

Milking extracellular vesicles for health benefit using a multi-omics approach

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Doctor of Philosophy

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Based on research carried out under the supervision of Prof. Lorraine O'Driscoll

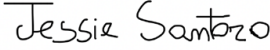
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“Ain't about how fast I get there, ain't about what's waiting on the other side. It's the climb”!

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Thesis Abstract

Milk is remarkable source of nutrients (*i.e.*, proteins, fat, and sugars) and extracellular vesicles (EVs). EVs are tiny vesicles release by cells into all biofluids including milk. Milk EVs have been gathered huge attention recently for their scalability and non-toxic effect. Milk EVs have been separated and characterised from different species such as human, cow, goat, horse *etc.* Furthermore, extracellular vesicles (EVs) in milk contribute to regulate biological processes and cellular communication. The first aim of this project was to establish a process for EVs separation from milk that could be applied to multiple milk sources. The intention was to find a method that would result in enriched EVs that were not contaminated with milk-derived proteins, because proteins like casein micelle have overlapping size as EVs. For this purpose, commercial milk was used. We investigated different milk pre-treatment, attempting to remove these contaminants, and two techniques for EVs separation, differential ultracentrifugation and density gradient ultracentrifugation. We discovered that acid pre-treatment coupled with density gradient ultracentrifugation was the optimal solution to obtain enriched EVs samples with minimal contamination. Raw milk at different stages of the lactation has variable composition in colostrum (COL), known as liquid gold, first milk (FM) and mature milk (MM). Milk was collected from three different Holstein-Friesian cows; COL after 24hrs, FM after 7 days and MM and then EVs were separated and characterised. We observed that COL and FM are highly enriched in EVs compared to mature milk; particularly colostrum samples had the highest concentration of EVs which decreased throughout the lactation period. Particularly, bovine milk is used essentially for the manufacture of infant milk formula, used as the best alternative of human milk. However, bovine milk is subjected to severe treatments during industrial manufacturing of infant milk formula. Thus, our objective was to investigate if loss of EVs may occur at some stages of milk processing by the dairy industry. Indeed, we observed that heat

treatments had detrimental consequences on EVs. EVs had disrupted structure and atypical morphology, while EVs yield was also significantly affected. Considering that, we are aware that millions of mothers worldwide are not breastfeeding. Therefore, we compared EVs characteristics in commercial IMF (liquid and powder formula) versus human milk (20 donors). EVs are transporters of important cargo such as proteins, nucleic acid and metabolites. Thus, human milk EVs contain cargo which have been associated with immune modulatory activity involved in shaping the newborn immune system. By investigating EVs quantity and quality from IMF and HM, we discovered that IMF had significant ($p < 0.0001$) lower concentration of EVs/particles. Using a multi-omics approach, we investigated the proteomics, metabolomics, and transcriptomics profile of milk EVs from infant milk formula and human milk. Mass spectroscopy analysis revealed that IMF EVs had lower concentration of 4 proteins (BTN1A1, CD36, SLC34A2, FABP3) in common with HM EVs, in which higher amount of proteins were detected. Similarly, for the metabolomic profiling of infant milk formula EVs compared to human milk EVs, we detected metabolites (*i.e.*, human milk oligosaccharides, free amino acids, and nucleotides) associated with development of intestine and the gut microbiota, growth and immune development, which were all less enriched in IMF EVs in comparison with HM EVs. Subsequently, we selected miRNAs known to be the most abundant in milk EVs and associated with immunoregulatory function and demonstrated that they (miR-200a-3p, miR-148a-3p, miR-200a-3p, miR-151a-5p, miR-148-3p, miR-30b-5p and miR-182-5p) had significantly lower levels in IMF EVs compared to HM EVs.

Overall, milk EVs have the potential to be a powerful tool for improving IMF, which might reduce the risk of obesity, diabetes, and cardiovascular diseases in adulthood for artificially fed infants. In fact, the role of breast milk in the modulation of the infant's immune system through the transfer of personalised microbial and immune factors is a topic of high scientific and commercial interest. Moreover, the essential role of milk in infant and adult nutrition along with

the abundance of EVs, makes milk EVs a particular candidate of interest with the potential therapeutic application. Their abundance and non-toxic nature make milk EVs an efficient vehicle for drug delivery.

