Bioactivity of Bovine Colostrum and Milk Exosomes from High, Average, and Low Immune Responders on Human Intestinal Epithelial Cells

by

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ABSTRACT

BIOACTIVITY OF BOVINE COLOSTRUM AND MILK EXOSOMES FROM HIGH, AVERAGE, AND LOW IMMUNE RESPONDERS ON HUMAN INTESTINAL EPITHELIAL CELLS

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Bovine colostrum and milk contain bioactive components that are nutritionally and immunologically important to calves and humans. While high (H) immune responder cows have improved colostrum and milk quality compared to average (A) and low (L) responders, bioactivity of colostrum and milk exosomes containing microRNAs on human intestinal epithelial cells was unknown. Therefore, the objectives of this research were to evaluate intestinal epithelial cell uptake, viability, and caspase 3 activity after co-culture with colostrum and milk exosomes from H, A, and L immune responders. Results indicated exosomes were taken up by epithelial cells, enhance cell viability, and activate the caspase 3 pathway. Further, this research provides novel insights into differences in bioactivity between colostrum and milk exosomes from H, A, and L immune responders, whereby cell viability was significantly greater after co-culture with H responder exosomes compared to L. Collectively, these findings suggest potential health benefits of cow’s milk.
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DECLARATION OF WORK PERFORMED

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I declare that all work presented in this thesis was performed by me with the exception of: ultracentrifugation protocol, electron microscopy images, immune response phenotyping of cows, and sample collection. Statistical analysis was performed with the assistance of William Sears.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>A</td>
<td>Average</td>
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<tr>
<td>AA</td>
<td>Average/Average</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute protein</td>
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<tr>
<td>AMIR</td>
<td>Antibody-mediated immune response</td>
</tr>
<tr>
<td>Anti-</td>
<td>Antibody</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human colorectal adenocarcinoma epithelial cells/human cancer intestinal epithelial cells</td>
</tr>
<tr>
<td>CAF1</td>
<td>Chromatin assembly factor 1</td>
</tr>
<tr>
<td>CCR4</td>
<td>C-C chemokine receptor type 4</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLDN1</td>
<td>Claudin-1</td>
</tr>
<tr>
<td>CMIR</td>
<td>Cell-mediated immune response</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DGCR8</td>
<td>Drosha-DiGeorge syndrome critical region in gene 8</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>EBV</td>
<td>Estimated breeding value</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex responsible for transport</td>
</tr>
<tr>
<td>EXP5</td>
<td>Exportin 5</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>H</td>
<td>High</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>HIR</td>
<td>High Immune Responder</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>ILVs</td>
<td>Intraluminal vesicles</td>
</tr>
<tr>
<td>IR</td>
<td>Immune responder</td>
</tr>
<tr>
<td>L</td>
<td>Low</td>
</tr>
<tr>
<td>LDLRAP1</td>
<td>LDL receptor adapter protein 1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDDCs</td>
<td>Monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MVBs</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>NKRF</td>
<td>Nuclear factor-kB–repressing factor</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pre-miRNA</td>
<td>Precursor microRNA</td>
</tr>
<tr>
<td>pri-miRNA</td>
<td>Primary microRNA</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline + tween</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TM</td>
<td>Trademark</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Tsg101</td>
<td>Tumor susceptibility gene 101</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>xg</td>
<td>Times gravity</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc finger E-box-binding homeobox 1</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
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CHAPTER 1
INTRODUCTION

1.1 Background and problem

The Canadian dairy industry ranks third in the Canadian agriculture sector in sales of agricultural commodities, generating over $17 billion in revenue (Canada’s Dairy Industry at a Glance, 2015). For a number of years, dairy cows have been under intense selection pressure for increased milk production. While this genetic approach to increase milk yield has been effective, it has also been associated with negative effects on reproduction and health (Oltenacu and Broom, 2010), resulting in substantial economic losses to the dairy industry due to poor milk quality, decreased milk production, veterinary costs, and culling (Rehbein et al., 2013). Selection objectives have now shifted to focus on breeding for both production and health traits in dairy cows (Miglior et al., 2012). Use of the High Immune Response (HIR™) technology is a suitable approach for improving the health of dairy cows, as well as their milk quality. Specifically, HIR colostrum and milk have greater concentrations of bioactive proteins including antibodies and beta-lactoglobulin than low (L) or average (A) responders (Fleming, 2014).

Along with changes in dairy cow selection objectives, there has also been growing interest in the potential health benefits of quality foods, such as milk, amongst Canadian consumers (Khamphoune, 2013). Colostrum and milk facilitate immune system development, provide local protection at the gastrointestinal barrier, and protect against diseases later in life (Pereira, 2014; Hassiotou & Geddes, 2015; Parigi et al., 2015; Hedegaard & Heegaard, 2016; Martin et al., 2016; Nissen et al., 2017). Given that the economic cost of human disease in Canada is substantial and increased 13.8% from 2005 to 2008 (Public Health Agency of Canada,
2014), consumption of quality milk products may be a potential solution to mitigate disease and maintain overall health of Canadians. Furthermore, while breast milk is the optimal food for infants, from 2011-2012 only 26% of mothers exclusively breastfed during the first 6 months postpartum, indicating many families rely on infant formula feeding (Statistics Canada, 2013). Since cow’s milk is used as the base of infant formula, it is important that bioactive components in cow’s milk closely mimic those of breast milk to ensure infant formula provides similar benefits to breastfeeding.

The health benefits of colostrum and milk to both calves and humans are proposed to be mediated by the presence of various biologically important molecules, many of which are found within milk-derived extracellular vesicles, conferring their protection from degradation during milk processing, storage, and digestion (Admyre et al., 2007; Chen et al., 2010; Hata et al., 2010; Kosaka et al., 2010; Weber et al., 2010; Gu et al., 2012; Zhou et al., 2012; Alsaweed et al., 2015; Pieters et al., 2015; Munagala et al., 2016; Chen et al., 2017; Sedykh et al., 2017; Yang et al., 2017). These extracellular vesicles are referred to as exosomes, which have an important role in intercellular communication by carrying and delivering cargo to recipient cells. One of the main types of cargo found in bovine colostrum and milk exosomes are a class of small, non-coding RNA molecules (21-23 nucleotides in length), known as microRNA (miRNA), which are involved in critical biological processes such as those that control intestinal epithelial proliferation and apoptosis (Biton et al., 2011; Zou et al., 2015; Zhou et al., 2015).

1.2 Rationale

Investigating the bioactivity of bovine colostrum and milk exosomes on human intestinal epithelial cells will advance the understanding of their regulatory role at the human intestinal barrier. Additionally, while it has been established that HIR cows have improved colostrum and
milk quality compared to A and L responders, the bioactivity of colostrum and milk exosomes from L, A, and HIRs on human intestinal epithelial cells is unknown. Exploring the functional role of colostrum and milk exosomes is expected to also provide a better understanding of how higher quality colostrum and milk maintains intestinal epithelial barrier. Furthermore, given the stability of exosomes and their cargo after processing and storage, along with their biological importance, information gained from this study could lead to the development of novel milk products, which support the health of individuals.

1.3 Hypothesis and objectives

The hypothesis of this study was that colostrum and milk exosomes containing miRNA isolated from HIR cows are stable and functionally active at the intestinal epithelial interface affecting cell viability and proliferation. This hypothesis was also examined in the context of L, A, and HIR cows. In other words, it was hypothesized that intestinal epithelial cells readily take up ingested miRNA in colostrum and milk exosomes from HIR cows improving cell viability and exerting less cytotoxicity, compared to exosomes from A or L responder’s colostrum and milk. Therefore, the specific objectives of this research were to: (1) characterize and confirm the presence of exosomes in colostrum and milk from L, A, and HIR cattle, (2) evaluate the ability of human colorectal adenocarcinoma epithelial (Caco-2; ATCC HTB-37) cells to take up exogenous exosomes from L, A, and HIR colostrum and milk, (3) determine whether exosomes from HIR colostrum and milk are cytotoxic to or promote the viability of Caco-2 cells compared to those from A and L responders, and (4) assess whether colostrum and milk exosomes induce apoptosis of Caco-2 cells.
1.4 Animals and design

The experimental design is outlined in Figure 1.1. Colostrum and milk samples (250 mL each) were collected (on the day of calving and six days post-calving, respectively) from Holstein cow’s previously classified as L, A, or HIR located at the University of Guelph’s Elora Dairy Research station. Exosomes were isolated by differential ultracentrifugation from 3 colostrum and 3 milk samples for each immune-response phenotype, for a total of 9 colostrum and 9 milk samples. Caco-2 cells were maintained in Eagle’s Essential Medium (EMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were performed at 37°C in an atmosphere of 5% CO₂. Prior to assessing the bioactivity of exosomes on Caco-2 cells, media was replaced with EMEM containing 10% exosome-depleted FBS, and the antibiotics mentioned previously. Exosome-depleted FBS was prepared by ultracentrifugation at 100,000 xg for 18 hours (h), as described by Shelke et al. (2014). Bioactivity of bovine colostrum and milk exosomes was assessed by (1) monitoring uptake of 0.16 μg/μL colostrum or milk exosomes after co-culture with Caco-2 cells for 2 and 24 h, (2) measuring Caco-2 cell metabolic activity by MTT assay after co-culture with 0.001, 0.005, 0.025, 0.125, 0.625 μg/μL colostrum or milk exosomes for 24, 48, and 72 h, and (3) evaluating apoptosis of Caco-2 cells by measuring caspase 3 activity after co-culture with 0.125 μg/μL colostrum or milk exosomes for 48 h.
Figure 1.1: Outline of experimental design; h=hours.

9 Holstein cows – 3 cows/immune response phenotype

250 mL colostrum and milk

Exosome isolation & co-culture with Caco-2 cells

Uptake assay:
0.16 μg/μL exosome co-culture for 2 and 24 h

MTT assay:
0.001, 0.005, 0.025, 0.125, 0.625 μg/μL exosome co-culture for 24, 48, and 72 h

Caspase assay:
0.125 μg/μL exosome co-culture for 48 h
CHAPTER 2
LITERATURE REVIEW

2.1 Bovine Colostrum and Milk Bioactive Components

Bovine colostrum and milk are distinct from each other in appearance, composition, and volume produced. Colostrum is the first secretion produced by the mammary gland, and compared to milk, is produced in lower quantities, contains relatively low concentrations of lactose, and is rich in immune and developmental factors (Gopal et al., 2000; Georgiev et al., 2008; Hurely & Theil, 2011). Nevertheless, both bovine colostrum and milk contain a wide range of bioactive components that hold significant nutritional value and provide immunological protection to both humans and calves (Haug et al., 2007; Hill and Newburg, 2015; Korst et al., 2017). Some of the immunological molecules found in colostrum and milk include large quantities immunoglobulins, leukocytes, lysozyme, lactoferrin, β-defensin, cathelicidin, and oligosaccharides (Hettinga et al., 2011; Nissen et al., 2017). These substances are critical in regulating tolerogenic and immunogenic responses at the gastrointestinal barrier. Additionally, the microbiome of colostrum and milk assists with microbial colonization of the gut, which acts to further support metabolism and immune system development (Meale et al., 2017).

Colostrum feeding is also important in ensuring passive transfer of immunoglobulins to the calf during the first 24-48 hours after birth, without which the calf would not survive (Hedegaard & Heegaard, 2016). Milk feeding thereafter is also important in ensuring calves receive sufficient nutrients and bioactive components, which support average daily gain, continued development of the immune system and gastrointestinal tract, as well as overall health and future productivity (Khan et al., 2011; Ballou et al., 2012; Ballou et al., 2015; Eckert et al., 2015; Azevedo et al., 2017; Korst et al., 2017; Meale et al., 2017).
Benefits of bovine colostrum and milk consumption by humans have also been reported. Bovine milk and milk products are a source of high quality protein, vitamins, and minerals for humans. Some studies have also suggested that milk consumption has a protective role against carcinogenesis, cardiovascular, metabolic, and inflammatory diseases (Lu et al., 2014; Pereira, 2014; Nongonierma and FitzGerald, 2015), however others suggest a possible association of milk consumption with increased risk for these diseases (Hu et al., 1999; Elwood et al., 2008; Melnik et al., 2009).

Bovine milk is also often used as the base of infant formula, and is therefore an important component of the diets of newborns requiring infant formula. For this reason, there has been growing interest in producing infant formula that more closely mimics the composition of breast milk in an attempt to better promote the intestinal and overall health of infants (Chen et al., 2010). Although less common than bovine milk consumption, bovine colostrum has also been used as a health food supplement for humans with beneficial effects on immune function and gastrointestinal health (Playford et al., 1999; He et al., 2001; Bagwe et al., 2015).

2.2 MicroRNA biogenesis and mode of action

Expression of colostrum and milk bioactive components are often controlled by microRNA (miRNA) (Ogorevc et al., 2009; Liao et al., 2010; Jaber et al., 2012; Li et al., 2012; Wang et al., 2016), which are single stranded RNAs (18-23 nucleotides in length) that do not encode proteins (Bartel et al., 2009). Typically, miRNA are processed from introns or exons of non-coding RNAs, as well as introns of protein coding genes (Kim and Kim, 2007). Biogenesis begins with RNA polymerase-II dependent transcription in the nucleus, creating primary miRNA (pri-miRNA) transcripts (Lee et al., 2004). Nuclear Drosha-DiGeorge syndrome critical region in gene 8 (DGCR8) complex is then responsible for cleaving pri-miRNA to release a 70 nucleotide,
hairpin-shaped pre-miRNA (Han et al., 2004). The pre-miRNA is then transported to the cytoplasm via the RanGTP-dependent nuclear transport receptor, exportin 5 (EXP5) (Bohsnack et al., 2004). Once in the cytoplasm, the pre-miRNA is processed by Dicer (Lee et al., 2003) and the mature strand of the double stranded miRNA is incorporated in the RNA-induced silencing complex (RISC). At this point the miRNA-RISC complex is guided by miRNA to its target mRNA to post-transcriptionally regulate gene expression (Gregory et al., 2005) (Figure 2.1).

Recognition of target mRNA by miRNA is based on complementarity between their sequences. Although perfect complementarity between miRNA and the 3’UTR of mRNA is beneficial to ensure target binding, it is not entirely necessary. Most miRNA can recognize a complimentary seed region (2-7 nucleotides in length) in the 3’UTR and maintain their post-transcriptional gene silencing function (Lewis et al., 2005). Upon recognition of target sites, miRNA mediate gene silencing by one of two processes. The first model describes destabilization of mRNA through removal of the mRNA-polyA tail by deadenylation factors in the RISC complex, resulting in increased susceptibility to exonucleolytic degradation (Fabian et al., 2011). The second model describes translational repression of mRNA, resulting in decreased protein expression while mRNA expression does not change (Mathonnet et al., 2007). While miRNA mainly function to repress gene expression, some studies have shown the ability of miRNA to activate certain genes by promoting translation of target mRNA (Vasudevan et al., 2007), which in some cases is mediated through binding of miRNA to the 5’-UTR of mRNA (Ørom et al., 2008).

The expression of miRNA in mammals is controlled by both genetic and epigenetic mechanisms. Single nucleotide polymorphisms (SNPs) have been located within miRNA genes, which influence the expression and function of miRNA (Georges et al., 2007). Jevsinek Skok et
al. (2013) conducted a genome-wide in silico screening of genomic sources and determined the genetic variability of miRNA in several livestock species. Results from the study suggest that identification of SNPs in miRNA genes may allow for selection of cattle based on their expression of specific miRNA. Another study by Geeleher et al. (2012) provided evidence that the regulatory effect of miRNA is a heritable trait in humans. Studies have also shown that some miRNA genes are regulated by CpG island methylation and histone modification (Chuang et al., 2007). Collectively, results from these studies suggest miRNA expression in bovine colostrum and milk is controlled by epigenetics, and may be heritable.

2.3 Classification of cell derived extracellular vesicles

Extracellular vesicles, such as microvesicles and exosomes, are typically isolated from biological fluids or cell culture supernatant by differential ultracentrifugation. Given that this procedure isolates various types of vesicles shed from cells, it is important to use specific criteria to distinguish between the types of vesicular structures in sample isolates (Lötval et al., 2014). Vesicles can be differentiated from one another based on size, morphology, buoyant density, and molecular composition (Table 2.1).

Electron microscopy images of milk exosomes indicate a round or cup-shaped morphology and sizes between 20 and 100 nm (Hata et al., 2010; Zonneveld et al., 2014; Pieters et al., 2015). In comparison to other vesicles, exosomes are quite small in size. For instance, apoptotic cells shed large vesicles, approximately 500 to 5000 nm in size (Dragovic et al., 2011; Crescitelli et al., 2013), and microvesicles, formed by budding of the cell’s plasma membrane, are approximately 50 to 1000 nm in size (Heijnen et al., 1999; Taraboletti et al., 2002; Xu et al., 2015). Additionally, microvesicles and apoptotic bodies do not share the same round or cup-shaped morphology as exosomes, but instead are irregular in shape (Heijnen et al., 1999; Théry
et al., 2001; Xu et al., 2015). Vesicles shed from cells are composed of a lipid membrane, allowing them to float on sucrose gradients at different densities. Exosomes isolated from human breast milk were shown to float at a density of 1.12-1.18 g/mL (Zonneveld et al., 2014), while microvesicles float at a density of 1.18-1.19 g/mL (Xu et al., 2015), and apoptotic bodies at a density of 1.16-1.28 g/mL (Théry et al., 2001).

