. Balcerak, and T. Przysucha. 2019. Composition and factors affecting quality of bovine colostrum: a review. Animals 9(12):1070.

Qin, J. and Q. Xu. 2014. Functions and application of exosomes. Acta Pol Pharm 71(4):537-543.

Quigley, J. D., A. Lago, C. Chapman, P. Erickson, and J. Polo. 2013. Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. Journal of Dairy Science 96(2):1148-1155. https://doi.org/10.3168/jds.2012-5823

Quigley, J. D., R. E. Strohbehn, C. J. Kost, and M. M. O'Brien. 2001. Formulation of Colostrum Supplements, Colostrum Replacers and Acquisition of Passive Immunity in Neonatal Calves1. Journal of Dairy Science 84(9):2059-2065. http://dx.doi.org/10.3168/jds.S0022-0302(01)74650-4

Rahman, K., J. Lovich, C. Lam, M. Camp, A. Wiley, F. Bartol, and C. Bagnell. 2014. Nursing supports neonatal porcine testicular development. Domestic animal endocrinology 48:84-92.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research 43(7):e47-e47.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139-140.

Robinson, M. D. and A. Oshlack. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome biology 11(3):1-9.

Robison, J. D. 1984. The Effects Of Passive Immunity On Growth And Survival In The Dairy Heifer (immunoglobulins, Morbidity, Neonate, Mortality, Colostrum). University Macrofilms International, ProQuest Dissertations & Theses Global.

Rodriguez, A., E. Vigorito, S. Clare, M. V. Warren, P. Couttet, D. R. Soond, S. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, D. Vetrie, K. Okkenhaug, A. J. Enright, G. Dougan, M. Turner, and A. Bradley. 2007. Requirement of bic/microRNA-155 for Normal Immune Function. Science 316(5824):608-611. 10.1126/science.1139253

Rosen, B. D., D. M. Bickhart, R. D. Schnabel, S. Koren, C. G. Elsik, E. Tseng, T. N. Rowan, W. Y. Low, A. Zimin, C. Couldrey, R. Hall, W. Li, A. Rhie, J. Ghurye, S. D. McKay, F. Thibaud-Nissen, J. Hoffman, B. M. Murdoch, W. M. Snelling, T. G. McDaneld, J. A. Hammond, J. C. Schwartz, W. Nandolo, D. E. Hagen, C. Dreischer, S. J. Schultheiss, S. G. Schroeder, A. M. Phillippy, J. B. Cole, C. P. Van Tassell, G. Liu, T. P. L. Smith, and J. F. Medrano. 2020. De novo assembly of the cattle reference genome with single-molecule sequencing. GigaScience 9(3). 10.1093/gigascience/giaa021

Ross, M., H. Atalla, and B. Mallard. 2016. Bioactivity of colostrum and milk exosomes containing microrna from cows genetically selected as high, average and low immune responders based on their estimated breeding values. Journal of Animal Science 94:65-66. 10.2134/jas2016.94supplement465x

RumimiR. 2020. http://rumimir.sigenae.org/

Schiessler, G., A. Nussbaum, H. M. Hammon, and J. W. Blum. 2002. Calves sucking colostrum and milk from their dams or from an automatic feeding station starting in the neonatal period: metabolic and endocrine traits and growth performance. Animal Science 74(3):431-444. 10.1017/S1357729800052589

Sharma, A., M. Kumar, J. Aich, M. Hariharan, S. K. Brahmachari, A. Agrawal, and B. Ghosh. 2009. Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. Proceedings of the National Academy of Sciences 106(14):5761-5766.

Shivley, C. B., J. E. Lombard, N. J. Urie, D. M. Haines, R. Sargent, C. A. Kopral, T. J. Earleywine, J. D. Olson, and F. B. Garry. 2018. Preweaned heifer management on US dairy operations: Part II. Factors associated with colostrum quality and passive transfer status of dairy heifer calves. Journal of Dairy Science 101(10):9185-9198. https://doi.org/10.3168/jds.2017-14008

Shrestha, A., R. T. Mukhametshina, S. Taghizadeh, E. Vásquez-Pacheco, H. Cabrera-Fuentes, A. Rizvanov, B. Mari, G. Carraro, and S. Bellusci. 2017. MicroRNA-142 is a multifaceted regulator in organogenesis, homeostasis, and disease. Developmental Dynamics 246(4):285-290.

Silverstein, A. M. 1996. History of Immunology. Cellular Immunology 174(1):1-6. http://dx.doi.org/10.1006/cimm.1996.0286

Skog, J., T. Würdinger, S. Van Rijn, D. H. Meijer, L. Gainche, W. T. Curry, B. S. Carter, A. M. Krichevsky, and X. O. Breakefield. 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nature cell biology 10(12):1470-1476.