List of Abbreviations

¹H-NMR: proton nuclear magnetic resonance

AA: acetic acid

ABC: ammonium bicarbonate

AFM: atomic force microscopy

ALB: albumin

ALDOA: aldolase

BCA: bicinchoninic acid assay

BM EVs: bovine milk extracellular vesicles

BM: bovine milk

BTN1A1: butyrophilin subfamily 1-member a1

CD36: glycoprotein iiib

CFL1: cofilin-1

CHRD2: chordin like protein 2

CL: cell lysate

COL: colostrum

CSN1S2: alpha-s2-casein

D₂O: deuterium oxide

DHA: docosahexaenoic acid

DLS: dynamic light scattering

DNMT1: dna methyltransferase 1

DNMT3A: dna methyltransferase 3

DTT: dithiothreitol

dTTP: thymidine 5'-triphosphate

DUC: differential ultracentrifugation

EDTA: ethylenediaminetetraacetic acid

EEF2: elongation factor 2

ESCRT: endosomal sorting complex required for transport

EVs: extracellular vesicles

FAAs: free amino acids

FABP3: fatty acid binding protein

FDA: food and drug administration

FITC: fluorescein isothiocyanate

FM: first milk

FOSs: fructoligosaccharides

FOXP3: forkhead box p3

GC-MS: gas chromatography mass spectrometry

GI: gastrointestinal

GOSs: galactoligosaccharides

GUC: gradient ultracentrifugation

HCl: hydrochloric acid

HM EVs: human milk extracellular vesicles

HM: human milk

HMOs: human milk oligosaccharides

HS: high sensitivity

IAA: iodoacetamide

ICA: icariin

IECs: intestinal epithelial cells

IFCM: imaging flow cytometric analysis

IMF EVs: infant milk formula extracellular vesicles

IMF: infant milk formula

IP: isoelectric precipitation

ISEV: international society on extracellular vesicles

KRT1: cytokeratin 1

KRT10: keratin type 10

KRT14: keratin type 14

LC-MS/MS: liquid chromatography tandem mass spectrometry

IEVs: large EVs

LFQ: label free quantitative

MET¹⁰: methionine 10

MFG-1: milk fat globule 1

MM: mature milk

MS WATER: mass spectrometry water

MUC 2: mucin 2

MUC-1: mucin 1

NADP: nicotinamide adenine dinucleotide phosphate

NEC: necrotising enterocolitis

NF- κ B: nuclear factor kappa b

NOESY: 1d nuclear overhauser effect spectroscopy

NTA: nanoparticle tracking analysis

OA: osteoarthritis

OPLS-DA: orthogonal projections to latent structures discriminant analysis

PAEP: lipocln cytosolic fa-bd dom domain-containing protein

PASEF: parallel accumulation serial fragmentation

PLS-DA: partial least squared discriminant analysis

PEG: polyethylene glycol

PPM: parts per million

PtC: phosphatidylcholine

qPCR: quantitative polymerase chain reaction

SCFAs: short fatty acids

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC: size exclusion chromatography

SEM: scanning electron microscopy

sEVs: small EVs

SIRT1: sirtuin 1

SLC34A2: sodium dependent phosphate transport protein 2b

SM: skim milk

SNR: signal to noise ratio

TEM: transmission electron microscopy

TFA: trifluoroacetic acid

TIMS: trapped ion mobility spectrometry

TLR4: toll-like receptor 4

TSG101: tumor susceptibility gene 101

TSP: trimethylsilylpropanoic acid

UHF: ultra-high field

UHPLC: ultra-high-performance liquid chromatography

UHPnLC: ultra-high pressure nanoflow chromatography system

UHT: ultra-high treatment

WHO: world health organisation

WPI: whey protein isolate

YWHAE: tyrosine-3-monooxygenase

1 Chapter 1: Introduction

Milk is a complete food, available easily and abundantly worldwide (Haug et al., 2007). Milk research is a well-established and attractive field of human health and nutrition science for over 50 decades now, due to its many benefits to neonates and adults. Foremost, human health is shaped also by the mother via breastfeeding as innate immunity and in shaping the adaptive immunity. Milk is a complex biochemical fluid, full of high-quality proteins, lipids, carbohydrates, lactose, vitamins, minerals, enzymes, hormones, immunoglobulin and growth factors (Carr et al., 2021). Additionally, minerals in milk such as calcium, potassium, magnesium, phosphorus, zinc and vitamins make milk unique (Reddy et al., 1990). Furthermore, milk is also known to be a source of commensal and probiotic bacteria found in infant gut (Chen et al., 2018; Sanwani et al., 2020). The principal components, such as water, proteins, fats are common across species, whereas their quantities vary widely. Water is the main constituent of milk, comprising ~ 87% of the total volume. Human breast milk has the highest lactose content followed by sheep, cow, goat and camel; whereas the protein content is higher in sheep milk followed by cow, goat, camel and human milk (Faten et al. 2012). Table 1.1 presents the composition (as g/100mL of milk) of the macronutrients present in human milk, bovine milk and infant milk formula, respectively, and it is evident that there are compositional differences between each source.

Macronutrient	Human milk	Bovine milk	IMF
Fat	4.2g	3.9g	3.6g
Protein	1.3g	3.2g	1.24g
Lactose	7.0g	4.9g	7.4g

Table 1.1: Milk nutrients

Macronutrient composition in human milk, bovine milk and IMF, presented as g/100mL of milk adapted from (Mølgaard et al., 2011)

1.1 Milk proteins composition

In Table 1.1 we have highlighted the % of the main constituent found in milk across species. Milk contains two major types of proteins: the caseins and the whey proteins and approximately 5% of fat in form of milk fat globules. Almost all casein is present in an aggregated form known as casein micelles with a size ranging from 20 to 300 nm (Morris 2002). In contrast, whey proteins are globular proteins whose structural characteristics are governed by disulphide bonding, co-operative hydrogen bonding and hydrophobic interactions (Camirand et al. 1996). Milk fats are present as emulsified globules, approximately 2 to 4 μm in diameter (Ferris et al. 1991). These differences observed are mostly correlated to genetic (inter- and intra-species), physiological (*e.g.* age, lactation curve, parity), nutritional factors and environmental conditions, making milk a complex matrix (Thorning, Bertram et al. 2017). Interestingly, in the gastrointestinal tract, casein becomes clotted after reacting with the gastric acids, while whey remains as a liquid and is easier to digest as it is broken down rapidly to free amino acids for absorption and utilisation (Martin et al., 2016). In regard to casein protein, there are three major groups: α -caseins, that are present in two sub-groups, *i.e.* $\alpha\text{s}1$ and $\alpha\text{s}2$ -like families (Holt, 1992). β -caseins, that are particularly rich in glutamines; and κ -casein that differ from the others because of its high solubility (Greenberg et al., 1979). Accordingly to the chemical properties of caseins in milk, they are present as macromolecular aggregates of proteins termed micelles, organised as $10^{14} - 10^{16}$ casein micelles tightly packed per mL of milk (Fox & Brodtkorb, 2008). How the relative proportion of the various sub-classes differ between bovine and human milk are summarised in Table 1.2 (Jensen, 1995). Similarly to caseins, the concentrations of whey proteins also differ in human milk compared to bovine milk, the latter being the starting material of infant milk formula (Hernell, 2011). Whey proteins included β -lactoglobulin, α -lactalbumin, serum albumin, immunoglobulin and lactoferrin (D'Alessandro et al., 2010). The differences in their ratio within

bovine and human milk are shown in Table 1.2. For instances, lactoferrin in human milk is the second most abundant glycoprotein (Conneely, 2001), at approximately a 10-fold higher concentration than bovine milk (Wakabayashi et al., 2006). Conversely, β -lactoglobulin is the predominant whey protein in bovine milk, but is completely absent from human milk whey (Fox, 2001).