Differences in lipid composition between exosomes, microvesicles, and apoptotic bodies exist as well. Exosomes are enriched in cholesterol, sphingomyelin, glycosphingolipids, and N-acetylneuraminyl-galactosylglucosylceramide, which contribute to the rigidity and delivery efficiency of exosomes (Parolini et al., 2009; Llorente et al., 2013). In comparison to microvesicles and apoptotic bodies, the amount of phosphatidylserine exposed on the outer leaflet of exosomes is much lower (Lai and Breakefield, 2012), providing another distinguishing characteristic of exosomes from other vesicles.

The proteome of vesicles is largely dependent on the cell from which they derive, contributing to the vast array of proteins identified in vesicles. With regards to bovine milk exosomes specifically, over 2100 proteins have been identified (Reinhardt et al., 2012; Wolf et al., 2015), many of which play important roles in various cellular pathways. For instance, proteome analysis revealed that exosomes were enriched in Rab and Snare proteins, which are important for vesicle trafficking and fusion. Additionally, exosomes expressed proteins involved in immunological functions such as antigen processing and presentation, chemokine signaling, and toll-like receptor signaling (Reinhardt et al., 2012).

While the proteome of vesicles is quite extensive, there are specific proteins that are commonly used to identify the presence of vesicles in samples. Tetraspanins (CD63, CD81, CD82, and CD53) were first identified in exosomes from B cells (Kleijmeer et al., 1998). To
date, studies have shown that tetraspanins are abundantly expressed in exosomes from any cell type (Théry et al., 2001), supporting their common use in exosome identification. Specifically, tetraspanins CD9, CD63, and CD81 have been identified in milk exosomes. A number of cytosolic proteins commonly expressed in exosomes such as tumor susceptibility gene (Tsg101), alix, heat shock proteins (such as Hsp70 and Hsp90), and flotillin-1 have also been identified in milk exosomes (Admyre et al., 2007; Lässer et al., 2011; Reinhardt et al., 2012; Zonneveld et al., 2014; Wolf et al., 2015; Yassin et al., 2015). With regards to other extracellular vesicles, studies have shown that microvesicles commonly express integrins (Dolo et al., 1998; Heijnen et al., 1999; Raposo and Stoorvogel, 2013), selectins (Heijnen et al., 1999; del Conde et al., 2005), and tetraspanins (CD9, CD63, CD81) (Crescitelli et al., 2013), while apoptotic bodies were shown to express histones, annexin-v, and tetraspanins (CD9, CD63, CD81) (Théry et al., 2001; Crescitelli et al., 2013).

In addition to the various lipids and proteins enriched in exosomes, several studies have reported on the presence of messenger RNAs (mRNA) and miRNA in milk exosomes across different species (Hata et al., 2010; Lässer et al., 2011; Kosaka et al., 2010; Gu et al., 2012; Zhou et al., 2012; Sun et al., 2013). Studies have shown that miRNA enclosed within exosomes are relatively stable under degradative conditions (Izumi et al., 2012; Zhou et al., 2012), and can be transferred to recipient cells where they are functionally active (Valadi et al., 2007; Wolf et al., 2015).

Insight into exosome composition may reflect their potential functions, and the manner by which they will affect other cells. Also, identifying key characteristics of exosomes will help distinguish them from other vesicles, as well as non-vesicular material in isolates, which is important to consider when conducting functional studies with exosomes (Lötvall et al., 2014).
2.4 Exosome biogenesis

Exosomes originate from the endocytic pathway within cells. Inward budding of the late endosome gives rise to intraluminal vesicles (ILVs) contained within multivesicular bodies (MVBs). Fusion of the MVB with the cell’s plasma membrane results in the release these vesicles into the extracellular space, which are then termed exosomes (Raposo and Stoorvogel, 2013) (Figure 2.2). There are many underlying mechanisms controlling the biogenesis of exosomes, some which are briefly described here.

Multiple mechanisms of ILV formation have been described. The endosomal sorting complex responsible for transport (ESCRT) dependent pathway is a well characterized pathway mediating the development of ILVs. There are four ESCRT multiprotein complexes: ESCRT-0, -I, and -II complexes recognize and sort ubiquitylated proteins to the perimeter of the endosomal membrane, as well as initiate budding of the membrane (Raiborg and Stenmark, 2009). ESCRT-II initiates scission of the vesicle (Hurley, 2010). Depletion of ESCRT proteins indicated there are several ESCRT-independent pathways controlling ILV formation (Stuffers et al., 2009). For instance, tetraspanins CD63 and CD81, promoted the formation of ILVs and the sorting of cargo into exosomes in an ESCRT-independent manner (van Niel et al., 2011; Perez-Hernandez et al., 2013; Edgar et al., 2014). Other known constituents of exosomes, such as ceramide and syntenin, also play a role in controlling the budding of ILVs independently of ESCRT proteins (Kosaka et al., 2010; Friand et al., 2015).

Rab GTPases are largely responsible for regulating cell trafficking, vesicle transport, as well as exosome biogenesis. A particular group of Rab proteins are important for mediating endosome maturation, sorting of cargo into endosomes, and release of exosomes from cells. Specifically, Rab5, Rab7, Rab11, Rab27, and Rab35 are all essential for the secretion of
exosomes from cells. Altering the expression of any of these Rab proteins has the potential to greatly affect progression through the endocytic pathway (Hsu et al., 2010; Ostrowski et al., 2010; Baietti et al., 2012; Alenquer and Amorim, 2015; Jaé et al., 2015). For instance, Baietti et al. (2012) showed overexpression of Rab5 significantly reduced the accumulation of syndecan, CD63, and Alix in exosomes. Overexpression of Rab7 restored the presence of these proteins in exosomes. Ostrowski et al. (2010) demonstrated knockdown of five Rab proteins (Rab2b, Rab9a, Rab5a, Rab27a and Rab27b) resulted in significant inhibition of exosome secretion in HeLa cells. Silencing of Rab27 specifically, reduced the expression of common exosomal proteins such as MHC-II, CD63, and Tsg101, and also decreased movement of multivesicular bodies to the cell periphery. These reports demonstrate the importance of Rab proteins in exosome secretion.

2.5 Bioactivity of milk exosomes

Exosomes have a multifaceted role as carriers that deliver cargo to recipient cells, thereby modifying the target cell’s gene expression, signaling, and function. Valadi et al. (2007) were one of the first to report that exosomal transfer of miRNA and mRNA is a mechanism of genetic regulation between cells. The study established that human and mouse mast cell derived exosomes were enriched in mRNA and miRNA, and were transferrable to other human and mouse mast cells. Further, the transferred mRNA from mouse mast cells was functional in recipient human cells as demonstrated by the expression of mouse specific proteins in these cells.

2.5.1 Uptake of milk exosomes

A number of studies have reported the ability of various human and rodent cell lines to take up milk exosomes, establishing that milk-derived exosomes can be delivered to recipient cells and are functional across species. For instance, Lässer et al. (2011) identified RNA-
containing exosomes in human breast milk and demonstrated their uptake by human macrophages using fluorescence microscopy and flow cytometry. The active uptake of human breast milk exosomes by human macrophages suggests a mechanism by which these exosomes facilitate cell-cell communication between mother and offspring, implicating their role in regulating infant development. A study by Izumi et al. (2015) reported similar results, but instead assessed the uptake of bovine milk-derived exosomes by human macrophage like cells (monocytic leukemia THP-1 cells).

Importantly, Wolf et al. (2015) demonstrated the uptake and transport of bovine milk exosomes containing miRNA across the basolateral membrane of human colon carcinoma and rat small intestinal cells. Uptake of these exosomes was mediated by endocytosis, and dependent on surface protein recognition between exosomes and intestinal epithelial cells, thereby establishing a mechanism by which bovine milk exosomes are transported across the human intestinal epithelium. These results, along with those reported by Izumi et al. (2015), suggest that bovine milk exosomes are bioavailable to humans, and may play a role similar to human breast milk exosomes.

### 2.5.2 Immune-modulation by milk exosomes

Studies have shown that in conjunction with uptake by recipient cells, milk exosomes also induce immune-modulatory effects on these cells. In a study conducted by Admyre et al. (2007), exosomes isolated from human breast milk and incubated with peripheral blood mononuclear cells (PBMCs) increased the number of Foxp3+CD4+CD25+ T regulatory cells. The study also indicated the ability of exosomes to inhibit anti-CD3-induced IL-2 and IFN-γ production by PBMCs. In addition to establishing that exosomes play a role in mediating responses of the immune system, this study provides evidence that milk exosomes may be
important for the development of infant’s immune system.

Bovine milk exosomes also elicit cross-species immune-modulatory effects. Sun et al. (2013) demonstrated that bovine colostrum, colostrum-powder, and milk exosomes enriched with immune-related miRNA were taken up by mouse macrophages (RAW264.7 cells). The colostrum-derived exosomes had several immune-modulatory effects on RAW264.7 cells. For instance, colostrum exosomes regulated cytokine production (increase in IL-1 and IL-6; decrease in IL-10) after stimulation with 10 and 100 ng/mL of lipopolysaccharide (LPS), as well as promoted phagocytosis and cell migration of RAW264.7 cells. Ultrasonication of the exosomes further showed these immune modulatory effects are significantly compromised when vesicle structure is destroyed. These results indicate that the physical structure of the vesicle is essential for uptake by RAW264.7 cells, delivery of miRNA, and immune modulation of these cells.

Another study by Munagala et al. (2016) also assessed the immune-modulatory effects of bovine milk exosomes in rodents and humans. The uptake and biocompatibility of milk exosomes was demonstrated using human lung and breast cancer cell lines, as well as rodent models. Following oral administration to mice, fluorescently labeled milk exosomes were detectable in circulation for up to 6 days, and also in various tissues including the liver, lungs, kidneys, pancreas, spleen, ovaries, colon, and brain, suggesting that these exosomes can cross the intestinal barrier, travel in the circulation, and regulate distant tissues. Further, oral administration of bovine milk exosomes to mice did not elicit adverse immune responses, indicating cross-species tolerance. Alternatively, an increase in the anti-inflammatory cytokine granulocyte-macrophage colony-stimulating factor was noted. Milk exosomes also exerted anti-inflammatory and anti-proliferative effects on human cells. For instance, treatment with 50 μg/mL of milk exosomal protein for 72 hours decreased survival of human lung (A549 and
H1299) and breast (T47D and MDA-MB-231) cancer cells between 15 and 45%, but did not exhibit such cytotoxicity towards normal human lung cells (Beas-2b). Additionally, both in vitro and in vivo studies demonstrated that milk exosomes decreased expression of NF-κB, further indicating their anti-inflammatory effects. These results suggest that bovine milk exosomes are non-toxic to humans, and also implicates their potential anti-inflammatory benefits in humans. Another study by Näslund et al. (2014) showed the ability of milk exosomes to decrease the transfer of human immunodeficiency virus from infected monocyte-derived dendritic cells to CD4+ T cells, suggesting a protective type function of human breast milk exosomes against the transmission of viruses.

2.5.3 Exosome depletion of fetal bovine serum

Fetal bovine serum (FBS) provides important nutrients that support the growth and survival of cultured cells. Several studies have shown the ability of exosomes isolated from FBS in influencing the phenotypes of cultured cells. A study by Ochieng et al. (2009) showed that exosomes isolated from FBS promoted the proliferation of human breast epithelial cells. A similar study by Shelke et al. (2014) determined that exosomes derived from FBS induce a migratory phenotype in recipient human lung epithelial cells. Additionally, the study demonstrated that FBS could be depleted of exosomes after 18 hours of ultracentrifugation at 100,000 xg, which in turn significantly decreased FBS-induced migration of human lung epithelial cells. These studies indicate the importance of using FBS depleted of exosomes when conducting research assessing the functional effects of exosomes isolated from other sources, on cultured cells.
2.6 Regulatory role of microRNA

Several miRNA, including let-7a, miR-29a, miR-125b, miR-148a, miR-155, miR-181a, miR-200c, and miR-223, are abundantly expressed in bovine milk (Chen et al., 2010; Hata et al., 2010; Izumi et al., 2012; Sun et al., 2013; Izumi et al., 2015), and play important roles in immune regulation, intestinal development, and colorectal cancer progression. An overview of how these miRNA regulate the immune system and intestinal development is provided herein.

2.6.1 Immune regulation by miRNA identified in bovine milk

Responses of the immune system are under the control of highly regulated gene expression in participating cells. MiRNA are one of the key factors involved in regulating gene expression in these cells, and thus involved in regulating the immune system as a whole. Commonly expressed miRNA in bovine milk have many implications in immune-related functions.

Cytokine expression is influenced by miRNA. For instance, let-7a inhibits Th17 differentiation by down-regulating IL-6 secretion (Zhang et al., 2013), suggesting that let-7a may mediate inflammation. Additionally, miR-181a may also function to regulate inflammatory responses by targeting and repressing translation of IL-8 transcripts (Galicia et al., 2014). MiR-29 also regulates cytokine expression by specifically targeting IFN-γ and suppressing its expression. Reduced expression of IFN-γ, in turn, suppressed Th1 responses in mice infected with Listeria monocytogenes or Mycobacterium bovis bacillus Calmette-Guérin (Ma et al., 2011).

Immune response signaling pathways are also controlled by miRNA expression. Tili et al. (2007) demonstrated that miR-125b represses TNF-α expression in Raw 264.7 macrophages after stimulation with LPS, while miR-155 has the opposite effect. The function of miR-125b in
macrophages may be to repress the LPS signal transduction pathway during the absence of microbial infection, while miR-155 activates the pathway to defend against infection. Additionally, miR-148a has been shown affect the antigen-presenting functions of dendritic cells by inhibiting MHC-II expression (Liu et al., 2010).

Studies have also shown how miRNA regulate activation and differentiation of various cells of the immune system. Li et al. (2012) demonstrated the importance of miR-181a in regulating T cell receptor (TCR) sensitivity during T cell maturation. Overexpression of miR-181a in mature T cells greatly reduced TCR sensitivity, while inhibition in immature T cells reduced sensitivity to antigens. Double positive thymocytes have high expression of miR-181a, increasing their sensitivity towards antigens, allowing them to undergo positive selection. These findings are further supported by an earlier report by Li et al. (2007), demonstrating that knockdown of miR-181a in mice resulted in impaired positive and negative selection. B cell lymphocyte differentiation is also mediated by miR-181a expression, whereby its overexpression positively regulates B lymphocyte differentiation in mouse bone marrow (Chen et al., 2004).

Similar to miR-181a, miR-223 is primarily expressed in bone marrow and functions to regulate myeloid and T cell differentiation (Chen et al., 2004). Using a mutant miR-223 mouse model, Johnnidis et al. (2008) demonstrated that miR-223 negatively regulates myeloid production by specifically targeting and repressing a transcription factor (Mef2c) responsible for promoting myeloid proliferation. Additionally, as granulocyte differentiation proceeds, miR-223 showed a steady increase, with highest expression in mouse peripheral blood neutrophils. Neutrophils lacking miR-223 were shown to be hypersensitive towards activating stimuli, suggesting that high expression of miR-223 at this stage of myeloid differentiation plays an important role in modulating neutrophil sensitivity.
Collectively, these findings highlight the importance of miRNA in regulating various aspects of the mammalian immune system. Additionally, these findings provide insight into how bovine colostrum and milk miRNA may function to regulate development of the immune system in new born calves and possibly humans.

### 2.6.2 Intestinal and colorectal cancer regulation by miRNA identified in bovine milk

The epithelial layer of the intestine forms an important physical barrier preventing dissemination of pathogens into underlying tissues. Similar to regulating gene expression networks in cells of the immune system, miRNA also regulate gene expression pathways in intestinal epithelial cells, thereby modulating their physiology and function.

The intestine requires proper signaling to maintain development, differentiation, and renewal of epithelial cells (Chivukula et al., 2014). Both miR-148a and let-7a are up-regulated during intestinal epithelial differentiation, implicating their roles in maintaining intestinal homeostasis. MiR-29 has also been suggested to regulate intestinal homeostasis. Using human colonic epithelial cells (FHC) and human small intestinal epithelial cells (FHs74Int), Zhou et al. (2009) demonstrated that overexpression of miR-29a increases intestinal permeability, while inhibition had the opposite effect. Further, expression of miR-29a in patients suffering from irritable bowel syndrome (IBS) increased intestinal permeability by targeting nuclear factor-kB–repressing factor (NKRF) and Claudin-1 (CLDN1) (Zhou et al., 2015).