Smith. 1946. THE IMMUNE PROTEINS OF BOVINE COLOSTRUM AND PLASMA. Journal of Biological Chemistry 164(1):345-358.

Smith. 1948. The Isolation and Properties of the Immune Proteins of Bovine Milk and Colostrum and their Role in Immunity: A Review. Journal of Dairy Science 31(2):127-138. http://dx.doi.org/10.3168/jds.S0022-0302(48)92187-0

Smythies, L. E. and J. R. Smythies. 2014. Exosomes in the gut. Frontiers in immunology 5:104.

Sonkoly, E., M. Ståhle, and A. Pivarcsi. 2008. MicroRNAs and immunity: Novel players in the regulation of normal immune function and inflammation. Seminars in Cancer Biology 18(2):131-140. 10.1016/j.semcancer.2008.01.005

Stewart, T. and M. Diekman. 1989. Effect of birth and fraternal litter size and cross-fostering on growth and reproduction in swine. Journal of animal science 67(3):635-640.

Stott, G. H., D. B. Marx, B. E. Menefee, and G. T. Nightengale. 1979. Colostral Immunoglobulin Transfer in Calves I. Period of Absorption1. Journal of Dairy Science 62(10):1632-1638. http://dx.doi.org/10.3168/jds.S0022-0302(79)83472-4

Sun, J., K. Aswath, S. G. Schroeder, J. D. Lippolis, T. A. Reinhardt, and T. S. Sonstegard. 2015. MicroRNA expression profiles of bovine milk exosomes in response to Staphylococcus aureus infection. BMC genomics 16(1):1-10.

Sun, Q., X. Chen, J. Yu, K. Zen, C.-Y. Zhang, and L. Li. 2013. Immune modulatory function of abundant immune-related microRNAs in microvesicles from bovine colostrum. Protein & Cell 4(3):197-210. 10.1007/s13238-013-2119-9

Tam, S., M.-S. Tsao, and J. D. McPherson. 2015. Optimization of miRNA-seq data preprocessing. Brief Bioinform 16(6):950-963. 10.1093/bib/bbv019

Tripathi, V. and B. Vashishtha. 2006. Bioactive compounds of colostrum and its application. Food Reviews International 22(3):225-244. 10.1080/87559120600694606

Trotz-Williams, L. A., K. E. Leslie, and A. S. Peregrine. 2008. Passive Immunity in Ontario Dairy Calves and Investigation of Its Association with Calf Management Practices. Journal of Dairy Science 91(10):3840-3849. http://dx.doi.org/10.3168/jds.2007-0898

Uruakpa, F. O., M. A. H. Ismond, and E. N. T. Akobundu. 2002. Colostrum and its benefits: a review. Nutrition Research 22(6):755-767. http://dx.doi.org/10.1016/S0271-5317(02)00373-1

Van Amburgh, M. E. 2017. Nutrition of the preweaned calf. Pages 409-419 in Large Dairy Herd Management. 3rd ed. D. K. Beede, ed. American Dairy Science Association, Champaign IL.

Van Hese, I., K. Goossens, L. Vandaele, and G. Opsomer. 2020. Invited review: MicroRNAs in bovine colostrum—Focus on their origin and potential health benefits for the calf. Journal of Dairy Science 103(1):1-15. https://doi.org/10.3168/jds.2019-16959

Vandeputte, S., J. Detilleux, and F. Rollin. 2011. Comparison of Four Refractometers for the Investigation of the Passive Transfer in Beef Calves. Journal of Veterinary Internal Medicine 25(6):1465-1469. 10.1111/j.1939-1676.2011.00816.x

Vejnar, C. E., M. Blum, and E. M. Zdobnov. 2013. miRmap web: Comprehensive microRNA target prediction online. Nucleic acids research 41(Web Server issue):W165-W168. 10.1093/nar/gkt430

Vejnar, C. E. and E. M. Zdobnov. 2012. MiRmap: comprehensive prediction of microRNA target repression strength. Nucleic Acids Res 40(22):11673-11683. 10.1093/nar/gks901

Vigorito, E., S. Kohlhaas, D. Lu, and R. Leyland. 2013. miR-155: an ancient regulator of the immune system. Immunological reviews 253(1):146-157.