Proteins	Human milk	Bovine milk
Casein	40% • high in β-casein	80% • high α-casein
Whey • Lactoferrin • β-lactoglobulin • α-lactalbumin	60% • Abundant • Absent • Highly abundant	20% • 10-fold lower than in human milk • Predominant • Lower amount than human milk

Table 1.2: Caseins and whey proteins

Comparison of protein content between human milk and bovine milk adapted from (R. G. Jensen, 2002)

1.2 Milk sources and their characteristics

1.2.1 The gold standard 'Human milk'

Human milk (HM) is a source of nourishment for infants because its composition synergistically interacts to stimulate and support the infant's immature immune system. Indeed the infants microbiota is nourished intensively while breastfeeding (Goldman, 2007) (Fig. 1.1). Hence, the World Health Organisation (WHO) (www.who.int) recommends that infants should be breastfed alone for the first six months of life (WHA63, 2010) and up to 2 years along with solid foods. As reported in Table 1.1, amongst all the nutrients, the maternal immunoglobulins have been recognised to be essential to sustain early growth, development and gut maturity (Hanson et al., 1972) and several studies confirmed this transfer from mother to child *via* milk, essentially building up the neonatal adaptive immune system (Medicine, 2004; Baer et al., 2011). Mostly,

HM bioactive molecules are synthesised by the mammary cells, maternal blood and adipose tissues and subsequently released in the milk (Perrella et al., 2021). However, during the lactation period from colostrum which is low in lactose but remarkably enriched in immunoglobulins and proteins, to transitional and mature milk represent a substantial change. HM mosaic of nutrients is stable while the immunoglobulins are significantly decreasing (Quinn, 2021). Furthermore, the nutritional buffet offered by human milk, including lactoferrin and several amino acids, are beneficial and associated with short and long-term health consequences of infant's nutritional intake, as reported in Fig. 1.1. Also hormones which are correlated to energy balance regulation in adults have been implicated in physical growth; particularly in breast-fed infants (Smith-Kirwin et al., 1998; Soori et al., 2020). However, it is relevant to explore in depth the composition of HM to further investigate how infant milk formula match the essential requirements for infant feeding.

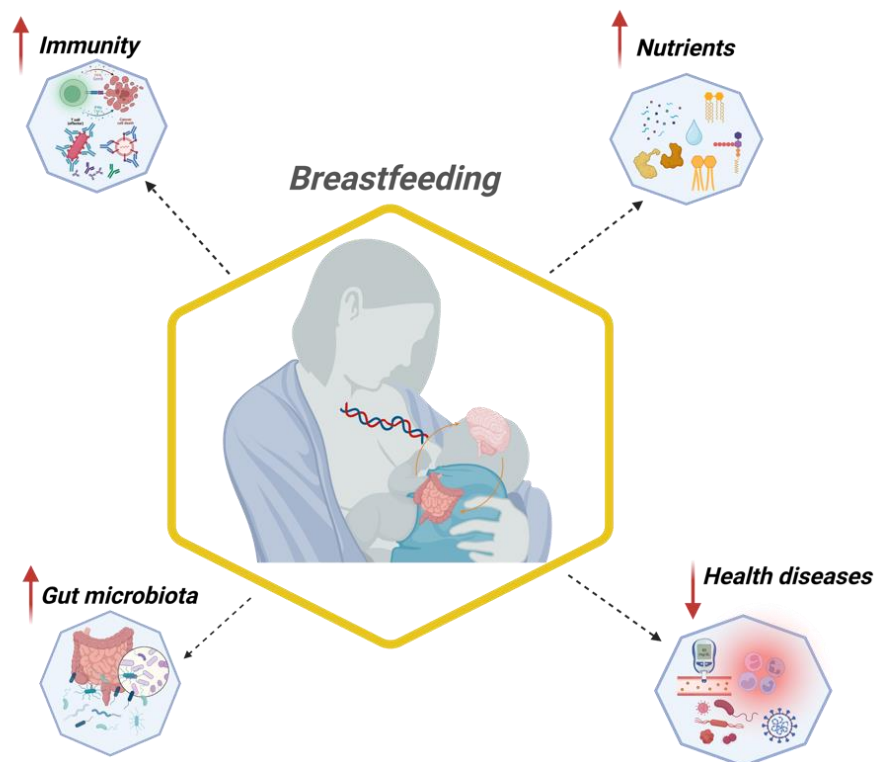


Figure 1.1: Human milk: The gold standard for human infants

The invaluable benefits of human breast milk: 1) nutrients; 2) defences against disease, such as necrotising enterocolitis, obesity, and allergies. 3) The gut microbiota is positively shaped by the mother's milk and numerous commensal bacteria are essential for host defense in gut microbiota; 4) also the adaptive immune system relies on breast milk (Carr et al., 2021).

1.2.2 Bovine milk

Humans are known to have consumed bovine milk as part of their diet since prehistoric times. As a convenient source of nutrition, bovine milk is the most-consumed milk worldwide because of its widespread availability and large production volumes (Dunne et al., 2012) (Fig. 1.2). Commercial skim milk is processed at medium temperature of ~71.7 °C for 15s, the standard procedure for milk pasteurisation. Therefore, potential microorganisms are eliminated without causing texture changes while protecting the essential components of bovine milk (Lund et al., 2002). Lipids, proteins, immunoglobulins, growth factors, hormones and many other bioactive components are essential in milk across species (Haug et al., 2007; Rahman et al., 2021). As in HM, bovine milk composition changes its dynamic accordingly to the stage of lactation, breed, and health status of the cattle. In fact throughout the lactation period, the most relevant difference is the concentration of immunoglobulins and fat is doubled up in colostrum then decreases and is maintained in the later stages (Ontsouka et al., 2003), accommodating all the essential needs for growth and development. However, within these factors, lactation has a huge impact on milk composition, because it is essentially, the most crucial part of the mammalian reproductive physiology, which will be described in Chapter 3. Colostrum (COL), secreted by mammary glands immediately after birth, is much thicker than subsequent milk and