Dysregulation of miRNA can disrupt signals necessary for maintaining intestinal homeostasis, potentially leading to development of colorectal cancer (Chivukula et al., 2014). Certain miRNA, such as let-7a, are considered to be tumor suppressors, which inhibit the growth or metastasis of cancerous cells. Akao et al. (2006) demonstrated this effect in human colon cancer cells whereby transfection of let-7a suppressed cell growth. Additionally, miR-200c
inhibited the metastatic ability of human colon cells (SW480/620) by targeting and decreasing expression of Zinc finger E-box-binding homeobox 1 (ZEB1) (Chen et al., 2012).

Together, with research on the immune-regulatory role of miRNA, these findings show the importance of miRNA in mediating intestinal homeostasis on both the epithelial and immune regulation level. Findings regarding modulation of intestinal disorders, such as colorectal cancer and IBS, suggest the therapeutic potential of miRNA, and perhaps the possible benefits of milk miRNA consumption in humans.

2.7 Dietary MicroRNAs

Many studies have reported on the bioavailability of dietary miRNA to mammalian organisms. Zhang et al. (2012) were the first to report cross-kingdom delivery of plant miRNA to recipient mammalian cells through food consumption, thereby facilitating post-transcriptional regulation of mammalian miRNA target genes. Specifically, Zhang et al. (2012) showed that miR-168a from rice (osa-miR-168a) was detectable in human plasma, as well as in mice plasma and tissues. Furthermore, osa-miR-168a decreased LDL receptor adapter protein 1 (LDLRAP1) mRNA expression in the liver, which in turn lowered LDL in the plasma. However, several recent reports suggest that plant miRNA are not bioavailable to humans, due to their absence in human or mouse plasma after consumption of a plant-based diet (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013; Baier et al, 2014).

Researchers have suggested factors which may contribute to the lack of plant miRNA uptake in mammalian hosts. For instance, while there is some evidence to suggest the presence of exosome-like vesicles in plants (Regente et al., 2012; Ju et al., 2013), it is still unclear whether plant miRNA are located within vesicles that confer their protection from degradation and
facilitate uptake by recipient cells, as mammalian exosomes do (Witwer et al., 2013; Baier et al., 2014).

A study by Baier et al. (2014) tested whether humans absorb bovine milk miRNA after consumption of nutritionally relevant doses of milk. The study showed a postprandial increase in plasma concentrations of miR-29b and miR-200c. These results suggest that these particular bovine milk-derived miRNA are bioavailable to humans and absorbed at the human intestinal barrier. Additionally, a miRNA depletion study was conducted in mice to determine if endogenous miRNA synthesis compensates for when a miRNA-depleted diet is fed. Results indicated that mice fed the depleted diet had a 61% decrease in miR-29b plasma concentrations, indicating that endogenous synthesis of miR-29b does not compensate for a diet depleted of miRNA. The study also assessed the bioavailability of plant-derived miRNA in humans. Results indicated that Brassica-specific miRNA are not detectable in prandial human plasma samples.

Another study by Gu et al. (2012) assessed the transfer of miRNA from sow colostrum and milk to their piglets. The study reported that immune-related miRNA within exosomes were more abundant in colostrum than milk. Accordingly, these immune-related miRNA were present at greater concentrations in the serum of colostrum-only fed piglets compared to those fed only milk. These results further support the idea that immune-related miRNA can cross the intestinal barrier and promote the development of the immune system in infants.

A study by Title et al. (2015) used mice knockout models to assess the transfer of exogenous milk-derived miRNA to offspring. The study showed that when miR-375 and miR-200c/141 knockout mice were fostered to wild-type dams, only trace amounts of these miRNA were found in the intestinal epithelium, blood, liver, and spleen of the offspring. In contrast to the conclusions made by previous studies showing the uptake of exogenous miRNA (Gu et al., 2012;
Sun et al., 2013; Baier et al., 2014; Wolf et al., 2015), Title et al. (2015) concluded that maternal milk miRNA are not bioavailable to offspring, as these miRNA do not cross the intestinal barrier and influence gene expression in distant tissues. However, future research should consider miRNA target expression in the intestinal epithelium or liver, which could account for low detection of miRNA in tissues reported by Title et al. (2015). Additionally, sample collection should occur soon after milk consumption to ensure accurate detection of miRNA in plasma and tissues (Gu et al., 2012; Baier et al., 2014).

While current methods suggest that dietary milk miRNA may be taken up by the intestine and affect distant tissues, studies assessing their bioavailability highlight the importance of designing protocols which distinguish between exogenous (ie. diet-derived) and endogenous miRNA to more accurately reflect the uptake.

2.8 References


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the human hepatoma HepG2 and lung cancer A549 cell lines. *Oncology reports*, 28(5), 1551-1558.


2.9 Figures and tables

Figure 2.1: miRNA biogenesis and mode of action. RNAPII: RNA polymerase II; TF: transcription factor; Pri-miRNA: primary miRNA; Pre-miRNA: precursor miRNA; DGCR8: DiGeorge syndrome critical region in gene 8; EXP5: exportin 5; AGO: Argonaute protein; CCR4: C-C chemokine receptor type 4; CAF1: Chromatin assembly factor 1. Both CCR4 and CAF1 are deadenylation factors. [Figure sourced with permission from Liang et al., 2015].
Figure 2.2: Exosome biogenesis through the endosomal pathway. [Figure sourced with permission from Kourembanas, 2015].
Table 2.1 Characteristics of extracellular vesicles

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<th>Exosomes</th>
<th>Microvesicles</th>
<th>Apoptotic Bodies</th>
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</thead>
<tbody>
<tr>
<td><strong>Size (nm)</strong></td>
<td>20-100</td>
<td>50-1000</td>
<td>500-5000</td>
</tr>
<tr>
<td><strong>Buoyant density on sucrose (g/mL)</strong></td>
<td>1.12-1.18</td>
<td>1.18-1.19</td>
<td>1.16-1.28</td>
</tr>
<tr>
<td><strong>Shape</strong></td>
<td>Round and cup-shaped</td>
<td>Heterogenous</td>
<td>Irregular</td>
</tr>
<tr>
<td><strong>Lipid composition</strong></td>
<td>High cholesterol, sphingomyelin, glycosphingolipids, and N-acetylneuraminyl-galactosylglucosylceramide. Low phosphatidylserine exposed on outer leaflet.</td>
<td>High phosphatidylserine exposed on outer leaflet.</td>
<td>High phosphatidylserine exposed on outer leaflet.</td>
</tr>
<tr>
<td><strong>Commonly expressed proteins</strong></td>
<td>Tetraspanins (CD9, CD63, CD81), integrins, flotillin-1, MHC-II, Hsp70/90, Alix, and Tsg101.</td>
<td>Tetraspanins (CD9, CD63, CD81), selectins, integrins, and CD40 ligand.</td>
<td>Tetraspanins (CD9, CD63, CD81), histones and annexin-v.</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td>Endosomal pathway</td>
<td>Budding from plasma membrane</td>
<td>Blebbing from apoptotic cells</td>
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CHAPTER 3

Bioactivity of bovine colostrum exosomes containing microRNA on human intestinal epithelial cells

Key words: bovine colostrum exosomes, microRNA, intestinal epithelial cells, viability, uptake
3.1 Abstract

Bovine colostrum contains bioactive components with immune-regulatory potential in newborn calves and humans. Expression of milk bioactive components is epigenetically regulated by microRNA (miRNA). Packaging of miRNA in exosomes reportedly protects them from degradation during milk processing and storage. The bioactivity of colostrum exosome-derived miRNA at the human intestinal epithelial barrier remains to be explored. Therefore, the purpose of this study was to evaluate the functional role of colostrum exosomes at the intestinal interface using human colorectal adenocarcinoma epithelial (Caco-2) cells. Exosomes were isolated by differential ultracentrifugation from bovine colostrum and characterized by Western blot analysis for the presence of common exosomal-proteins (CD9, CD63, CD81, and Hsp70). To assess bioactivity, exosomes were co-cultured with Caco-2 cells. Fluorescent labeling of exosomes using PKH67 dye confirmed their uptake by Caco-2 cells, suggesting that bovine colostrum exosomes are likely bioavailable to humans. MTT assay further demonstrated the bioactivity of exosomes, whereby co-culture with colostrum exosomes for 48 and 72 hours significantly increased Caco-2 cell metabolic activity compared to 24 hours co-culture ($p < 0.0001$). This confirms colostrum exosomes are not only non-cytotoxic but also enhance viability of Caco-2 cells supplemented with exosome-depleted media. Caspase 3 activity, an indicator of apoptosis, was significantly greater after co-culture with colostrum exosomes, compared to cells co-cultured without exosomes ($p < 0.0006$), although to a small extent, indicating bovine colostrum exosomes contain molecules that may induce apoptosis of cancer cells. This study adds further understanding to the potential functional importance of bovine colostrum on gastrointestinal health of humans.
3.2 Introduction

Bovine colostrum is the first secretion produced by the mammary gland immediately after parturition. It is a nutrient rich fluid that contains bioactive components that are immunologically significant to calves, which do not receive cross-placental transfer of immunoglobins in utero (Dunn et al., 2017; Nissen et al., 2017). Due to its high concentration of bioactive components, bovine colostrum has also been used as a health food supplement for humans with beneficial effects on immune function and gastrointestinal health, although further research is needed to assess the ability of bovine colostrum to treat or mitigate disease in humans (Playford et al., 1999; He et al., 2001; Bagwe et al., 2015).

The process of lactation in dairy cows, including the volume of milk produced along with quality and quantity of bioactive components, is epigenetically regulated by microRNA (miRNA), which are small, non-coding RNA molecules (18-23 nucleotides in length) (Ogorevc et al., 2009; Li et al., 2012; Wang et al., 2016). MiRNA act as post-transcriptional regulators of gene expression by binding the 3’-untranslated region of target messenger RNA (mRNA), resulting in repressed translation or degradation of the target mRNA, thereby regulating protein expression (He & Hannon, 2004). They have been identified in a variety of biological fluids and regulate a wide range of processes, such as those controlling cell differentiation, proliferation, and apoptosis (Bartel, 2004), indicating their potential functional importance.

Bovine colostrum miRNA are abundantly expressed in colostrum-derived extracellular vesicles, referred to as exosomes (20-100 nm in size) (Hata et al., 2010; Sun et al., 2013). Exosomes originate via the endocytic pathway in a variety of cells such as reticulocytes, dendritic cells, macrophages, T cells, mast cells, epithelial cells, and adipocytes (Théry et al., 2002). Exosomes have multifaceted roles in intercellular communication by acting as carriers
that deliver cargo to recipient cells, resulting in modification of the target cell’s signaling and function (Admyre et al., 2007; Hata et al., 2010; Lässer et al., 2011). Reports have shown that colostrum miRNA are stable under degradative conditions such as RNase digestion, low pH, high temperature, and freeze/thaw cycles, which is attributed to protection provided by the exosomes carrying them. Packaging of colostrum miRNA within exosomes suggests their potential to withstand processing, storage, and digestion, potentially facilitating their interaction with intestinal cells (Zhou et al., 2012; Pieters et al., 2015; Melnik et al., 2016).

Several studies have demonstrated the biological effects of bovine colostrum and milk exosomes on human cells. Bovine milk exosomes have been shown to be taken up by and modulate the function of human monocytic leukemia cells, monocyte-derived dendritic cells, colon carcinoma cells, lung, and breast cancer cells (Näslund et al., 2014; Izumi et al., 2015; Wolf et al., 2015; Munagala et al., 2016). While the bioactivity of bovine milk exosomes on human cells has been demonstrated, the role of these bovine colostrum-derived exosomes and their cargo in regulating human intestinal cell function remains unclear. Based on previous knowledge of biological effects of bovine colostrum exosomes, it was hypothesized that bovine colostrum exosomes containing miRNA would interact with and promote maintenance of the human intestinal epithelial barrier. The objectives of the study were to: (1) characterize and confirm the presence of exosomes in bovine colostrum, (2) evaluate the ability of intestinal epithelial cells to take up exogenous bovine colostrum exosomes, (3) determine whether colostrum exosomes are cytotoxic to or promote intestinal cell viability, and (4) determine whether colostrum exosomes induce apoptosis of cancerous human intestinal epithelial cells. This study will help to better understand the functional role of bovine colostrum exosomes in maintaining the human intestinal barrier.
3.3 Materials and methods

3.3.1 Samples

Colostrum samples (250 mL) were collected on the day of calving from healthy Holstein cows located at the University of Guelph’s Elora Dairy Research Station. Samples were stored at -80°C until the time of analysis. A total of 9 colostrum samples were used for this study.

3.3.2 Isolation of colostrum exosomes

Colostrum exosomes containing miRNA were isolated through differential ultracentrifugation according to a protocol established Atalla et al. (2016, manuscript in preparation). Colostrum samples were first centrifuged at 5,000 xg for 30 minutes at 4°C to remove fat and cell debris. The supernatant was collected and diluted 2:1 in phosphate buffered saline (PBS) and centrifuged again at 12,000, 35,000, and 70,000 xg for 1 hour (h) at 4°C to further remove cell debris and larger vesicles such as apoptotic bodies and microvesicles. Exosomes were pelleted by centrifuging at 100,000 xg for 1 h at 4°C. Resultant pellets were resuspended in approximately 38 mL PBS and centrifuged again at 100,000 xg for 1 h at 4°C to wash the pellet. After washing, pellets were weighed and resuspended in a volume of PBS equivalent to their weights and total exosomal protein was quantified using Pierce BCA protein assay (ThermoFisher Scientific). Isolated exosomes were then stored in 100 μL aliquots at -80°C until use in future experiments (Yamada et al., 2012; Momen-Heravi et al., 2013).

3.3.3 Enzyme linked immunosorbent assay of colostrum exosomes

CD63 ExoELISA kit (System Biosciences) was used for detection of CD63 in colostrum exosomes isolated from nine different cows, and were tested in duplicate. Exosomes were resuspended in 200 μL exosome binding buffer, followed by adding 50 μL of each exosome sample to wells of a microtiter plate. The plate was incubated overnight at 37°C. After washing,
50 μL of anti-CD63 (1:100) was added to the wells and incubated for 1 h at 37°C. The plate was washed again and then incubated with 50 μL horseradish peroxidase enzyme-linked secondary antibody (goat anti-rabbit, 1:5000) for 1 h at room temperature. After washing, 50 μL colorimetric substrate was added to each of the wells for 45 minutes followed by the addition of stop buffer. Absorbance was read at 450 nm using a microplate spectrophotometer (BioTek PowerWave XS2).

3.3.4 Western blotting of colostrum exosomes

Exosomes isolated from colostrum were first identified by another member of our group, Dr. H. Atalla, using transmission electron microscopy and immunogold labeling for CD63 (Atalla et al., 2016, manuscript in preparation). Colostrum exosomes were further characterized by Western blot analysis for commonly expressed exosomal proteins (CD9, CD63, CD81, and heat shock protein 70; Hsp70). Exosomes were first lysed in Triton-X-100 cell lysis buffer containing Roche mini complete protease inhibitor for 5 minutes on a shaker. To further lyse the exosomes, samples were sonicated in a water bath at room temperature for 3 x 5 minutes with brief vortexing in between. Samples were then centrifuged for 5 minutes at 13,000 xg at room temperature. Exosome lysate was transferred to clean microfuge tubes and stored on ice while total protein in the lysate was quantified using Pierce BCA protein assay (ThermoFisher Scientific). Laemmli buffer was added to samples at a 1:1 ratio and heated to 100°C for 10 minutes. Samples (10-20 μg of protein per well) were loaded onto a 12% acrylamide gel and proteins were separated by electrophoresis, followed by transfer to 0.22 μm polyvinylidene difluoride (PVDF) membranes. To ensure equal loading of protein per well, PVDF membranes were stained with Ponceau red for 5 minutes, gently rinsed with distilled water for 30 seconds and imaged using ChemiDoc MP System (BioRad). Membranes were then washed in tris-
buffered saline + tween (TBS-T) for 15 minutes to remove the Ponceau stain. Membranes were blocked with 0.2% fish skin gelatin in tris buffered saline (TBS) for 1 h at room temperature, followed by washing with TBS-T. Exosomal proteins were detected using either mouse anti-cow CD9 (ab3923, Abcam), mouse anti-human CD63 (ab193349, Abcam), mouse anti-human TAPA1 (ab79559, Abcam), mouse anti-human Hsp70 (ab2787, Abcam) diluted 1:1000 in 0.2% fish skin gelatin in TBS-T. Membranes were incubated with primary antibodies for approximately 18 h at 4°C. After extensive washing with TBS-T, membranes were incubated with donkey anti-mouse IgG-HRP (ab6820, Abcam) secondary antibody diluted 1:10,000 in 0.2% fish skin gelatin in TBS-T for 1 h at room temperature. Concentration of antibody as reported by the manufacturer are included in Table 3.1. Detected proteins were then imaged using the ChemiDoc MP system (Bio-Rad) (Lässer et al., 2011; Zonneveld et al., 2014).