Virtala, A. M. K., Y. T. Gröhn, G. D. Mechor, and H. N. Erb. 1999. The effect of maternally derived immunoglobulin G on the risk of respiratory disease in heifers during the first 3 months of life1This report presents a portion of a dissertation submitted by the first author to the graduate school of Cornell University as partial fulfillment of the requirements for the Ph.D. degree.1. Preventive Veterinary Medicine 39(1):25-37. https://doi.org/10.1016/S0167-5877(98)00140-8

Wang, M., S. Moisá, M. Khan, J. Wang, D. Bu, and J. J. Loor. 2012. MicroRNA expression patterns in the bovine mammary gland are affected by stage of lactation. Journal of dairy science 95(11):6529-6535.

Wang, X., Z. Gu, and H. Jiang. 2013. MicroRNAs in farm animals. animal 7(10):1567-1575. 10.1017/S1751731113001183

Weaver, D. M., J. W. Tyler, D. C. VanMetre, D. E. Hostetler, and G. M. Barrington. 2000. Passive Transfer of Colostral Immunoglobulins in Calves. Journal of Veterinary Internal Medicine 14(6):569-577. 10.1111/j.1939-1676.2000.tb02278.x

Wheeler, T. T., A. J. Hodgkinson, C. G. Prosser, and S. R. Davis. 2007. Immune Components of Colostrum and Milk—A Historical Perspective. Journal of Mammary Gland Biology and Neoplasia 12(4):237-247. 10.1007/s10911-007-9051-7

Wienholds, E. and R. H. A. Plasterk. 2005. MicroRNA function in animal development. FEBS Letters 579(26):5911-5922. 10.1016/j.febslet.2005.07.070

Williams, A. E. 2007. Functional aspects of animal microRNAs. Cell Mol Life Sci 65(4):545-562. 10.1007/s00018-007-7355-9

Williams, D. R., P. Pithua, A. Garcia, J. Champagne, D. M. Haines, and S. S. Aly. 2014. Effect of Three Colostrum Diets on Passive Transfer of Immunity and Preweaning Health in Calves on a California Dairy following Colostrum Management Training. Veterinary Medicine International 2014:698741. 10.1155/2014/698741

Wooding, F. 1992. The synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 13(2):101-113.

Wu, C., Y. Cao, Z. He, J. He, C. Hu, H. Duan, and J. Jiang. 2014. Serum levels of miR-19b and miR-146a as prognostic biomarkers for non-small cell lung cancer. The Tohoku journal of experimental medicine 232(2):85-95.

Xiao, C., D. P. Calado, G. Galler, T.-H. Thai, H. C. Patterson, J. Wang, N. Rajewsky, T. P. Bender, and K. Rajewsky. 2007. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131(1):146-159.

Xu, D.-d., P.-j. Zhou, Y. Wang, Y. Zhang, R. Zhang, L. Zhang, S.-h. Chen, W.-y. Fu, B.-b. Ruan, and H.-p. Xu. 2016. miR-150 suppresses the proliferation and tumorigenicity of leukemia stem cells by targeting the nanog signaling pathway. Frontiers in pharmacology 7:439.

Yan, W., A. A. Wiley, R. A. Bathgate, A.-L. Frankshun, S. Lasano, B. D. Crean, B. G. Steinetz, C. A. Bagnell, and F. F. Bartol. 2006. Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. Endocrinology 147(9):4303-4310.

Yang, M., Y. Zou, Z. H. Wu, S. L. Li, and Z. J. Cao. 2015. Colostrum quality affects immune system establishment and intestinal development of neonatal calves. Journal of Dairy Science 98(10):7153-7163. https://doi.org/10.3168/jds.2014-9238

Ying, X., W. Zhang, M. Fang, W. Zhang, C. Wang, and L. Han. 2019. miR-345-5p regulates proliferation, cell cycle, and apoptosis of acute myeloid leukemia cells by targeting AKT2. Journal of cellular biochemistry 120(2):1620-1629.

Yuan, X., N. Berg, J. W. Lee, T. T. Le, V. Neudecker, N. Jing, and H. Eltzschig. 2018. MicroRNA miR-223 as regulator of innate immunity. Journal of leukocyte biology 104(3):515-524.

Zhang, Y., D. Liu, X. Chen, J. Li, L. Li, Z. Bian, F. Sun, J. Lu, Y. Yin, and X. Cai. 2010. Secreted monocytic miR-150 enhances targeted endothelial cell migration. Molecular cell 39(1):133-144.

Zhou, Q., M. Li, X. Wang, Q. Li, T. Wang, Q. Zhu, X. Zhou, X. Wang, X. Gao, and X. Li. 2012. Immune-related microRNAs are abundant in breast milk exosomes. International journal of biological sciences 8(1):118.

Ziemann, M., A. Kaspi, and A. El-Osta. 2016. Evaluation of microRNA alignment techniques. Rna 22(8):1120-1138.