is of a yellowish colour. It is a rich source of growth and immunological factors and increased level of vitamins and minerals (Sanwlani et al., 2020). COL is rich in immunoglobulins (particularly IgA) and immunomodulatory cytokines (especially IL-1, IL-6 and TNF- α) (Gu et al., 2012). The mammary gland has a short window of COL production which makes it even more precious. Furthermore, it is important for the dairy industry to monitor milk yield. Especially because bovine milk, in its pure form or derivative dairy products (cream, butter, cheese, and yogurt) are a major source of nutrition for humans (Vincent et al., 2016), as illustrated in Fig. 1.2. In fact, milk proteins content is directly affected by heat treatment, during the preparation of dairy products, leading to potential structural changes of proteins, especially whey proteins (Haug et al., 2007). Thus, bovine milk and its components are of interest for human health and most importantly its invaluable use to make infant milk formula for infants.

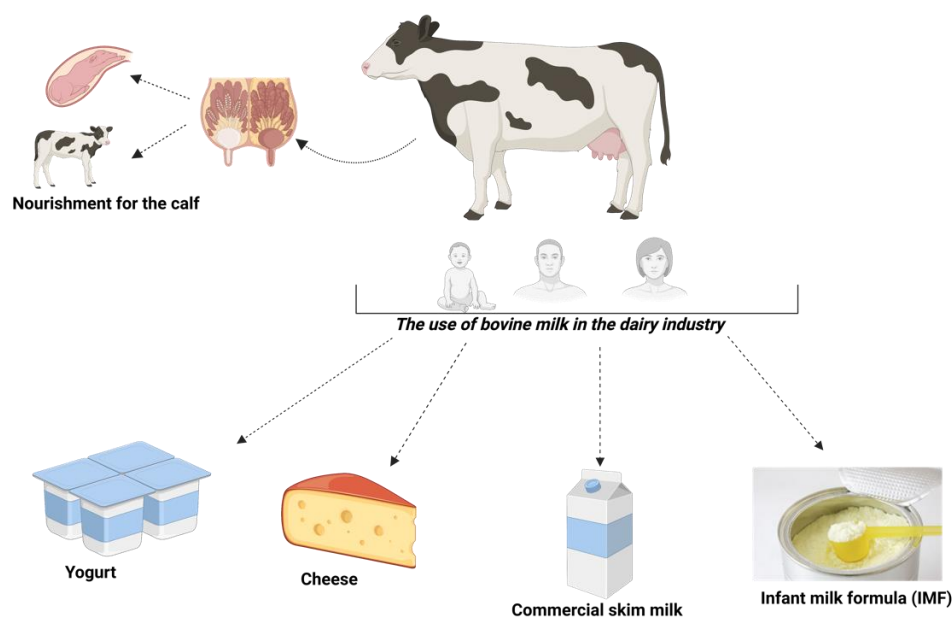


Figure 1.2: Bovine milk and its derivatives

First, source of nourishment for the calves. Then, an overview of the many uses of bovine milk in the dairy industry for human consumption

1.2.3 Infant Milk Formula (IMF) and the dairy industry

Infant milk formula (IMF) is developed using bovine milk as replacement for human milk (Hernell, 2011). However in order to process bovine milk to IMF, heat treatment is commonly utilised to destroy bacteria and several microorganisms (Vasbinder & de Kruif, 2003). IMF is available in powder and ready-to-feed forms. Ready-to-feed formula can be used as it is, while powder formula is mixed with water prior to use (Jiang & Guo, 2014). Therefore it is crucial that IMFs' starting material is of its finest quality (Jiang & Guo, 2014).

Similar manufacturing process are applied for both types of formula (Fig. 1.3). However, for the liquid formula two standardisation are required to guarantee sterility, so it can be stored for 6-12 months without facing textural changes. Furthermore, liquid formula is usually stored in an aseptic storage, hermetically sealed, and finally prepared for packaging, whereas the powder formula is more stable and less sensitive to contamination compared to liquid formula (Jiang & Guo, 2014). Finally, both formulae undergo rigorous testing for composition; for sterility to guarantee long-term quality of the product; and for shelf-life, before released for sale. In fact, it has been proposed there might be an increase of casein micelle size due to its association with whey proteins during the process (Anema & Li, 2003; Wijayanti et al., 2014). Thus, some bioactive components are added during IMF manufacture to restore the functional ingredients that are naturally found in human milk. These include polyunsaturated fatty acids, vegetable oils, human oligosaccharides (Joeckel et al., 2009).