3.3.5 Caco-2 cell culture

Human colorectal adenocarcinoma epithelial cells (Caco-2, HTB-37) were purchased from American Type Culture Collection (ATCC, Cedarlane, Burlington, ON), and maintained in Eagle’s Essential Medium (EMEM; ATCC), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were performed at 37°C in an atmosphere of 5% CO₂. Media was changed every 2-3 days and cells were passaged once they reached approximately 70% confluence (4-5 days), and seeded at a density of approximately 1.0 x 10⁶ cells per T-75 cm² flask (Lea, 2015). Prior to assessing bioactivity of colostrum exosomes on Caco-2 cells, media was replaced with EMEM containing 10% exosome-depleted FBS and the previously mentioned antibiotics. FBS was depleted of exosomes by centrifuging at 100,000 xg for 18 h at 4°C as previously reported (Chiba et al., 2012; Shelke et al., 2014). Caco-2 cells from passages 4-30 were used for bioactivity assays.
3.3.6 Exosome uptake assay

Exosomes were labeled with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich). Immediately prior to labeling, 4 μL of PKH67 dye was added to 1 mL Diluent C to prepare the dye reagent (Lässer et al., 2011). A 100 μL aliquot of colostrum exosomes was added to the PKH67 dye reagent. As a control, the same volume of PBS was mixed with 1 mL Diluent C and dye. The samples were gently mixed for 4 minutes on a shaker, followed by the addition of 2 mL 1% exosome depleted FBS (diluted in PBS) to bind excess dye for 1 minute (Khatua et al., 2009; Lässer et al., 2011). The samples were then transferred to Beckman Ultra-Clear 13 x 51 mm ultracentrifuge tubes (Beckman Coulter) and filled with exosome-depleted EMEM. Using the Beckman Coulter Swinging Bucket rotor (SW 55 Ti, 6 x 5 ml), the samples were ultracentrifuged at 100,000 xg at 4°C for 1 h. After ultracentrifugation, the supernatant was discarded and the pellet was resuspended in exosome-depleted EMEM and transferred to clean ultracentrifuge tubes. The tubes were filled with exosome-depleted EMEM and ultracentrifuged once again at 100,000 xg at 4°C for 1 h to remove unbound dye. After ultracentrifugation, the supernatant was discarded and the pellet was resuspended in the appropriate volume of exosome-depleted EMEM to give a concentration of 0.16 μg/μL of labeled exosomes.

Prior to labeling, Caco-2 cells were seeded at a density of 60,000 cells/well in 24 well plates using exosome-depleted EMEM. Cells were allowed to adhere for ~18 h, at which point they reached the desired confluence (~70% confluence). Once exosomes were labeled and prepared for co-culture, media was removed from each of the wells and cells were washed with Dulbecco’s phosphate buffered saline (DPBS; Gibco). Labeled exosomes (0.16 μg/μL) or the same volume of PKH67-PBS control was added to the appropriate wells and incubated for 2 or
24 h at either 37°C or 4°C. Colostrum exosomes isolated from 3 cows were tested in triplicate. After co-culture, cells were harvested using 0.25% trypsin-0.53 M EDTA solution (Gibco) to remove surface bound exosomes (Franzen et al., 2014). Trypsinized cells were fixed in 4% formaldehyde for 10 minutes (Lässer et al., 2011; Izumi et al., 2015), followed by fixing cells to microscope slides (25,000 cells/slide) using the Shandon Cytospin 2 Centrifuge. Once adhered to slides, cells were washed twice with DPBS and 100 μL 0.1 M ammonium chloride solution was added to the cells for 10 minutes to quench the formaldehyde reaction (Davis et al., 2014). Cells were gently washed again with DPBS. To label the nuclei of cells, 5 μL of 7-AAD dye (Molecular Probes) was diluted in 200 μL DPBS and added to the cells for 15 minutes. Cells were then rinsed with DPBS and mounted using Vectashield (Vector Laboratories) (Izumi et al., 2015). Cellular uptake of exosomes was then visualized by fluorescence microscopy. Images were captured using the Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies). Images were analyzed using AxioVision software from Carl Zeiss (release 4.8.2).

3.3.7 MTT cell viability and proliferation assay

Caco-2 cells were seeded at a density of 6.0 x 10⁴ cells/well in collagen coated 24 well plates using exosome-depleted media and allowed to adhere for ~18 h. Exosomes from 9 cows were co-cultured with Caco-2 cells at various exosomal protein concentrations (0, 0.001, 0.005, 0.025, 0.125, 0.625 μg/μL) for 24, 48, and 72 h (Ahmed et al., 2015; Wolf et al., 2015; Munagala et al., 2016) in exosome-depleted media, and tested in triplicate.

Prior to adding MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen) at each time point, exosome co-culture media was removed and replaced with exosome-depleted Minimum Essential Medium containing no phenol red (Gibco) because
phenol red interacts with the MTT dye. Once the media was changed, 0.5 mg/mL of MTT solution was added to each of the wells and also to wells containing only media to serve as a blank control. Plates were then incubated at 37°C/5% CO₂ for 4 h, followed by incubation of 0.01 M SDS-HCl solution to solubilize formazan crystals for another 4 h. Absorbance was read at 570 nm using a microplate spectrophotometer (BioTek PowerWave XS2).

**3.3.8 Caspase 3 in vitro apoptosis assay**

A colorimetric caspase 3 assay kit (Sigma) was used to detect caspase 3 activity in Caco-2 cells after co-culture with colostrum exosomes. Caco-2 cells were seeded at a density of 7.5 x 10⁵ cells/flask in collagen coated T-25 cm² flasks using exosome depleted media and allowed to adhere for ~18 h. Cells were co-cultured with 0.125 μg/μL exosomal protein for 48 h in exosome depleted media (Hosseini et al., 2014; Ahmed et al., 2015; Wolf et al., 2015; Munagala et al., 2016). According to the manufacturers protocol and modified methods from Hosseini et al. (2014), 3.0 x 10⁶ cells were lysed in lysis buffer for 15 minutes on ice. Samples were then centrifuged for 10 minutes at 18,000 xg at 4°C. Cell lysates were collected and transferred to new microfuge tubes and stored at -80°C until time of analysis. Using 96 well plates, 5 μL cell lysate and 10 μL caspase 3 substrate was added to the appropriate wells to measure caspase 3 activity in the cells after exosome co-culture. Each sample (n=9) and control (no exosomes) was tested in triplicate. After 18 h of incubation at 37°C, absorbance was read at 405 nm using a microplate spectrophotometer (BioTek PowerWave XS2).

**3.3.9 Statistical analysis**

Data from the MTT assay were analyzed using a linear mixed model (PROC MIXED, SAS Version 9.4, SAS Institute, Cary, North Carolina, USA). The statistical model was:
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\[ y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \kappa(X_{ijkl}) + \epsilon_{ijkl} \]

where \( y_{ijkl} \) = treatment optical density (570 nm) as an indicator of cell viability; \( \mu \) = the grand population mean; \( \alpha_i \) = immune response phenotype (low, average, or high); \( \beta_j \) = sample type (colostrum or milk); \( \gamma_k \) = duration of exosome co-culture (24, 48, or 72 h); \( \delta_l \) = exosome treatment concentration (\( \mu g/\mu L \)); \( \kappa(X_{ijkl}) \) = relationship between treatment optical density and control optical density (covariate); \( \epsilon_{ijkl} \) = residual error. Holstein cows were classified by immune response phenotype as described previously (Thompson-Crispi et al., 2012) and included in the statistical model as it may explain some of the variability in cell viability, albeit determining the effect of immune response phenotype was not the focus of this experiment. Normality was tested using Shapiro-Wilk test statistic and did not require a log transformation. Interactions were tested and removed if non-significant in a hierarchal fashion. Values for F-tests are summarized in Table 3.2. Results are presented as least square means +/- their standard errors. Significant differences between least square means was determined by Student’s t test and \( p \leq 0.05 \) were considered significant. Data is presented using GraphPad Prism version 7.0 for Mac OS X.

Data from the caspase 3 assay were analyzed using a linear mixed model (PROC MIXED, SAS Version 9.4, SAS Institute, Cary, North Carolina, USA). The statistical model was:

\[ y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \epsilon_{ij} \]

where \( y_{ij} \) = treatment optical density (405 nm) as an indicator of caspase 3 activity; \( \mu \) = the grand mean; \( \alpha_i \) = immune response phenotype (low, average, or high); \( \beta_j \) = sample type (colostrum or milk); \( \epsilon_{ij} \) = residual error. Immune response phenotypes (LL, AA, or HH)
associated with each cow were included in the statistical model as it may explain some of the variability in cell caspase 3 activity, although determining the effect of immune response phenotype was not the focus of this experiment. Values for F-tests are summarized in Table 3.3. Normality was tested using Shapiro-Wilk test statistic and data was log transformed. Results are presented as medians with 95% confidence intervals. Significant differences between immune response groups were determined by Student’s t test. Differences $p \leq 0.05$ were considered significant. Data is presented using GraphPad Prism version 7.0 for Mac OS X.

3.4 Results

3.4.1 Isolation and characterization of bovine colostrum exosomes

Exosomes isolated from the colostrum of 9 cows were shown to express Hsp70, CD9, and CD63, but not CD81 (Figure 3.1). CD63 was also detected in all colostrum exosome isolates by ELISA (Figure 3.2). Western blot images depicting the entire blot for each antibody tested, along with the corresponding Ponceau staining images to show equal loading of protein per well are included as supplementary data (Appendix, Figure 3.2 A-H). Bovine colostrum exosomes used for this study were previously identified by another member of our group, Dr. H. Atalla, using electron microscopy and immunogold labeling for CD63 (Appendix, Figure 3.1 A-C).

3.4.2 Uptake of bovine colostrum exosomes

Fluorescence microscopy demonstrated the uptake of labeled bovine colostrum exosomes by Caco-2 cells after co-culture for 2 and 24 h at 37°C (Figure 3.3, E-F), and after co-culture with sonicated or proteinase K treated exosomes (Appendix, Figure 3.3 A-H). Cells cultured with PKH67-PBS control for 2 and 24 h at 37°C showed an observable decrease in green fluorescence (Figure 3.3 C-D). Similarly, co-culture at 4°C with labeled exosomes for 2 and 24 h resulted in decreased green fluorescence (Figure 3.3, G-H).
3.4.3 Cell viability after co-culture with bovine colostrum exosomes

The least square means were calculated for the different experimental time points, using a set control optical density as a covariate. The resulting least square means show the effect of bovine colostrum exosomes on Caco-2 cell viability over time (Figure 3.4). Compared to 24 h co-culture with colostrum exosomes (0.21 ± 0.02), Caco-2 cell viability was significantly greater after co-culture with colostrum exosomes for 48 and 72 h (0.29 ± 0.02 and 0.31 ± 0.02, respectively, p <0.0001). While a general increase in cell viability was observed over time, there was no significant difference in viability after co-culture with colostrum exosomes for 48 and 72 h (p = 0.37). Trypan blue cell counts also showed an increase in cell number over time after co-culture with colostrum exosomes and that co-culture with colostrum exosomes did not result in the Caco-2 cells reaching their proposed population doubling time as stated in the product manual for the cells (Figure 3.5).

3.4.4 Caspase 3 activity after co-culture with bovine colostrum exosomes

Caspase 3 activity was significantly higher in cells co-cultured with colostrum exosomes (0.0153, 95% CI: 0.0117, 0.0199; p = 0.0006) compared to the control (0.0049, 95% CI: 0.0028, 0.0086) (Figure 3.6). Optical densities (OD) corresponding to p-nitroaniline (pNA) standards provided with the caspase 3 assay kit (highest OD_{pNA standard curve} = 0.262 corresponds to 200 μM pNA in solution) demonstrate caspase 3 activity to be relatively low in Caco-2 cells after colostrum exosome co-culture (OD_{colostrum exosome co-culture} = 0.0153 corresponds to 12.331 μM pNA released by caspase 3 activity in Caco-2 cells) (Appendix, Figure 3.4).

3.5 Discussion

Exosomes play a significant role in intercellular communication by delivering biologically important proteins, mRNA, and miRNA to recipient cells, thereby modifying
signaling and function of the target cell (Admyre et al., 2007; Hata et al., 2010; Kosaka et al., 2010; Weber et al., 2010; Gu et al., 2012; Munagala et al., 2016; Chen et al., 2017; Yang et al., 2017). The focus of this study was to evaluate the bioactivity of bovine colostrum exosomes on human intestinal epithelial cells. In the present study, exosomes were characterized by Western blot analysis for commonly expressed exosomal proteins. Previous reports have demonstrated exosomes isolated from colostrum and milk of various species express Hsp70, CD9, CD63, and CD81 as detected by Western blot (Lässer et al., 2011; Reinhardt et al., 2012; Yamada et al., 2012; Zonneveld et al., 2014; Wolf et al., 2015; Yassin et al., 2015). In agreement with these reports, Hsp70, CD9, and CD63 were detected by Western blot, as well as CD63 by ELISA, in colostrum exosome isolates in this study. The detection of CD63 by Western blot and ELISA supports an earlier observation whereby CD63 was detected by immunogold labeling (Atalla et al., 2016). The presence of CD81 was also tested, however was not detected in colostrum exosomes, which could be due to the lack of specificity between bovine CD81 in our samples and the available CD81 antibody (human specific) used for analysis. To further confirm purity of our colostrum exosomes isolates, future work will aim to detect whether isolates contain non-exosomal proteins, such as calnexin, cytochrome c, or histone 3 (Lässer et al., 2011; Lötvall et al., 2014; Wolf et al., 2015).

Recent studies showed that exosomes can be taken up by and deliver biologically functional cargo to a variety of cells (Lässer et al., 2011; Izumi et al., 2015; Pieters et al., 2015; Wolf et al., 2015; Munagala et al., 2016; Chen et al., 2017). In agreement with these reports, this study showed that bovine colostrum exosomes are taken up by human intestinal epithelial cells, as demonstrated by fluorescence microscopy. These findings suggest the potential importance of bovine colostrum exosomes containing miRNA in vivo. Previous studies reported that cells
cultured at 4°C have significantly reduced ability to take up exosomes, suggesting that uptake is not passive, but rather an energy-dependent process (Lässer et al., 2011, Franzen et al., 2014; Izumi et al., 2015; Pieters et al., 2015; Wolf et al., 2015). In agreement with these results, Caco-2 cells cultured with labeled bovine colostrum exosomes at 4°C showed an observable decrease in green fluorescence, suggesting that uptake was greatly inhibited.

Mechanisms of exosome uptake typically involve protein interactions and intact vesicle membranes (Danzer et al., 2012; Mulcahy et al., 2014). While several reports have demonstrated proteinase K treatment or sonication significantly reduced exosome uptake (Escrevente et al., 2011; Salomon et al., 2014; Shelke et al., 2014; Smyth et al., 2014; Rice et al., 2015; Wolf et al., 2015; Lundberg et al., 2016), results from this study demonstrated otherwise. Uptake after sonication of bovine colostrum exosomes showed similar observable green fluorescence as co-culture with labeled exosomes without sonication. A proposed reason for this is that sonication may have disaggregated exosomes, better facilitating their uptake.

The ability of milk exosomes to alter cellular function (Näslund et al., 2014; Pieters et al., 2015; Munagala et al., 2016) or specifically, to enhance intestinal epithelial cell viability (Chen et al., 2016; Hock et al., 2017) has been previously demonstrated. In agreement with these findings, the current study showed that exposure to bovine colostrum exosomes resulted in significantly increased Caco-2 cell metabolic activity and cell number over time, as demonstrated by analysis using MTT assay and cell counting using trypan blue. Further, cell counting data demonstrate that co-culture with colostrum exosomes did not cause Caco-2 cells to reach their proposed population doubling time according to the Caco-2 cell product manual from ATCC. Together, these results suggest bovine colostrum exosomes do not stimulate oncogenic proliferation of Caco-2 cells, while at the same time, they are also not cytotoxic to these cells.
These results could be attributed to the expression of specific miRNA (miR-21, -26a, -29b, 148a, and -155) in colostrum exosomes previously identified by Atalla et al. (2016, manuscript in preparation), which have been shown by others to stimulate cell proliferation and death pathways after transfection to various colorectal cancer cell lines (Zhang et al., 2011; Konishi et al., 2015; Qu et al., 2015; Yuan et al., 2015; Nedaeinia et al., 2016).