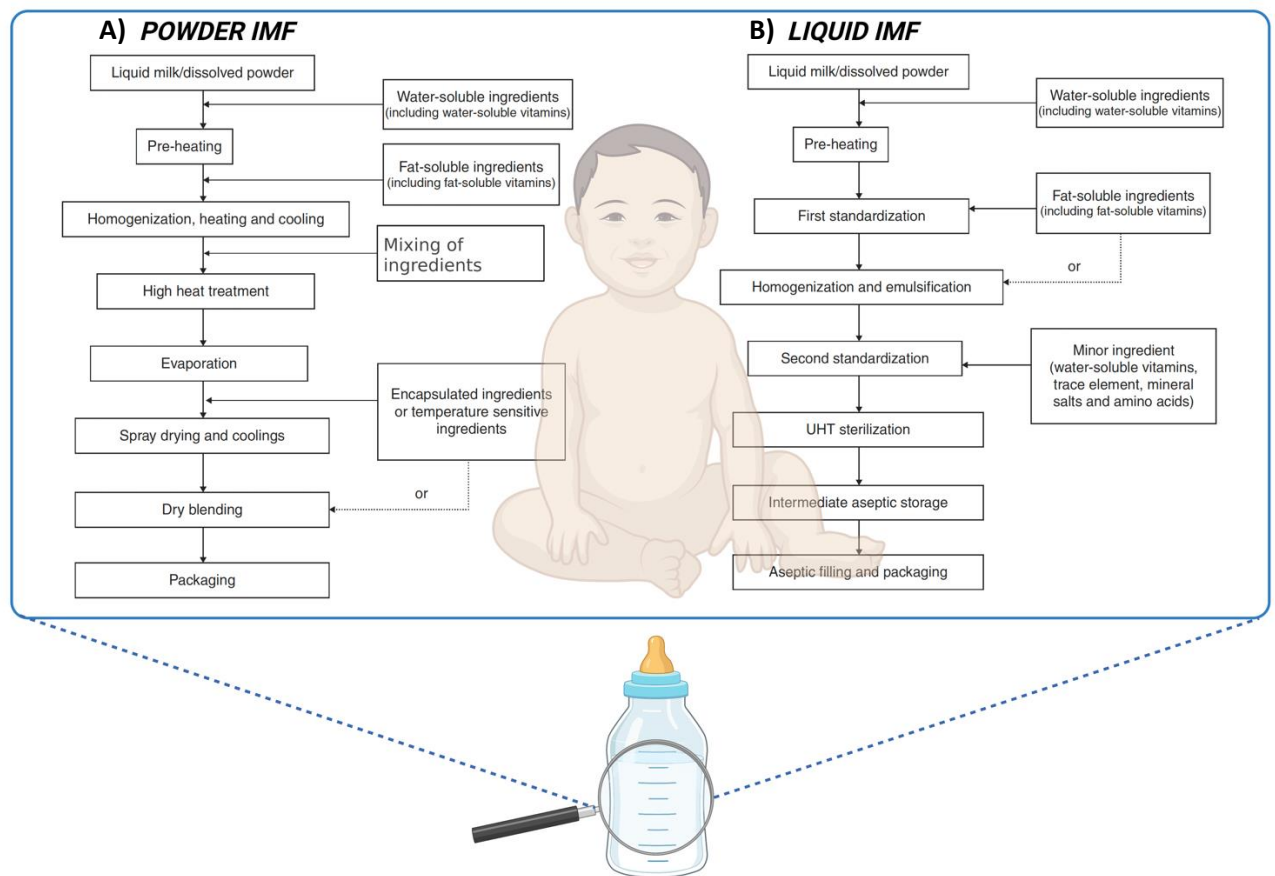


Figure 1.3: IMF manufacturing flowchart

Overview of the manufacture: A) powder IMF; B) liquid IMF adapted from (Jiang & Guo, 2014)

1.3 Extracellular vesicles

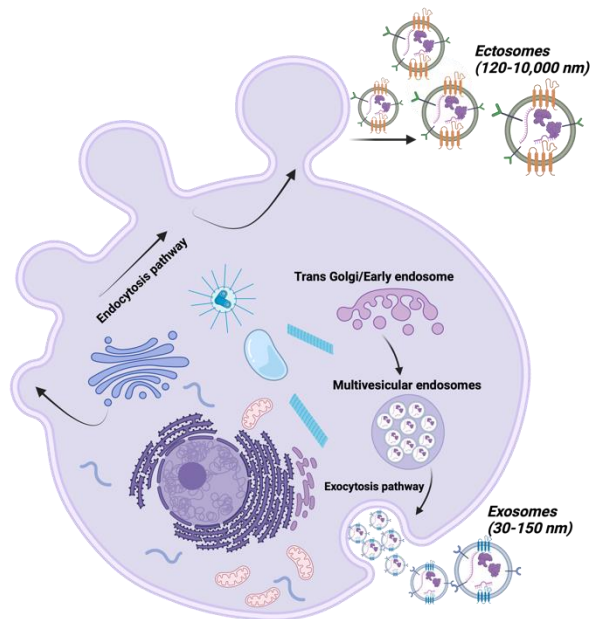
1.3.1 General definition and function

Extracellular vesicles (EVs) are membrane-surrounded vesicles which are released from most, if not all, type of cells into physiological fluids including plasma, urine, saliva and milk. Initially, EVs were observed by Wolf in 1967 as a formation of pro-coagulant “dust” around activated blood platelets (Wolf, 1967). Since then it has been well established by our research group and others that EVs are remarkably involved in cell-cell communication and in many other functions. EVs

represent a heterogeneous population of nanoparticles and their biogenesis share basic steps generally observed by various intracellular trafficking processes (van Niel et al., 2022). Generally EVs subtypes are defined by their cargoes which identify their origins and sorting fate and they are listed as follows: ectosomes (including microvesicles and oncosomes known as large EVs) which are 120-10,000 nm in diameter; exosomes (small EVs) with a diameter of 30-150 nm and apoptotic bodies which are 500-5,000 nm in diameter (Colombo et al., 2013; Mathieu et al., 2021; Santavanond et al., 2021), summarised in Fig. 1.4a. Specifically, ectosomes are released by outward budding of the cell membranes (Raposo et al., 2013; Mc Namee et al., 2018), while exosome are generated as intraluminal vesicles (ILVs) by a process which involves inward budding of the endosomal membrane either via endosomal sorting complex required for transport (ESCRT) dependent or independent mechanisms of cargo sorting. EVs are released upon fusion of the multivesicular bodies with the cell membrane and so subsequently released as exosomes by exocytosis. Conversely, apoptotic bodies are formed from apoptotic cells upon activation of apoptosis-related transduction pathways (van Niel et al., 2022) (Fig. 1.4b).