The present study also tested whether bovine colostrum and milk exosomes induce apoptosis of Caco-2 cells by measuring caspase 3 in cell lysate after exosome co-culture. While previous reports have assessed caspase activity after co-culture with exosomes isolated from cell culture supernatant (Yang et al., 2013; Hosseini et al., 2014; Ahmed et al., 2015; Wang et al., 2015; Ahsan et al., 2016; Rivoltini et al., 2016), to the best of our knowledge, the present study is the first to evaluate the ability of bovine colostrum exosomes to induce caspase 3 activity in cells. Results from this study demonstrate caspase 3 activity was significantly greater after co-culture with colostrum exosomes compared to the control. While these results, along with reports by Atalla et al. (2016, manuscript in preparation) suggest bovine colostrum exosomes contain molecules, including specific miRNA, that activate the caspase 3 pathway and induce apoptosis in cancerous human intestinal cells, it should be noted that little caspase 3 activity was detected in Caco-2 cells based on optical densities corresponding to p-nitroaniline standards provided with the caspase 3 assay kit. This observation could be attributed to a number of reasons including (1) the presence of Hsp70 in colostrum exosomes, which has been shown to inhibit the caspase 3 pathway in cells (Li et al., 2000; Takayama et al., 2003), (2) the potential release of caspase 3 enriched vesicles from cells, thereby preventing cellular apoptosis (Böing et al., 2013), and (3) overexpression of oncogenic proteins and miRNA in Caco-2 cells (Ruemmele et al.,)
2003; Li et al., 2008; Kern et al., 2012) making it difficult to induce apoptosis of these cells after co-culture with colostrum exosomes containing a variety of cargo.

In summary, this study characterized exosomes in bovine colostrum and demonstrated their ability to be taken up by human intestinal epithelial cells, maintain their metabolic activity, while at the same time not being cytotoxic to these cells. To our knowledge, this is the first study to identify bovine colostrum exosomes by Western blot analysis for the expression of CD9, CD63, and Hsp70. The present study is also the first to report the bioactivity of bovine colostrum exosomes on human intestinal epithelial cells. While efforts were made to maintain the cancerous phenotype of Caco-2 cells by passaging the cells before reaching confluence and limiting the number of passages, future work should evaluate whether Caco-2 cells maintained their cancerous phenotype, given their propensity to differentiate into enterocyte-like cells (Lea et al., 2015). In doing so, the functional role of bovine colostrum exosomes on cancerous human intestinal epithelial cells can be better established. Nonetheless, this study provides important findings regarding the potential bioavailability of bovine colostrum exosomes to the human gut and highlights their potential implications on human intestinal health.

3.6 Acknowledgments

This research was funded by grants to B.A. Mallard by the Ontario Ministry of Agriculture, Food and Rural Affairs, Dairy Farmers of Ontario, and Natural Sciences and Engineering Research Council of Canada. This paper is also a contribution to the Food from Thought research program supported by the Canada First Research Excellence Fund. The authors thank Laura Wright and staff at the Elora Research Station for collecting samples used in this study and members of the Mallard lab for delivering samples, and technical assistance with experiments. The authors also thank William Sears for his assistance with the statistical analyses.
3.7 References


3.8 Figures and tables

**Figure 3.1:** Western blot analysis of bovine colostrum exosome isolates for commonly expressed exosomal proteins. 10 μg of exosomal protein was loaded per well. Hsp70, CD63, and CD9 were detected in colostrum exosome isolates.

![Western blot analysis](image)
Figure 3.2: Enzyme-linked immunosorbent assay (ELISA) demonstrating the detection of CD63 in bovine colostrum exosomes, but not in bovine milk exosomes. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates per sample type, \(*p < 0.05\) was considered significant.
**Figure 3.3 (A-H):** Uptake of bovine colostrum exosomes by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μL of PKH67 labeled colostrum exosomes (E-H), PKH67-PBS control (C-D), or no PKH67 dye (A-B) were incubated with Caco-2 cells at 37°C or 4°C for 2 or 24 h. The uptake of fluorescently labeled bovine colostrum exosomes was detected by fluorescence microscopy after co-culture for 2 and 24 h (E and F, respectively). Uptake decreased at 4°C (G and H, respectively). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 3 colostrum samples. Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
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Exosomes 37°C

Exosomes 4°C

2 hours

24 hours

E

F

G

H
**Figure 3.4:** Human cancer intestinal epithelial (Caco-2) cell viability after treatment with 0.001-0.625 μg/μL bovine colostrum exosomes for 24, 48, and 72 h. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates, *p < 0.05 was considered significant.
Figure 3.5: Human cancer intestinal epithelial (Caco-2) cell count after co-culture with bovine colostrum exosomes. Cells were counted 0, 42, 66, and 90 h post-seeding. At 42, 66, and 90 h post-seeding, colostrum exosomes had been co-cultured with Caco-2 cells for 24, 48, and 72 h, respectively. Data are presented as mean cell number ± SEM using GraphPad Prism 7.0, n = 3 biological replicates for Caco-2 cells co-cultured with colostrum exosomes. Dashed line represents the proposed population doubling time of Caco-2 cells according to the ATCC product manual.
**Figure 3.6:** Caspase-3 activity in human cancer intestinal epithelial (Caco-2) cells after co-culture with 0.125 μg/μL of bovine colostrum exosomes for 48 h. Data are presented at medians with 95% confidence intervals using GraphPad Prism 7.0, n = 9 biological replicates, *p < 0.05 was considered significant.
Table 3.1: Information on primary and secondary antibodies used for Western blot analysis of bovine colostrum exosomes; CD = cluster differentiation, TAPA1 = target of the antiproliferative antibody 1, Hsp70 = heat shock protein 70; IgG-HRP = immunoglobulin G-horseradish peroxidase.

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<td></td>
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<td>Abcam</td>
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Table 3.2: Analysis of covariance table for fixed effects summarizing the variables and their interactions included in the mixed effects model for analyzing the MTT data. Output obtained using SAS software Version 9.4, SAS Institute, Cary, North Carolina, USA; DF = degrees of freedom, F = F value, Pr = probability.

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**Table 3.3:** Analysis of variance table for fixed effects summarizing the variables and their interactions included in the mixed effects model for analyzing the caspase 3 data. Output obtained using SAS software Version 9.4, SAS Institute, Cary, North Carolina, USA; DF = degrees of freedom, F = F value, Pr = probability.

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CHAPTER 4

The Functional Role of Milk Exosomes from High, Average, and Low Immune responder Cows on Human Intestinal Epithelial Cells Compared to Colostrum Exosomes

Key words: High immune responders, milk and colostrum exosomes, microRNA, Caco-2 cells, uptake assay, MTT assay, caspase 3, cell viability, apoptosis.
4.1 Abstract

Bovine milk contains bioactive components that are nutritionally and immunologically important to calves and humans. Dairy cows classified as high (H) immune responders using the patented High Immune Response™ (HIR) technology have higher concentrations of immunoglobulin and beta-lactoglobulin compared to average (A) and low (L) responders. MicroRNA (miRNA) post-transcriptionally regulates expression of milk bioactive components and are enriched in extracellular vesicles known as exosomes. Recently, the bioactivity of bovine colostrum exosomes containing miRNA on human colorectal adenocarcinoma epithelial (Caco-2) cells was assessed. This study further explored the bioactivity of bovine milk exosomes compared to colostrum exosomes from H, A and L responders. Exosomes were isolated by differential ultracentrifugation and confirmed by Western blot analysis for the presence of common exosomal-proteins (CD9, CD63, CD81, and Hsp70). Fluorescent labeling of exosomes using PKH67 dye confirmed their uptake by Caco-2 cells, demonstrating their potential bioavailability. Here, MTT assays showed bovine colostrum and milk exosomes maintain Caco-2 metabolic activity and are not cytotoxic to these human cells. Specifically, Caco-2 metabolic activity after co-culture with colostrum and milk exosomes from H responder cows was significantly greater than after co-culture with exosomes from L responders (p = 0.0198). Caspase 3 activity, an indicator of apoptosis, was significantly lower after co-culture of Caco-2 cells with milk exosomes compared to colostrum exosomes (p < 0.0001), suggesting that unlike colostrum exosomes, particularly those from L responders, milk exosomes do not activate the caspase 3 pathway in Caco-2 cells. This study helps to better understand the functional importance of colostrum and milk exosomes from dairy cows, and emphasizes there are different
effects that relate to immune response phenotype, in maintaining human intestinal epithelial
cells. These findings suggest potential health benefits of cow’s milk.
4.2 Introduction

Bovine milk contains bioactive components of significant nutritional and immunological value to humans and calves (Haug et al., 2007; Hill and Newburg, 2015; Korst et al., 2017). Although produced in lower quantities than colostrum, milk still serves as a rich source of immune and developmental factors, and functions primarily in gastrointestinal development and supporting long-term health and production of calves (Khan et al., 2011; Eckert et al., 2015; Meale et al., 2017). Health benefits of bovine milk to humans and calves are proposed to be mediated by the presence of various bioactive components (Haug et al., 2007; Mills et al., 2011; Korst et al., 2017), which are found at varying concentrations in milk from dairy cows classified using the patented High Immune Response (HIR™) technology. The HIR™ technology identifies dairy cattle as high (H), average (A), or low (L) immune responders using estimated breeding values for cell- and antibody-mediated immune responses. Studies have shown HIR™ cows have more balanced and robust immune responses, less disease occurrence (Thompson-Crispi et al., 2014), and improved colostrum and milk quality compared to A and L responders (Mallard et al., 2015). Specifically, HIR™ cows have significantly greater concentrations of bioactive components in their milk including immunoglobulin and beta-lactoglobulin (Fleming, 2014).

Expression of milk bioactive components is post-transcriptionally regulated by small, non-coding RNA molecules (18-23 nucleotides in length) known as microRNA (miRNA) (Ogorevc et al., 2009; Liao et al., 2010; Jabled et al., 2012; Li et al., 2012; Wang et al., 2016). MiRNA have been identified in a variety of biological fluids and are involved in critical biological processes, such as those controlling cell differentiation, proliferation, and apoptosis (Bartel, 2004), indicating their functional importance.
Bovine colostrum and milk miRNA are particularly enriched in colostrum- and milk-derived exosomes (20-100 nm in size) (Hata et al., 2010; Izumi et al., 2012; Sun et al., 2013; Izumi et al., 2015; Atalla et al., 2016, manuscript in preparation), which are extracellular vesicles originating via the endocytic pathway in a variety of cells (Théry et al., 2009). Exosomes have an important role in intercellular communication by delivering their cargo to recipient cells, thereby modifying the target cell’s signaling and function (Admyre et al., 2007; Hata et al., 2010; Lässer et al., 2011). Reports have shown that colostrum and milk miRNA are stable under degradative conditions such as RNAse digestion, low pH, high temperature, and freeze/thaw cycles (Izumi et al., 2012; Pieters et al., 2015). Their stability under these conditions can be attributed to their packaging within exosomes. Further, reports have demonstrated the beneficial biological effects of milk exosomes and their miRNA on various human cell lines including monocytic leukemia cells, monocyte-derived dendritic cells, colon, lung, and breast cancer cells (Näslund et al., 2014; Izumi et al., 2015; Wolf et al., 2015; Munagala et al., 2016).

While the bioactivity of bovine milk exosomes on human cells has been demonstrated, the role of bovine colostrum and milk-derived exosomes from different IR phenotypes in regulating human intestinal cell function is undetermined. Based on previous knowledge of the biological effects of bovine colostrum and milk exosomes containing miRNA, along with established differences in H responder colostrum and milk quality, it was hypothesized that colostrum and milk exosomes containing miRNA isolated from HIR™ cows are functionally active at the gut mucosal interface, and promote the maintenance of the intestinal epithelial barrier compared to exosomes from A and L responder’s colostrum and milk. The specific objectives of this study were to: (1) characterize and confirm the presence of exosomes in colostrum and milk from H, A, and L responders, (2) evaluate the uptake of exogenous exosomes
from H, A, and L responder colostrum and milk by human cancer intestinal epithelial cells, (3) determine whether exosomes from H responder colostrum and milk promote intestinal cell viability compared to those from A and L responders, and (4) evaluate whether exosomes from H responders are apoptotic to human cancer intestinal epithelial cells. This study will help to better understand the functional role of colostrum and milk exosomes from dairy cows with different immune response phenotypes in regulating human intestinal epithelial cells.

4.3 Materials and methods

4.3.1 Samples

Colostrum and milk samples (250 mL each) were collected (on the day of calving and five-six days post-calving, respectively) from healthy Holstein cows previously classified using estimated breeding values (EBVs) as H, A, or L responders from Elora Dairy (Table 4.1). Briefly, EBVs for both antibody- (AMIR) and cell-mediated immune responses (CMIR) allowed identification of cows as HH, AA, or LL for both traits (Thompson-Crispi et al., 2012). For this study, HH cows were selected for EBVs around +1.0 and LL cows were selected for EBVs around -1.0. AA cows were selected for EBVs as close to zero as possible, while attempting to say within the range of +0.5 and -0.5. Samples were stored at -80°C until the time of analysis. Three samples of both colostrum and milk for each immune-response phenotype were used for subsequent experiments, for a total of 9 colostrum and 9 milk samples.

4.3.2 Isolation of colostrum and milk exosomes

Colostrum and milk exosomes containing miRNA were isolated through differential ultracentrifugation, using a protocol by Atalla et al. (2016, manuscript in preparation). Colostrum and milk samples were first centrifuged at 5,000 xg for 30 minutes at 4°C to remove fat and cell debris. The supernatant was collected and diluted 2:1 in phosphate buffered saline (PBS) and
centrifuged again at 12,000, 35,000, and 70,000 xg for 1 hour (h) at 4°C to further remove cell
debris and larger vesicles such as apoptotic bodies and microvesicles. Exosomes were pelleted
by centrifuging at 100,000 xg for 1 h at 4°C. Resultant pellets were resuspended in
approximately 38 mL PBS and centrifuged again at 100,000 xg for 1 h at 4°C to wash the pellet.
After washing, pellets were weighed and resuspended in a volume of PBS equivalent to their
weights and total exosomal protein was quantified using Pierce BCA protein assay
(ThermoFisher Scientific). Isolated exosomes were then stored in 100 μL aliquots at -80°C until
use in future experiments (Yamada et al., 2012; Momen-Heravi et al., 2013).

4.3.3 Enzyme linked immunosorbent assay of colostrum and milk exosomes

CD63 ExoELISA kit (System Biosciences) was used for detection of CD63 in colostrum
and milk exosomes from H, A, and L responders (n=3 per immune response phenotype), and
were tested in duplicate. Exosomes were resuspended in 200 μL exosome binding buffer,
followed by adding 50 μL of each exosome sample to wells of a microtiter plate. The plate was
incubated overnight at 37°C. After washing, 50 μL of anti-CD63 (1:100) was added to the wells
and incubated for 1 h at 37°C. The plate was washed again and then incubated with 50 μL
horseradish peroxidase enzyme-linked secondary antibody (goat anti-rabbit, 1:5000) for 1 h at
room temperature. After washing, 50 μL colorimetric substrate was added to each of the wells
for 45 minutes followed by the addition of stop buffer. Absorbance was read at 450 nm using a
microplate spectrophotometer (BioTek PowerWave XS2).

4.3.4 Western blotting of colostrum and milk exosomes

Colostrum and milk exosomes were characterized by Western blot analysis for
commonly expressed exosomal proteins (CD9, CD63, CD81, and heat shock protein 70; Hsp70).
Exosomes were first lysed in Triton-X-100 cell lysis buffer containing Roche mini complete
protease inhibitor for 5 minutes on a shaker. To further lyse the exosomes, samples were sonicated in a water bath at room temperature for 3 x 5 minutes with brief vortexing in between. Samples were then centrifuged for 5 minutes at 13,000 xg at room temperature. Exosome lysate was transferred to clean microfuge tubes and stored on ice while total protein in the lysate was quantified using Pierce BCA protein assay (ThermoFisher Scientific). Laemmli buffer was added to samples at a 1:1 ratio and heated to 100°C for 10 minutes. Samples (10-20 μg of protein per well) were loaded onto a 12% acrylamide gel and proteins were separated by electrophoresis, followed by transfer to 0.22 μm polyvinylidene difluoride (PVDF) membranes. To ensure equal loading of protein per well, PVDF membranes were stained with Ponceau red for 5 minutes, gently rinsed with distilled water for 30 seconds and imaged using ChemiDoc MP System (BioRad). Membranes were then washed in tris-buffered saline + tween (TBS-T) for 15 minutes to remove the Ponceau stain. Membranes were blocked with 0.2% fish skin gelatin in tris buffered saline (TBS) for 1 h at room temperature, followed by washing with TBS-T. Exosomal proteins were detected using either mouse anti-cow CD9 (ab3923, Abcam), mouse anti-human CD63 (ab193349, Abcam), mouse anti-human TAPA1 (ab79559, Abcam), mouse anti-human Hsp70 (ab2787, Abcam) diluted 1:1000 in 0.2% fish skin gelatin in TBS-T. Membranes were incubated with primary antibodies for approximately 18 h at 4°C. After extensive washing with TBS-T, membranes were incubated with donkey anti-mouse IgG-HRP (ab6820, Abcam) secondary antibody diluted 1:10,000 in 0.2% fish skin gelatin in TBS-T for 1 h at room temperature. Concentration of antibody as reported by the manufacturer are included in Table 4.2. Detected proteins were imaged using the ChemiDoc MP system (Bio-Rad) (Lässer et al., 2011; Zonneveld et al., 2014). Exosomes isolated from colostrum and milk samples were first
identified by Dr. H. Atalla using transmission electron microscopy and immunogold labeling for CD63 (Atalla et al., 2016, manuscript in preparation).