Other subpopulations of EVs have been reported. These include migrasomes (500-3,000nm) released during cell migration and generated from retraction fibres (Ma et al., 2015). Similarly, vesicles associated to the autophagy pathways such as amphisomes and exophers (1,000–10,000 nm) (Hessvik et al., 2016; Melentijevic et al., 2017), were also included in the EVs family. On the other hand exomeres, lower than < 50 nm in diameter, should not be considered as part of EVs subfamily, because they don't have a membrane (Zhang et al., 2018).

a)



b)

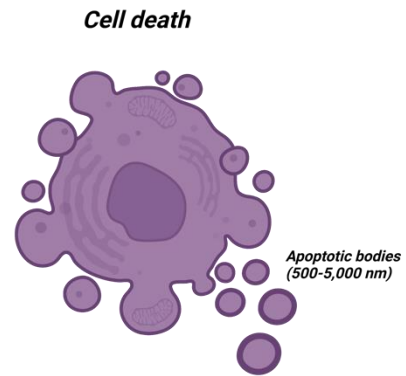


Figure 1.4 : EVs biogenesis and secretion

a) Exosomes (30-150nm) are assembled in multivesicular endosomes from the trans Golgi network, where the cargo sorting happens. Subsequently, exosomes are released via exocytosis in the extracellular space. Instead, ectosomes (120-1,000nm) and microvesicles are formed from budding of the cell membrane via the endocytosis pathway. b) Apoptotic bodies (500-5,000nm) are generated during cell death via apoptosis. Adapted from (van Niel et al., 2022).

EVs enable cells to pass information to cell recipient, even at long distance, while protecting the information provided (Izumi et al., 2015; Yáñez-Mó et al., 2015). Such signals can be transmitted by a wide categories of biomolecules, including proteins, lipids, nucleic acids and metabolites. Studies focussing on the smallest EVs of up to ~ 150 nm in diameter have shown them to include a wide range of cargo including coding and noncoding RNAs (ncRNAs), lipids, and proteins (Thai et al., 2007;Huang et al., 2013) (Fig. 1.5). An ongoing challenge is to define methods that will allow us to discriminate between different types of EVs as it is almost impossible to discern them

based on intrinsic properties, such as size, structure, buoyant density, or protein composition (Colombo et al., 2014). Based on density characterisation of EVs, (Colombo et al., 2014) described exosomes as equilibrating at densities ranging from 1.13 to 1.19 g/mL in sucrose. In 2018 guidelines were published by some members (including our research group) of the International Society on Extracellular Vesicles (ISEV) (www.isev.org) on the minimal information required regarding EVs separation and fundamental characterisation in order to accurately designate the term “EVs” (Fig. 1.5). These are intended to more clearly define EVs and help with their nomenclature based on characteristics, including protein markers associated with EVs (Colombo et al., 2014; Théry et al., 2018). After performing collection techniques, to confirm what is collected are EVs, ISEV recommends identification of those markers for EVs, using widely accepted techniques such as immunoblotting, imaging flow cytometry analysis (IFCM), immunoelectron microscopy, as well as estimation of particle sizes and concentration using method such nanoparticle tracking analysis (NTA) and analysis of EVs morphology by transmission electron microscopy (TEM) (Witwer et al., 2017). Moreover, as the EVs field continuously gathered more insights worldwide, the ISEV community led by Lorraine O’Driscoll, Joshua Welsh and Deborah Goberdhan is aiming to improve the current guidelines by exchanging knowledges and discoveries on EVs in a document which will be published in 2023.

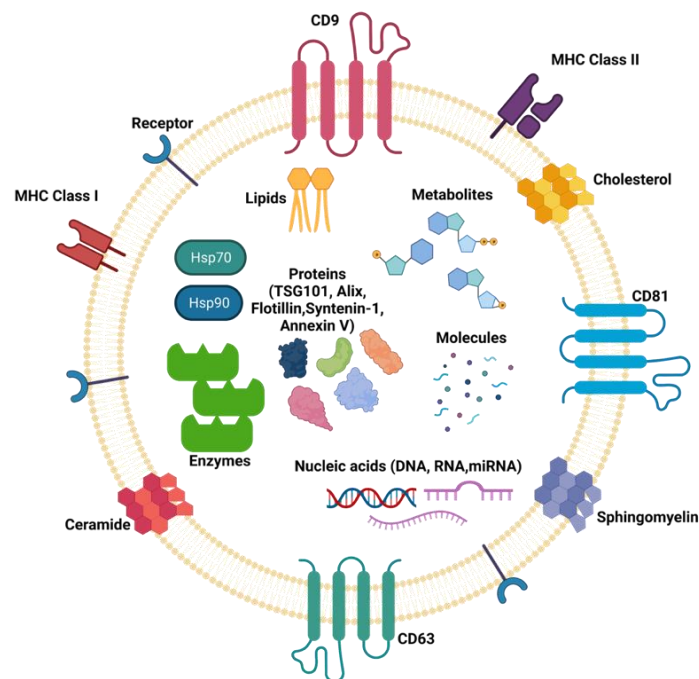


Figure 1.5: EVs structure and composition

A representation of EVs with their cargo such as proteins, nucleic acid, enzymes and metabolites. The EVs corona has transmembrane proteins like CD9, CD63, whereas typical molecules of phospholipidic bilayer are sphingomyelin and ceramide. EVs have also numerous receptors (MHC I and II) which are essential to mediate cell to cell communication.

1.3.2 Extracellular vesicles in milk

Milk EVs have been collected from multiple species such as human, bovine, camel, goat, sheep, donkey and many others (Admyre et al., 2007; Hata et al., 2010; Zonneveld et al., 2014). Milk also contain milk fat globules (MFGs) which are bigger than EVs ($> 300\text{nm}$); they are usually removed by skimming followed by filtration (Hu et al., 2021). However, human milk EVs and bovine milk EVs are the most studied because of their potential invaluable contribution to nourishment in feeding infants, adults, and calves, respectively. Milk EVs molecular composition largely depends on the mother/cattle lifestyle and their lactation stage (Torregrosa et al., 2014). Indeed, mammary gland epithelial cells release EVs into milk, which substantially mediate cell to cell communication between the mother and the new-born (Valadi et al., 2007). It has been reported the use of bovine milk in the dairy industry which include the production of IMF, used

worldwide as the best alternative to breast milk (Sobti et al., 2002). Therefore, we investigated the composition of IMF. Breast milk and bovine milk share similar characteristics (Haug et al., 2007), as reported in sections 1.2, but what is unknown and so important is to establish whether IMF contains EVs similar to human or bovine milk. In our study, described in Chapter 2, we reported that harsh conditions during IMF manufacturing, such as spray-drying at very high temperature, seem to damage EVs in IMF (Mukhopadhyaya et al., 2021). Therefore, the use of high-throughput characterisation techniques of the milk EVs content across species (proteomic, transcriptomic and metabolomics) may be useful to analyse the differences in characteristics that continuously coexist between HM and IMF (Mecocci et al., 2020, 2021).

1.3.3 Method of EVs separation from milk

Once EVs are in biofluids such as milk and then collected, they can be classified according to size, density and surface antigen, but definitive knowledge on other site of biogenesis and so their identification as exosomes or ectosomes is challenging, as these populations overlap in terms of composition and size (van Niel et al., 2022). In fact, because absolute EVs purification from other entities is recognised to be an unrealistic goal, the MISEV2018 preference is to replace “isolation” with the term “separation” (Théry et al., 2018). In accordance with a survey carried by the ISEV community (www.isev.org) in 2016, differential ultracentrifugation (DUC) was the most commonly used EVs separation methodology, with various other alternative techniques being used to varying degree such as density gradients, precipitation, filtration, size exclusion chromatography (SEC), and immunoisolation (Gardiner et al., 2016; Théry et al., 2018), summarised in Fig. 1.6. Of note SEC was one of the first technique applied to evaluate the diversity of protein cargo carried by EVs (Pan & Johnstone, 1983). Recently, field-flow and tangential flow filtration have been considered highly promising because they can overcome to

some of the main challenges for the EVs field, which are time and large-scale production (Mecocci et al., 2022). Instead, precipitation based protocols, including polyethylene glycol (PEG) is cheaper than many other separation methodologies but, especially for milk EVs, it is not suitable due to the contamination with PEG and this lower EVs' purity, whereas immunoaffinity might be useful in some medical fields, but it is particularly expensive (Mecocci et al., 2022). One of the initial studies to recover EVs from biological fluids was published by (Théry et al., 2006) detailing the sequential ultracentrifugation steps involved in the separation of EVs from cell culture supernatants or body fluids with additional EVs purification using density gradient. Moreover, our group also contributed to optimising some of these methods .