4.3.5 Caco-2 cell culture

Human colorectal adenocarcinoma epithelial (Caco-2, HTB-37) cells were purchased from American Type Culture Collection (ATCC, Cedarlane, Burlington, ON), and maintained in Eagle’s Essential Medium (EMEM; ATCC) at 37°C, 5% CO₂ supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Media was changed every 2-3 days and cells were passaged once they reached approximately 70% confluence (4-5 days), and seeded at a density of approximately 1.0 x 10⁶ cells per T-75 cm² flask (Lea, 2015).

Prior to assessing colostrum and milk exosome bioactivity on Caco-2 cells, media was replaced with EMEM containing 10% exosome-depleted FBS and the previously mentioned antibiotics. FBS was depleted of exosomes by centrifuging at 100,000 xg for 18 hours at 4°C as previously reported (Chiba et al., 2012; Shelke et al., 2014). Caco-2 cells from passages 4-30 were used for bioactivity assays.

4.3.6 Exosome uptake assay

Uptake of colostrum or milk exosomes by Caco-2 cells was assessed by labeling exosomes with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich). Immediately prior to labeling, 4 μL of PKH67 dye was added to 1 mL Diluent C to prepare the dye reagent (Lässer et al., 2011). A 100 μL aliquot of colostrum exosomes was added to the PKH67 dye reagent. As a control, the same volume of PBS was mixed with 1 mL Diluent C and dye. The samples were gently mixed for 4 minutes on a shaker, followed by the addition of 2 mL 1% exosome depleted FBS (diluted in PBS) to bind excess dye for 1 minute (Khatua et al., 2009; Lässer et al., 2011). The samples were then transferred to Beckman Ultra-
Clear 13 x 51 mm ultracentrifuge tubes (Beckman Coulter) and filled with exosome-depleted EMEM. Using the Beckman Coulter Swinging Bucket rotor (SW 55 Ti, 6 x 5 ml), the samples were ultracentrifuged at 100,000 xg at 4°C for 1 h. After ultracentrifugation, the supernatant was discarded and the pellet was resuspended in exosome-depleted EMEM and transferred to clean ultracentrifuge tubes. The tubes were filled with exosome-depleted EMEM and ultracentrifuged once again at 100,000 xg at 4°C for 1 h to remove unbound dye. After ultracentrifugation, the supernatant was discarded and the pellet was resuspended in the appropriate volume of exosome-depleted EMEM to give a concentration of 0.16 μg/μL of labeled exosomes.

Prior to labeling, Caco-2 cells were seeded at a density of 60,000 cells/well in 24 well plates using exosome-depleted EMEM. Cells were allowed to adhere for ~18 h, at which point they reached the desired confluence (~70% confluence). Once exosomes were labeled and prepared for co-culture, media was removed from each of the wells and cells were washed with Dulbecco’s phosphate buffered saline (DPBS; Gibco). Labeled exosomes (0.16 μg/μL) or the same volume of PKH67-PBS control was added to the appropriate wells and incubated for 2 or 24 h at either 37°C or 4°C. Colostrum or milk exosomes from 3 cows (1 per immune response phenotype) were tested in triplicate. After co-culture, cells were harvested using 0.25% trypsin-0.53 M EDTA solution (Gibco) to remove surface bound exosomes (Franzen et al., 2014). Trypsinized cells were fixed in 4% formaldehyde for 10 minutes (Lässer et al., 2011; Izumi et al., 2015), followed by fixing cells to microscope slides (25,000 cells/slide) using the Shandon Cytospin 2 Centrifuge. Once adhered to slides, cells were washed twice with DPBS and 100 μL 0.1 M ammonium chloride solution was added to the cells for 10 minutes to quench the formaldehyde reaction (Davis et al., 2014). Cells were gently washed again with DPBS. To label the nuclei of cells, 5 μL of 7-AAD dye (Molecular Probes) was diluted in 200 μL DPBS and
added to the cells for 15 minutes. Cells were then rinsed with DPBS and mounted using Vectashield (Vector Laboratories) (Izumi et al., 2015). Cellular uptake of exosomes was then visualized by fluorescence microscopy. Images were captured using the Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excellitas Technologies). Images were analyzed using AxioVision software from Carl Zeiss (release 4.8.2).

4.3.7 MTT cell viability and proliferation assay

Caco-2 cells were seeded at a density of 6.0 x 10^4 cells/well in collagen coated 24 well plates using EMEM containing 10% exosome-depleted FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (exosome-depleted media). After allowing cells to adhere for ~18 hours, colostrum or milk exosomes from 9 cows (3 cows per immune response phenotype) were co-cultured with Caco-2 cells at various exosomal protein concentrations (0, 0.001, 0.005, 0.025, 0.125, 0.625 μg/μL) for 24, 48, and 72 hours (Ahmed et al., 2015; Wolf et al., 2015; Munagala et al., 2016) in exosome depleted media. After each of the respective co-culture time points, media was replaced with Minimum Essential Medium containing no phenol red (Gibco) and 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution for 4 h, followed by solubilization of formazan crystals with 0.01 M SDS-HCl for another 4 h. Absorbance was read at 570 nm using a microplate spectrophotometer (BioTek PowerWave XS2). Each sample and control (no exosomes) was tested in triplicate.

4.3.8 Caspase-3 in vitro apoptosis assay

Caspase 3 activity in Caco-2 cells after co-culture with 0 and 0.125 μg/μL colostrum or milk exosomal protein for 48 hours was assessed using a colorimetric caspase 3 assay kit (Sigma). Caco-2 cells were seeded at a density of 7.5 x 10^5 cells/flask in collagen coated T-25 cm² flasks using exosome depleted media and allowed to adhere for ~18 hours. Cells were co-
cultured with 0.125 μg/μL exosomal protein for 48 h in exosome depleted media (Hosseini et al., 2014; Ahmed et al., 2015; Wolf et al., 2015; Munagala et al., 2016). According to the manufacturers protocol and modified methods from Hosseini et al. (2014), 3.0 x 10^6 cells were lysed in lysis buffer for 15 minutes on ice. Samples were then centrifuged for 10 minutes at 18,000 xg at 4°C. Cell lysates were collected and transferred to new microfuge tubes and stored at -80°C until time of analysis. Using 96 well plates, 5 μL cell lysate and 10 μL caspase 3 substrate was added to the appropriate wells to measure caspase 3 activity in the cells after exosome co-culture. Each sample (n=9) and control (no exosomes) was tested in triplicate. After 18 h of incubation at 37°C, absorbance was read at 405 nm using a microplate spectrophotometer (BioTek PowerWave XS2).

**4.3.9 Statistical analysis**

Data from the MTT assay were analyzed using a linear mixed model (PROC MIXED, SAS Version 9.4, SAS Institute, Cary, North Carolina, USA). The statistical model was:

\[
y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \kappa(X_{ijkl}) + \epsilon_{ijkl}
\]

where \(y_{ijkl}\) = treatment optical density (570 nm) as an indicator of cell viability; \(\mu\) = the grand population mean; \(\alpha_i\) = immune response phenotype (LL, AA, or HH); \(\beta_j\) = sample type (colostrum or milk); \(\gamma_k\) = duration of exosome co-culture (24, 48, or 72 hours); \(\delta_l\) = exosome treatment concentration (μg/μL); \(\kappa(X_{ijkl})\) = relationship between treatment optical density and control optical density (covariate); \(\epsilon_{ijkl}\) = residual error. Normality was tested using Shapiro-Wilk test statistic and did not require a log transformation. Interactions were tested and removed if non-significant in a hierarchal fashion. Values for F-tests are summarized in Table 4.3. Results
are presented as least square means +/- standard errors. Significant differences between least square means was determined by Student’s t test and p≤0.05 were considered significant. Data is presented using GraphPad Prism version 7.0 for Mac OS X.

Data from the caspase 3 assay were analyzed using a linear mixed model (PROC MIXED, SAS Version 9.4, SAS Institute, Cary, North Carolina, USA). The statistical model was:

\[ y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ij} \]

where \( y_{ij} \) = treatment optical density (405 nm) as an indicator of caspase 3 activity; \( \mu \) = the grand population mean; \( \alpha_i \) = immune response phenotype (LL, AA, or HH); \( \beta_j \) = sample type (colostrum or milk); \( \varepsilon_{ij} \) = residual error. Values for F-tests are summarized in Table 4.4. Normality was tested using Shapiro-Wilk test statistic and data was log transformed. Results are presented as medians with 95% confidence intervals. Significant differences between immune response groups were determined by Student’s t test. Differences \( p \leq 0.05 \) were considered significant. Data is presented using GraphPad Prism version 7.0 for Mac OS X.

4.4 Results

4.4.1 Characterization of bovine milk exosomes

Exosomes isolated from the milk of 9 cows were characterized by Western blotting for the commonly expressed exosomal proteins Hsp70, CD9, CD63, and CD81. Similar to colostrum, exosomes isolates from milk were shown to express Hsp70 and CD9, but not CD81. However, unlike colostrum exosomes, CD63 was not detected in milk by Western blot (Figure 4.1), or by ELISA. Further, CD63 expression in colostrum exosomes (OD = 0.842) was significantly higher than expression in milk exosomes, as detected by ELISA (OD = 0.0334, \( p <0.0001 \)) (Figure 4.2). CD63 expression was also higher in colostrum exosomes from H (OD =
0.877) and A responders (OD = 1.337) compared to L responders (OD = 0.312), with CD63 expression being significantly higher in A responder colostrum exosomes compared to L (p = 0.0019) (Figure 4.3, A). With regards to milk exosomes, there were no significant differences between IR phenotypes (Figure 4.3, B). Images of Ponceau staining to confirm equal loading of protein per well, along with images depicting the entire blot for each antibody tested are included as supplementary data (Appendix, Figure 3.2 A-H). Bovine milk exosomes used for this study were previously identified by Dr. Atalla using electron microscopy (Appendix, Figure 3.1 A-C).

4.4.2 Uptake of bovine colostrum and milk exosomes

Fluorescence microscopy demonstrated the uptake of labeled bovine milk exosomes from each IR phenotype by Caco-2 cells after co-culture for 24 h at 37°C (Figure 4.5 C, E, and G), and after co-culture with sonicated milk exosomes (Appendix, Figure 4.3 A-B). Colostrum exosomes from each IR phenotype were also taken up by Caco-2 cells under the same conditions (Figure 4.4 C, E, and G and Appendix, Figure 3.3 E-F). Cells cultured with PKH67-PBS control for 24 h at 37°C showed an observable decrease in green fluorescence (Figure 4.3 B). Similarly, co-culture at 4°C with labeled colostrum or milk exosomes for 24 h resulted in decreased green fluorescence (Figure 4.4 D, F, H and Figure 4.5 D, F, H, respectively).

4.4.3 Cell viability after co-culture with bovine colostrum or milk exosomes

The least square means have been calculated for the different experimental time points, using a set control optical density as a covariate (OD = 0.31). The resulting least square means show the effect of bovine milk exosomes on Caco-2 cell viability over time (Appendix, Figure 4.4). Caco-2 cell viability was significantly greater after co-culture with milk exosomes for 72 h (0.38 ± 0.02), compared to 24 and 48 h co-culture (0.29 ± 0.02, p < 0.0001 and 0.31 ± 0.02, p = 0.0016, respectively), however there was no significant difference in viability between 24 and 48
h (p = 0.2573) (Appendix, Figure 4.4). Trypan blue cell counts also showed an increase in cell number over time after co-culture with milk exosomes, in addition to demonstrating that cells co-cultured with milk exosomes did not reach their proposed population doubling time (Figure 4.6). Compared to culture with colostrum exosomes, Caco-2 cell viability was significantly higher after culture with milk exosomes (0.27 ± 0.02 and 0.32 ± 0.02, respectively; p < 0.0001) (Appendix, Figure 4.5), and remained significantly higher after 24 (0.21 ± 0.02 and 0.29 ± 0.02, respectively; p < 0.0001) and 72 h co-culture (0.31± 0.02 and 0.38 ± 0.02, respectively; p < 0.0001) (Figure 4.7).

There was also an overall main effect of bovine colostrum and milk exosome protein treatment concentration and co-culture time. Cell viability after co-culture with 0.025 (0.33 ± 0.02), 0.125 (0.32 ± 0.02), 0.625 μg/μL exosomal protein (0.30 ± 0.02) was significantly greater than co-culture with 0.001 (0.27 ± 0.02; p <0.0001, p = 0.0001, and p = 0.0074, respectively) and 0.005 μg/μL exosomal protein (0.27 ± 0.02; p <0.0001, p = 0.0002, and p = 0.0151, respectively). Co-culture with 0.025 and 0.125 μg/μL exosomal protein resulted in significantly greater cell viability compared co-culture with 0.625 μg/μL exosomal protein (p = 0.041) (Appendix, Figure 4.6). A significant increase in cell viability occurred over time, whereby co-culture for 48 (0.30 ± 0.02) and 72 h (0.35 ± 0.02) resulted in significantly greater cell viability compared to 24 h (0.25 ± 0.02; p = 0.0116 and p = 0.0007, respectively), and viability after 72 h co-culture was significantly greater than co-culture for 48 h (p = 0.0383) (Appendix, Figure 4.7).

MTT data was also analyzed for differences in cell viability due to co-culture with bovine colostrum and milk exosomes isolated from different IR phenotypes. When comparing the main effect of IR phenotype on Caco-2 cell viability, co-culture with bovine colostrum and milk exosomes from H responders (0.35 ± 0.03) resulted in greater cell viability compared to co-
culture with A (0.31 ± 0.03) and L responder exosomes (0.23 ± 0.03), with a significant difference between L and H responder exosomes (p = 0.0198) (Figure 4.8). Co-culture with milk exosomes from H responders resulted in significantly greater cell viability than co-culture with L responder milk exosomes for 24 (0.36 ± 0.03 and 0.21 ± 0.03; p = 0.001) and 72 h (0.44 ± 0.04 and 0.28 ± 0.04; p = 0.0048), but not 48 h (0.29 ± 0.03 and 0.29 ± 0.03, p = 0.9886). Cell viability after co-culture with A responder milk exosomes (0.43 ± 0.04) was also significantly greater than co-culture with L responder milk exosomes for 72 h (p = 0.0051) (Figure 4.9, B).

With regards to co-culture with colostrum exosomes, co-culture with those from H responders resulted in significantly greater cell viability than after co-culture with L responder colostrum exosomes for 24 (0.26 ± 0.03 and 0.17 ± 0.03; p = 0.0442), 48 (0.38 ± 0.03 and 0.22 ± 0.03; p = 0.0008), and 72 h (0.40 ± 0.04 and 0.24 ± 0.04; p = 0.0024) (Figure 4.9, A).

### 4.4.4 Caspase 3 activity after co-culture with bovine colostrum or milk exosomes

Caspase 3 activity in control cells (0.0049, 95% CI: 0.0028, 0.0086) was not significantly different from caspase 3 activity in cells co-cultured with milk exosomes (0.0031, 95% CI: 0.0024, 0.004; p = 0.1298). Compared to caspase 3 activity in cells cultured with colostrum exosomes (0.0153, 95% CI: 0.012, 0.020), activity after co-culture with milk exosomes was significantly lower (p < 0.0001) (Appendix, Figure 4.10).

Data were also analyzed for differences in caspase 3 activity due to co-culture with bovine colostrum and milk exosomes from different IR phenotypes. Caspase 3 activity after co-culture with milk exosomes from H (0.0036, 95% CI: 0.0024, 0.0056), A (0.0045, 95% CI: 0.0030, 0.0070), or L responders (0.0017, 95% CI: 0.0011, 0.0027) was not significantly different from the control (0.0036, 95% CI: 0.0017, 0.0074; p = 0.0973, p = 5647, and p = 0.9546, respectively) (Figure 4.10, B).
Caspase 3 activity after co-culture with L responder colostrum exosomes (0.018, 95% CI: 0.012, 0.027) was significantly greater than the control (0.0068, 95% CI: 0.0033, 0.014; p = 0.0298), but there were no significant differences between the control and H (0.013, 95% CI: 0.0083, 0.019; p = 0.1505) or A responders (0.016, 95% CI: 0.010, 0.024; p = 0.052), or between the IR phenotypes (Figure 4.10, A). In general, optical densities (OD) corresponding to p-nitroaniline (pNA) standards provided with the caspase 3 assay kit (highest ODpNA standard curve = 0.262 corresponds to 200 μM pNA in solution) demonstrate caspase 3 activity to be relatively low in Caco-2 cells after colostrum exosome co-culture from H (OD = 0.0083 corresponds to 7.385 μM pNA released), A (OD = 0.0104 corresponds to 9 μM pNA released), or L responders (OD = 0.0115 corresponds to 9.846 μM pNA released) (Appendix, Figure 3.4).

4.5 Discussion

Exosomes play a significant role in intercellular communication and immune functions that have important implications on gut health (Admyre et al., 2007; Pieters et al., 2015; Arntz et al., 2015). The focus of this study was to evaluate the bioactivity of bovine milk exosomes in comparison to colostrum exosomes, particularly those isolated from H, A, and L immune responders, on the human intestinal epithelial barrier. Exosomes isolated from H, A, and L responder milk were characterized by Western blot analysis for commonly expressed exosomal proteins. In agreement with previous reports (Reinhardt et al., 2012; Yamada et al., 2012; Benmoussa et al., 2016; Yu et al., 2017), expression of CD9 and Hsp70 was detected by Western blot analysis of colostrum and milk exosome isolates. The presence of CD81 was also tested but not detected in either colostrum or milk exosomes, which could be due to lack of specificity between bovine CD81 in our samples and the available CD81 antibody (human specific) used for analysis. The detection of CD63 in colostrum, but not milk exosomes by Western blot analysis.
and ELISA, supports an earlier observation whereby CD63 was not detected on milk exosomes by immunogold labeling (Atalla et al., 2016), however these results are contrary to previous reports characterizing bovine milk exosomes by CD63 expression (Reinhardt et al., 2012; Izumi et al., 2015; Wolf et al., 2015; Munagala et al., 2016; Yu et al., 2017). Furthermore, ELISA data indicated differential CD63 expression in colostrum exosomes between the different IR phenotypes, whereby CD63 expression was higher in H and A responder colostrum exosomes, compared to L. The purpose of the ELISA kit used in this study is to quantify exosomes by using a set of standards that have been calibrated using NanoSight Nanoparticle Tracking Analysis. Studies have shown this quantification method to be unreliable (Jeppesen et al., 2014; van der Pol et al., 2014; Maas et al., 2015), which is why exosome number was not reported in this study, but instead the expression of CD63 in isolates was reported. Differential expression of CD63 between exosomes from colostrum, milk, and different IR phenotypes could be due to the presence of exosomes with various surface markers other than CD63 and suggests that CD63 expression is not the gold standard for detecting exosomes in biological fluids (Jørgensen et al., 2013; He et al., 2014; Oksvold et al., 2014).

Fluorescent microscopy images show that bovine colostrum and milk exosomes from H, A, and L responders are internalized by Caco-2 cells, suggesting exosomes and their cargo can interact with and potentially modulate the human intestinal epithelium. The results are supported by recent studies demonstrating the uptake of bovine milk exosomes by human macrophages (Izumi et al., 2015), lung cancer cells (Munagala et al., 2016), and Caco-2 cells (Wolf et al., 2015). Similar to the present study, uptake of bovine milk exosomes has previously been assessed by fluorescence microscopy (Izumi et al., 2015; Munagala et al., 2016), as well as other methods measuring changes in fluorescence intensity by flow cytometry, fluorescence
microscopy image analysis, and microplate readers (Izumi et al., 2015; Wolf et al., 2015; Munagala et al., 2016). Also in line with previous reports, this study showed that when Caco-2 cells are incubated at 4°C, their ability to internalize exosomes is inhibited suggesting uptake is an energy-dependent process (Lässer et al., 2011, Franzen et al., 2014; Izumi et al., 2015; Pieters et al., 2015; Wolf et al., 2015). Milk exosomes were also sonicated in an attempt to disrupt vesicle membranes and inhibit uptake by Caco-2 cells (Danzer et al., 2012; Salomon et al., 2014; Rice et al., 2015). Similar to results obtained from sonication of colostrum exosomes (Chapter 3), uptake was not inhibited when milk exosomes received the same treatment. These results suggest sonication may disaggregate colostrum and milk exosomes, potentially facilitating uptake.

Once taken up, exosomes have been shown to modulate the function of recipient cells (Biton et al., 2011; Näslund et al., 2014; Izumi et al., 2015; Wolf et al., 2015; Zou et al., 2015; Zhou et al., 2015; Munagala et al., 2016). In this study, there were no visually observable differences in uptake of bovine colostrum and milk exosomes from H, A, and L responders. However, Caco-2 cell metabolic activity was differentially affected by co-culture with exosomes from different sample types (colostrum and milk), IR phenotypes (H, A, and L), and exosomal protein treatment concentrations. Similar to results reported for colostrum exosomes (Chapter 3), as well as previous studies assessing the effects of milk exosomes on intestinal viability (Chen et al., 2016; Hock et al., 2017; Yu et al., 2017), Caco-2 cell viability and cell number increased over time, as demonstrated by MTT analysis and cell counting using trypan blue. Similar to results obtained after colostrum exosome co-culture, milk exosomes did not cause Caco-2 cells to reach their proposed population doubling time. These results suggesting bovine milk exosomes are not only non-cytotoxic to Caco-2 cells, but also enhance cell viability and without
stimulating oncogenic proliferation when supplemented with exosome-depleted media. In view of a previous observation that extracellular vesicle-depleted fetal bovine and human sera have reduced capacity to support cell growth (Eitan et al., 2015), it is plausible that exogenous supplementation of bovine colostrum and milk exosomes were adequate to support Caco-2 survival and proliferation up to 72 h.

Compared to co-culture with bovine colostrum exosomes, Caco-2 metabolic activity after bovine milk exosome co-culture was significantly greater. Differences in Caco-2 metabolic activity when co-cultured with bovine colostrum or milk exosomes isolated from different IR phenotypes was also observed, whereby metabolic activity after co-culture with colostrum or milk exosomes from H responder cows was significantly greater than co-culture with L responder exosomes. Recently, our group demonstrated differential expression of miRNA between colostrum and milk exosomes, as well as those isolated from different IR phenotypes (Atalla et al. 2016, manuscript in preparation). Additionally, others have reported on the differential expression of miRNA and proteins between colostrum and milk exosomes (Admyre et al., 2007; Chen et al., 2010; Hata et al., 2010; Kosaka et al., 2010; Weber et al., 2010; Gu et al., 2012; Alsaweed et al., 2015; Munagala et al., 2016; Yang et al., 2017). Based on these studies, differences in Caco-2 metabolic activity between colostrum, milk, and IR phenotypes in this study could be attributed to differential expression of exosomal cargo, particularly the miRNA: miR-21, -26a, -29b, 148a, and -155 identified by Atalla et al. (2016, manuscript in preparation), which have been shown to regulate proliferation and apoptosis of various colon cancer cell lines (Zhang et al., 2011; Konishi et al., 2015; Qu et al., 2015; Yuan et al., 2015; Nedaeinia et al., 2016). Specifically, results from this study suggest milk exosomes, particularly those from H responders, contain cargo that support growth of the human intestinal epithelial
cells. To better elucidate whether colostrum or milk exosomes, particularly those from certain IR phenotypes, increase the risk of proliferative cancer in humans, future work should consider whether Caco-2 cells maintained their cancerous phenotype given their tendency to differentiate into normal enterocyte-like cells of the small intestine.

A concentration dependent increase in cellular metabolic activity after co-culture with colostrum and milk exosomes was also observed in this study. As described in the results, Caco-2 metabolic activity after co-culture with the highest concentration of exosomal protein (0.625 μg/μL) was significantly greater than after co-culture with 0.001 and 0.005 µg/µL exosomal protein. In agreement with previous studies, metabolic activity after co-culture with 0.625 μg/μL was significantly lower than after co-culture with 0.025 and 0.125 μg/µL (Munagala et al., 2016; Song et al., 2016). Together, these results suggest bovine colostrum and milk exosomes may affect Caco-2 cells in a dose dependent manner and may increase cell viability up to a certain co-culture protein concentration.

The present study also tested whether bovine colostrum and milk exosomes contain molecules that induce apoptosis of Caco-2 cells. Caspase 3 activity in Caco-2 cells co-cultured with milk exosomes was not significantly different from the control, however was significantly lower compared to cells co-cultured with colostrum exosomes (Chapter 3), suggesting that unlike colostrum exosomes, milk exosomes do not activate the caspase 3 pathway in human intestinal cells. Results from this study also show bovine colostrum and milk exosomes from different IR phenotypes do not induce apoptosis of Caco-2 cells, with the exception of L responder colostrum exosomes whereby caspase activity after co-culture with these exosomes was significantly greater than the control, suggesting L responder colostrum exosomes potentially express molecules, including specific miRNA, that activate the caspase 3 pathway and induce apoptosis.
in cancer cells. While these results suggest bovine colostrum exosomes from different IR phenotypes have the ability to activate the caspase 3 pathway in human intestinal epithelial cells, little caspase 3 activity was detected in Caco-2 cells based on optical densities corresponding to p-nitroaniline standards provided with the caspase 3 assay kit. Low detection to almost lack of caspase activity after co-culture with colostrum or milk exosomes from different IR phenotypes could be attributed to a number of reasons including, (1) the presence of Hsp70 in colostrum and milk exosomes, which has been shown to inhibit the caspase 3 pathway in cells (Li et al., 2000; Takayama et al., 2003), (2) the potential release of caspase 3 enriched vesicles from cells, thereby preventing cellular apoptosis (Böing et al., 2013), and (3) over expression of oncogenic proteins and miRNA in Caco-2 cells (Ruemmele et al., 2003; Li et al., 2008; Kern et al., 2012) making it difficult to induce apoptosis of these cells after co-culture with colostrum and milk exosomes containing a variety of cargo. Of note, we are currently evaluating the effect of inhibitors on Hsp70 expressed by colostrum and milk exosomes as well as assessing caspase 3 activity in culture supernatant compared to cell lysate.

In summary, this study characterized expression of exosome surface markers in both the colostrum and milk of H, A, and L responder cows, and demonstrated their ability to be taken up human intestinal epithelial cells, maintain their metabolic activity, while at the same time not being cytotoxic to these cells. To our knowledge, this is the first study to identify colostrum exosomes by Western blot analysis for the expression of CD9, CD63, and Hsp70. Furthermore, Western blot analysis for the presence of CD63 demonstrated that bovine milk exosomes from this study do not express CD63, highlighting the importance of using various detection methods to confirm the presence of exosomes in biological fluids prior to assessing their bioactivity. The present study is also the first to report regulation of human intestinal cells by bovine colostrum
exosomes, particularly in comparison to milk exosomes. Importantly, these findings provide novel insights into differences in functionality between bovine colostrum and milk exosomes from H, A, and L immune responders, highlighting that dairy products derived from cows with diverse immune responses could differ in their impact on gut health. Given that development of the intestinal epithelium and healthy gut is essential for the overall health of humans and newborns, these results will provide a better understanding of the health benefits of bovine colostrum and milk, potentially improving nutrition for humans.

4.6 Acknowledgments
This research was funded by grants to B.A. Mallard by the Ontario Ministry of Agriculture, Food and Rural Affairs, Dairy Farmers of Ontario, and Natural Sciences and Engineering Research Council of Canada. This paper is also a contribution to the Food from Thought research program supported by the Canada First Research Excellence Fund. The authors thank Laura Wright and staff at the Elora Research Station for collecting samples used in this study and members of the Mallard lab for immune response phenotyping the animals, delivering samples, and technical assistance with experiments. The authors also thank William Sears for his assistance with the statistical analyses.

4.7 References


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4.8 Figures and tables

Figure 4.1: Western blot analysis of bovine colostrum and milk exosome isolates for commonly expressed exosomal proteins. 10 μg of exosomal protein was loaded per well. Hsp70, CD63, and CD9 were detected in colostrum exosome isolates, while Hsp70 and CD9 were detected in milk exosome isolates.
Figure 4.2: Enzyme-linked immunosorbent assay (ELISA) demonstrating the detection of CD63 in bovine colostrum exosomes, but not in bovine milk exosomes. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates per sample type, *p <0.05 was considered significant.
**Figure 4.3 (A-B):** Enzyme-linked immunosorbent assay (ELISA) demonstrating the detection of CD63 in bovine colostrum exosomes from H, A, and L immune responders (A), but not in bovine milk exosomes from H, A, and L immune responders (B). Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 3 biological replicates per immune response phenotype, *p <0.05 was considered significant.
**Figure 4.4 (A-H):** Uptake of bovine colostrum exosomes isolated from high, average, and low immune responders by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μL of PKH67 labeled colostrum exosomes (C-H), PKH67-PBS control (B), or no PKH67 dye (A) were incubated with Caco-2 cells at 37°C or 4°C for 24 h. The uptake of fluorescently labeled bovine colostrum exosomes was detected by fluorescence microscopy after co-culture for 24 h (C, E, and G). Uptake decreased at 4°C (D, F, and H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 3colostrum samples (1 per immune response phenotype to simply demonstrate uptake is possible for all phenotypes). Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelsitas Technologies) at 200x magnification.
Figure 4.4 continued:
Figure 4.5: (A-H): Uptake of bovine milk exosomes isolated from high, average, and low immune responders by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μL of PKH67 labeled milk exosomes (C-H), PKH67-PBS control (B), or no PKH67 dye (A) were incubated with Caco-2 cells at 37°C or 4°C for 24 h. The uptake of fluorescently labeled bovine milk exosomes was detected by fluorescence microscopy after co-culture for 24 h (C, E, and G). Uptake decreased at 4°C (D, F, and H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 3 milk samples (1 per immune response phenotype to simply demonstrate uptake is possible for all phenotypes). Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
Figure 4.5 continued:
Figure 4.6: Human cancer intestinal epithelial (Caco-2) cell count after co-culture with bovine milk exosomes. Cells were counted 0, 42, 66, and 90 h post-seeding. At 42, 66, and 90 h post-seeding, milk exosomes had been co-cultured with Caco-2 cells for 24, 48, and 72 h, respectively. Data are presented as mean cell number ± SEM using GraphPad Prism 7.0, n = 3 biological replicates for Caco-2 cells co-cultured with milk exosomes. Dashed line represents the proposed population doubling time of Caco-2 cells according to the ATCC product manual.
Figure 4.7: Human cancer intestinal epithelial (Caco-2) cell viability after treatment with 0.001-0.625 μg/μL bovine colostrum or milk exosomes for 24, 48, and 72 h. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates per sample type, *p < 0.05 was considered significant.
**Figure 4.8:** Human cancer intestinal epithelial (Caco-2) cell viability after treatment with bovine colostrum and milk exosomes from low, average or high immune responders. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, LL = low/low, AA = average/average, HH = high/high, n = 6 biological replicates per immune response phenotype, *p<0.05 was considered significant.
Figure 4.9 (A-B): Human cancer intestinal epithelial (Caco-2) cell viability after treatment with 0.001-0.625 μg/μL bovine colostrum (A) or milk exosomes (B) from low, average, or high immune responders for 24, 48, and 72 h. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, LL = low/low, AA = average/average, HH = high/high, n = 3 biological replicates per immune response phenotype, *p <0.05 was considered significant.

A)
Figure 4.10 (A-B): Caspase-3 activity in human cancer intestinal epithelial (Caco-2) cells after co-culture with 0.125 μg/μL of bovine colostrum (A) or milk exosomes (B) from low, average, or high immune responders for 48 h. Data are presented at medians with 95% confidence intervals using GraphPad Prism 7.0, LL = low/low, AA = average/average, HH = high/high, n = 3 biological replicates per immune response phenotype, control represents caspase-3 activity in cells cultured without exosomes, *p < 0.05 was considered significant.

A)

B)
Table 4.1: Cows used for colostrum (C) and milk (M) collection and their corresponding identification numbers (ID), estimated breeding values (EBVs) for antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR), and Immune response phenotype.

<table>
<thead>
<tr>
<th>Cow ID/Sample type</th>
<th>Immune response phenotype (according to EBV for AMIR and CMIR)</th>
<th>EBV for AMIR/CMIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>4056 C/M</td>
<td>High/High</td>
<td>0.94/1.67</td>
</tr>
<tr>
<td>4238 C/M</td>
<td>High/High</td>
<td>0.91/1.13</td>
</tr>
<tr>
<td>4290 C/M</td>
<td>High/High</td>
<td>1.43/0.92</td>
</tr>
<tr>
<td>4172 C/M</td>
<td>Average/Average</td>
<td>0.43/-0.27</td>
</tr>
<tr>
<td>4222 C/M</td>
<td>Average/Average</td>
<td>0.11/-0.35</td>
</tr>
<tr>
<td>4312 C/M</td>
<td>Average/Average</td>
<td>0.72/0.59</td>
</tr>
<tr>
<td>4089 C/M</td>
<td>Low/Low</td>
<td>-1.51/-1.14</td>
</tr>
<tr>
<td>4335 C/M</td>
<td>Low/Low</td>
<td>-1.26/-0.8</td>
</tr>
<tr>
<td>4233 C/M</td>
<td>Low/Low</td>
<td>-1.06/-1.95</td>
</tr>
</tbody>
</table>
Table 4.2: Information on primary and secondary antibodies used for Western blot analysis of bovine colostrum exosomes; CD = cluster differentiation, TAPA1 = target of the antiproliferative antibody 1, Hsp70 = heatshock protein 70; IgG-HRP = immunoglobulin G-horseradish peroxidase.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Antibody name/ catalog number</th>
<th>Supplier</th>
<th>Type of antibody/ concentration reported by manufacturer</th>
<th>Species reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD9</td>
<td>Anti-CD9 [IVA50]/ab3923</td>
<td>Abcam</td>
<td>Monoclonal/ 1 mg/mL</td>
<td>Cow</td>
</tr>
<tr>
<td>CD63</td>
<td>Anti-CD63 [MX-49.129/ ab193349</td>
<td>Abcam</td>
<td>Monoclonal/ 0.2 mg/mL</td>
<td>Human, mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Predicted to react with rabbit, cow, cat</td>
<td></td>
</tr>
<tr>
<td>TAPA1</td>
<td>Anti-TAPA1 [M38]/ab79559</td>
<td>Abcam</td>
<td>Monoclonal/ 1 mg/mL</td>
<td>Human, rabbit, cat</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Anti-Hsp70 [5A5]/ab2787</td>
<td>Abcam</td>
<td>Monoclonal/ 1 mg/mL</td>
<td>Mouse, rat, rabbit, cow, human, saccharomyces cerevisiae, bird, drosophila melanogaster, fish, amphibian</td>
</tr>
<tr>
<td>IgG-HRP</td>
<td>Donkey anti-mouse IgG H&amp;L (HRP)/ab6820</td>
<td>Abcam</td>
<td>Polyclonal/ 2 mg/mL</td>
<td>Mouse</td>
</tr>
</tbody>
</table>
Table 4.3: Analysis of covariance table for fixed effects summarizing the variables and their interactions included in the mixed effects model for analyzing the MTT data. Output obtained using SAS software Version 9.4, SAS Institute, Cary, North Carolina, USA; DF = degrees of freedom, \( F = F \) value, \( Pr = \) probability.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Numerator DF</th>
<th>Denominator DF</th>
<th>F Value</th>
<th>( Pr &gt; F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response phenotype</td>
<td>2</td>
<td>6</td>
<td>5.09</td>
<td>0.0510</td>
</tr>
<tr>
<td>Time point</td>
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<td>12</td>
<td>10.53</td>
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</tr>
<tr>
<td>Sample type</td>
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<td>229</td>
<td>46.38</td>
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</tr>
<tr>
<td>Exosome treatment concentration</td>
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<td>229</td>
<td>8.80</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Immune response phenotype*Time point</td>
<td>4</td>
<td>12</td>
<td>0.97</td>
<td>0.4587</td>
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<tr>
<td>Immune response phenotype*Sample type</td>
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<td>229</td>
<td>8.14</td>
<td>0.0004</td>
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<tr>
<td>Time point*Sample type</td>
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<td>229</td>
<td>8.08</td>
<td>0.0004</td>
</tr>
<tr>
<td>Immune response phenotype<em>Time point</em>Sample type</td>
<td>4</td>
<td>229</td>
<td>8.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Control (no exosomes)</td>
<td>1</td>
<td>229</td>
<td>0.39</td>
<td>0.5314</td>
</tr>
</tbody>
</table>
Table 4.4: Analysis of variance table for fixed effects summarizing the variables and their interactions included in the mixed effects model for analyzing the caspase 3 data. Output obtained using SAS software Version 9.4, SAS Institute, Cary, North Carolina, USA; DF = degrees of freedom, F = F value, Pr = probability.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Numerator DF</th>
<th>Denominator DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response phenotype</td>
<td>3</td>
<td>52</td>
<td>1.84</td>
<td>0.1511</td>
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<tr>
<td>Sample type</td>
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<td>52</td>
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<tr>
<td>Immune Response Phenotype*Sample type</td>
<td>3</td>
<td>52</td>
<td>3.82</td>
<td>0.0150</td>
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</tbody>
</table>
CHAPTER 5
SYNOPSIS AND FUTURE DIRECTIONS

5.1 Synopsis

The research in this thesis provides new findings related to the characterization of bovine colostrum and milk exosomes, along with novel insights into their bioactivity on human intestinal epithelial cells, as well as differences in functionality of exosomes isolated from the colostrum and milk of cows classified as high (H), average (A), and low (L) responders. While the importance of bovine colostrum and milk to newborn calves, infants who require infant formula, and humans who consume dairy products, has been well established (Hettinga et al., 2011; Ballard and Morrow, 2013; Lu et al., 2014; Pereira, 2014; Hassiotou & Geddes, 2015; Parigi et al., 2015; Martin et al., 2016; Dunn et al., 2017; Nissen et al., 2017), the specific functional role of bovine colostrum and milk exosomes containing microRNA (miRNA), especially those from different immune responder cows, on human intestinal epithelial cells needed to be elucidated.

Exosomes isolated from colostrum and milk of H, A, and L responders were previously identified by electron microscopy and immunogold labeling. In the current study, Western blot analysis confirmed the presence of commonly expressed exosomal proteins. Exosomes displayed characteristic size and shape, however expression of exosomal proteins differed between colostrum and milk isolates. While CD63 is one of the most frequently reported proteins used to identify exosomes, including those isolated from milk (Admyre et al., 2007; Kosaka et al., 2010; Andreu and Yáñez-Mó, 2014; Wolf et al., 2015; Munagala et al., 2016; Yu et al., 2017), its expression in milk exosomes was not detected in this study. These findings provide evidence for the potential existence of colostrum and milk exosome subtypes and also highlight the
importance of testing for expression of several proteins, such as CD9 and Hsp70, to identify exosomes.

Bovine colostrum and milk exosomes from H, A, and L responders were taken up by human colorectal adenocarcinoma epithelial (Caco-2) cells as visualized by fluorescent microscopy, suggesting these exosomes and their associated cargo can be delivered to and possibly modulate the function of the human intestinal epithelium. Further, co-culture of labeled exosomes with Caco-2 cells at 4°C prevented internalization by Caco-2 cells, suggesting uptake is energy-dependent rather than a passive process (Lässer et al., 2011, Franzen et al., 2014; Izumi et al., 2015; Pieters et al., 2015; Wolf et al., 2015). Additionally, the present study is one of the few to report the uptake of colostrum and milk exosomes by non-phagocytic cells (Lässer et al., 2011; Izumi et al., 2015), highlighting exosome uptake may occur by various mechanisms.

An interesting finding of this research was that bovine colostrum and milk exosomes are not only non-cytotoxic but also enhance the viability of Caco-2 cells. This finding was demonstrated by increased metabolic activity of Caco-2 cells co-cultured with colostrum and milk exosomes over time. A concentration dependent increase in Caco-2 metabolic activity was also observed, whereby cell metabolic activity after co-culture with the highest concentrations of exosomal protein was significantly higher than after co-culture with the lowest concentrations. These findings suggest bovine colostrum and milk exosomes regulate Caco-2 cells in a dose dependent manner. Metabolic activity of Caco-2 cells after milk exosome co-culture was also found to be significantly greater than after co-culture with colostrum exosomes. Further, differences in bioactivity of colostrum and milk exosomes isolated from different IR phenotypes were observed in this study, whereby co-culture with colostrum or milk exosomes from H responders resulted in significantly greater viability compared to co-culture with L responder
exosomes, suggesting milk exosomes, as well as colostrum and milk exosomes from H responders, contain cargo that support growth of the human intestinal epithelial barrier. Differences in Caco-2 viability after co-culture with exosomes from different sample types (colostrum or milk) and IR phenotypes (H, A, or L) also supports the idea that exosomal cargo is differentially expressed between colostrum, milk, and IR phenotypes, thus having different effects on cell viability.

Additionally, the potential of colostrum exosomes to initiate apoptosis of Caco-2 cells was shown, whereby caspase 3 activity in Caco-2 cell lysate was greater after co-culture with colostrum exosomes than milk exosomes. Furthermore, increased caspase 3 activity was higher after co-culture with colostrum exosomes from L responders compared to A and H. Although this observation warrants further investigation, it could be related to high expression of molecules, including specific miRNA, in colostrum from L responders that activate the caspase 3 pathway and induce apoptosis in cancer cells.

Taken together, these findings demonstrate the bioactive potential of bovine colostrum and milk exosomes on the human intestinal epithelium. Importantly, this research provides novel insights into differences in functionality of bovine colostrum and milk exosomes from H, A, and L responders, which has further downstream implications for production of colostrum or milk products that support gastrointestinal and overall health of calves and humans.

5.2 Future directions

- Evaluate the bioactivity and functional role of colostrum and milk exosomes from cows with different IR phenotypes on primary human and calf intestinal epithelial cells to assess their role in the development of healthy gut mucosa.
• Evaluate the ability of bovine colostrum and milk exosomes to promote wound healing of the intestinal epithelium by performing a migration or scratch assay, whereby a gap in the cell monolayer is created by scratching and monitoring cell migration and growth towards the gap. This will help to further understand how colostrum and milk exosomes modulate intestinal epithelial cells.

• In view of the lack of specific antibodies for detecting common bovine exosomal proteins, there is a pressing need for the development of bovine specific antibodies for detection of exosomal proteins from different biological fluids. This will allow for more accurate identification and characterization of exosomes isolated from bovine colostrum and milk.

• Sonicate colostrum and milk exosomes prior to co-culture with Caco-2 cells and re-evaluate exosome bioactivity. Evidence presented in this thesis and from others suggests exosomes tend to aggregate which may affect their functionality, and sonication is a viable method to disaggregate exosomes.

• Quantify colostrum and milk exosome uptake by flow cytometry under the conditions evaluated in this thesis (sonication, proteinase K treatment, and co-culture at 4 and 37°C). This will allow for determination of whether certain conditions increase or decrease exosome uptake, and also evaluate whether exosomes from colostrum, milk, or different IR phenotypes are taken up more readily.

• Consider more accurate methods for quantifying exosomes in biological fluids. Many researchers currently rely on total protein quantification of exosome isolates, however this does not quantify the number of isolated vesicles. Others rely on methods such as
NanoSight to quantify vesicles of a certain size, however this can also prove to be unreliable due exosome aggregation.

- Consider other variables that may influence colostrum and milk quality, such as cow parity, herd, age, and number of lactations, and include in the statistical models used to analyze the MTT and caspase 3 data. Given that these variables have been shown to affect colostrum and milk quality, it may be reasonable to postulate they also influence the quality of exosomes and their cargo and should be considered in future statistical models.

- In view of the previous and ongoing exosomal miRNA studies in the Mallard lab, including genome wide characterization studies of colostrum and milk exosomal miRNA from heat-treated colostrum and milk of H, A, and L responders, it would be of particular interest to assess the bioactivity of exosomes containing miRNA from heat-treated colostrum and milk on calf and human intestinal epithelial cells. Similarly, differentially expressed immune-related miRNA in raw and heat-treated colostrum and milk exosomes can be identified and transfected to calf and human intestinal epithelial cells to evaluate their specific role in epithelial barrier integrity.

5.3 References


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Figure 3.1 (A-C): Transmission electron microscopy images courtesy of Dr. Atalla, indicate bovine colostrum and milk contain exosomes displaying characteristic morphology and shape. Exosomes ranged in size from 20-100 nm (A-B). Immunogold labeling for anti-CD63 further confirmed the presence of exosomes in colostrum isolates (C). Red arrows indicate exosomes. Scale bars represent 100 nm.
**Figure 3.2 (A-H):** Western blot images depicting the entire blot for each antibody tested, along with the corresponding Ponceau staining images to show equal loading of protein per well.

A) Ponceau stain – Milk samples  
B) Ponceau stain – Milk samples  
Anti-Hsp70 – Milk samples  
Anti-CD9 – Milk samples  
C) Ponceau stain – Milk samples  
D) Ponceau stain – Milk samples  
Anti-CD63 – Milk samples  
Anti-CD81 – Milk samples
Figure 3.3 (A-H): Uptake of bovine colostrum exosomes by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μl of PKH67 labelled colostrum exosomes after sonication (E-F) or proteinase K treatment (G-H), PKH67-PBS control (C-D), or no PKH67 dye (A-B) were incubated with Caco-2 cells at 37°C for 2 or 24 h. The uptake of fluorescently labelled bovine colostrum exosomes after sonication or proteinase K treatment was detected by fluorescence microscopy after co-culture for 2 and 24 h (E-H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 1 colostrum sample per treatment (PKH67 dye, sonication, or proteinase K). Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
**Figure 3.4:** p-Nitroaniline standard curve for caspase 3 assay. Data are presented as mean optical densities corresponding to specific concentrations of p-nitroaniline standards ± SEM using GraphPad Prism 7.0, n = 3 technical replicates.
**Figure 4.1 (A-H):** Uptake of bovine colostrum exosomes isolated from low, average, and high immune responders by human cancer intestinal epithelial cells (Caco-2). Concentrations 0.16 μg/μl of PKH67 labelled colostrum exosomes (C-H), PKH67-PBS control (B), or no PKH67 dye (A) were incubated with Caco-2 cells at 37°C or 4°C for 2 h. The uptake of fluorescently labelled bovine colostrum exosomes was detected by fluorescence microscopy after co-culture for 2 h (C, E, and G). Uptake decreased at 4°C (D, F, and H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 1 colostrum sample per immune response phenotype (low, average, or high). Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
**Figure 4.2 (A-H):** Uptake of bovine milk exosomes isolated from low, average, and high immune responders by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μl of PKH67 labelled milk exosomes (C-H), PKH67-PBS control (B), or no PKH67 dye (A) were incubated with Caco-2 cells at 37°C or 4°C for 2 h. The uptake of fluorescently labelled bovine milk exosomes was detected by fluorescence microscopy after co-culture for 2 h (C, E, and G). Uptake decreased at 4°C (D, F, and H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 1 sample for each immune response phenotype (low, average, or high) Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
Figure 4.3 (A-B): Uptake of bovine milk exosomes by human cancer intestinal epithelial cells (Caco-2). Concentrations of 0.16 μg/μl of PKH67 labelled milk exosomes after sonication (A-B) were incubated with Caco-2 cells at 37°C for 2 or 24 h. The uptake of fluorescently labelled bovine milk exosomes after sonication was detected by fluorescence microscopy after co-culture for 2 and 24 h (E-H). 7-AAD dye was used to label the nuclei of Caco-2 cells (red) and PKH67 was used to label exosomes (green). N = 1 sample per treatment. Scale bars represent 50 μm. Photographs obtained using Axio Observer.A1 Inverted Microscope (Carl Zeiss) and X-Cite®120Q Microscope Illumination System (Excelitas Technologies) at 200x magnification.
**Figure 4.4:** Human cancer intestinal epithelial (Caco-2) cell viability after treatment with 0.001-0.625 μg/μL bovine milk exosomes for 24, 48, and 72 h. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates per time point, *p<0.05 was considered significant.
**Figure 4.5**: Human cancer intestinal epithelial (Caco-2) cell viability after treatment with bovine colostrum or milk exosomes. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 9 biological replicates per sample type, *p<0.05 was considered significant.
Figure 4.6: Human cancer intestinal epithelial (Caco-2) cell viability after treatment with different doses of bovine colostrum and milk exosomes. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 18 biological replicates per exosome protein treatment concentration, different letters indicate significance (p<0.05).
**Figure 4.7:** Human cancer intestinal epithelial (Caco-2) cell viability after treatment with bovine colostrum and milk exosomes for 24, 48, and 72 h. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, n = 18 biological replicates per time point, different letters indicate significance ($p<0.05$).
Figure 4.8 (A-C): Human cancer intestinal epithelial (Caco-2) cell viability after treatment with bovine colostrum or milk exosomes from low, average or high immune responders for 24 (A), 48 (B), and 72 h (C). Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, LL = low/low, AA = average/average, HH = high/high, n = 3 biological replicates per immune response phenotype, *p<0.05 was considered significant.
**Figure 4.9 (A-B):** Differences in human cancer intestinal epithelial (Caco-2) cell viability between 24, 48, and 72 h treatment with bovine colostrum (A) or milk exosomes (B) from low, average or high immune responders. Data are presented as mean optical density ± SEM using GraphPad Prism 7.0, LL = low/low, AA = average/average, HH = high/high, n = 3 biological replicates per immune response phenotype, *p<0.05 was considered significant.

A)
**Figure 4.10:** Caspase-3 activity in human cancer intestinal epithelial (Caco-2) cell after co-culture with 0.125 μg/μl of bovine colostrum or milk exosomes for 48 h. Data are presented at medians with 95% confidence intervals using GraphPad Prism 7.0, n = 9 biological replicates per colostrum or milk, control represents caspase-3 activity in cells cultured without exosomes, *p<0.05 was considered significant.