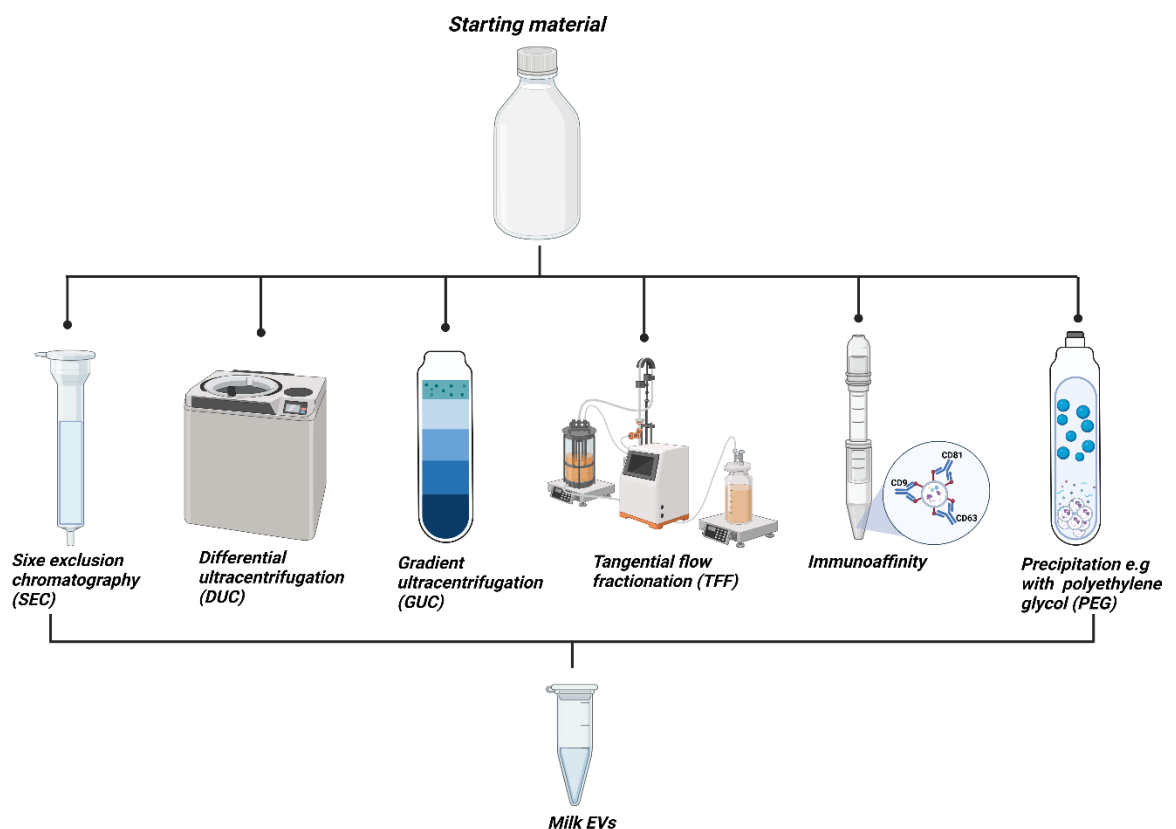


Figure 1.6: Summary of different EVs separation methods

All biofluids are different in composition and, compared to medium conditioned by cultured cells, many are more complex. As milk is composed of proteins, carbohydrates, lipids, cells and other biologically important components, a few protocols for EVs separation have been explored. These included ultracentrifugation on density gradients and SEC (Yamada et al., 2012; Zonneveld et al., 2014). Table 1.3, summaries some studies on milk EVs separation methods. The first report of the presence of EVs in HM was published by Admyre et al (2007). This study aimed to understand the role of EVs in breast milk in developing infants' immune system. EVs were collected by differential centrifugation (samples were collected from eight women) as an approach to clear all 'cells' and 'cell debris, before confirming the presence of a surface marker CD63 by immunoblotting. Density gradient ultracentrifugation (GUC) has been considered suitable to separate and characterise milk EVs using a combination of ultracentrifugation and SEC. In one study, milk was first centrifuged at 10,000 g and the supernatant underwent an additional step of purification by density gradient ultracentrifugation for 14–21 hours at 192,000 g (Zonneveld et al., 2014). Furthermore using GUC, it has been reported that milk EVs within a density range of 1.12–1.18 g/mL after pooling, were positive for CD9, CD63 and flotillin-1 and their sizes was approximately between 50-150 nm. Another group used DUC to collect EVs from skim milk, where they analysed isolates obtained after low speed ultracentrifugation at 12,000 g and 35,000 g and these subsets of ~ 100 nm in diameter were enriched in EVs markers (TSG101, alix) (Benmoussa et al., 2016). Similarly Samuel et al (2021) adopted DUC and further density gradient ultracentrifugation to separate EVs from bovine milk and they obtained milk EVs within the density range of 1.08–1.24 g/mL which were found to be enriched in EVs marker TSG101.

Separation methods	Milk source	Reference
Differential ultracentrifugation	Human	(Admyre et al., 2007)
Differential ultracentrifugation	Bovine	(Hata et al., 2010)

Differential ultracentrifugation and density gradient	Bovine	(Benmoussa et al., 2016, 2017, 2019, 2020)
Differential ultracentrifugation and density gradient	Human	(Zonneveld et al., 2014, 2021)
Differential ultracentrifugation and density gradient	Bovine	(Samuel et al., 2017, 2021)

Table 1.3: Summary of EVs separation methods from milk

1.3.4 Relevance of milk pre-treatment prior to EVs separation

Generally, milk fat is removed by centrifugation of milk at 12,000 g for 30min at 4°C (Lock et al. 2004), a method that is widely used in the dairy industry to produce skim (defatted) milk. Based on these traditional methods, dilution of skim milk using a urea-based buffer, acetone precipitation of full cream milk, ammonium sulfate and acetic acid removal of caseins to isolate whey proteins, or low-speed centrifugation were also explored (Vincent et al., 2016). Interestingly, the use of these methods also indicates the complexity of the milk matrix that attributes to not having an optimal separation method for milk fats and proteins. However, as explained in Section 1.2 caseins are organised in $10^{14} - 10^{16}$ micelles tightly packed per mL of milk and are 50–500 nm (av. 120 nm) in diameter. Kunz et al (1990) reported that the most common procedure to remove casein micelles is precipitation at pH 4.6. Conversely, without this pH adjustment, high residues of casein were found in the whey fraction. Hence, a combination of precipitation and filtration techniques should be employed to effectively remove milk proteins and fats, which may act as contaminants in downstream analyses of EVs. Therefore, to optimise milk EVs separation, Somiya et al (2018) described the positive effect of low pH on EVs yield and enrichment. By adding acetic acid before EVs separation from bovine milk, they achieved a higher yield of EVs compared to non-treated milk by promoting casein

removal. Yamauchi et al (2019) compared isoelectric precipitation versus non pre-treatment of raw bovine milk followed by differential ultracentrifugation. Hydrochloric acid (HCl) was used to promote casein aggregation and facilitate the separation process. Their study concluded that the acidification by isoelectric precipitation does not negatively affect EVs yields and size. Another study used acidification of bovine milk followed by differential ultracentrifugation, milk EVs collected were 125 nm in diameter and TEM analysis did not show any breakage of the EVs membrane (Rahman et al., 2019). Immunoblotting confirmed enrichment of the EVs marker CD63 in the EVs separated following HCl treatment of milk (Rahman et al., 2019). Therefore, because Somiya et al (2018) and Yamamuchi et al (2019) did not report any modifications of EVs, milk pre-treatment using acetic acid or isoelectric precipitation prior to separate EVs are highly promising techniques to obtain pure EVs at increased levels. We considered these approaches to obtain enriched EVs samples from milk, thus we developed our own methodology described in Chapter 2.

1.4 Characterisation of milk EVs

1.4.1 Particle size and concentration

Milk EVs, like EVs from other sources can be characterised using a combination of optical techniques commonly used to determine the size distribution of nanometre-scale particles in solution. Dynamic light scattering (DLS) is based on the principle of analysis of the fluctuations in the intensity of light scattered by nanoparticles upon illumination with a laser beam (Szatanek et al., 2017). These fluctuations are caused by the Brownian motion of the particles in solution. However, there are some limitations to this technique most commonly associated with polydisperse samples, where the size determination by DLS is questionable (Claudio et al. 2017). For this reason, few research articles have used DLS as a characterisation technique for milk EVs

(Arntz et al. 2015, Benmoussa et al. 2016, Benmoussa et al. 2017). The most commonly used by the EVs community is the nanoparticle tracking analysis (NTA), which is considered more reliable, compared to the DLS, for determination of size distribution and concentration of EVs particles. NTA technology uses a conventional microscope for direct visualisation of individual scattering particles, in Brownian motion, illuminated by a laser beam, measuring the hydrodynamic radius. Some advantages of NTA are its precision to measure particles down to 30 nm in size, ease of use, and its suitability for polydisperse samples (Krzyworzeka et al. 2017). It is interesting to note that EVs measure approximately 50-265 nm in human milk and 44-200 nm in bovine milk (Hu et al., 2021). Therefore, based on the MISEV2018 guidelines (Thery et al. 2018), vesicles of this range are considered small to medium vesicles. Furthermore, for the identification of EVs sub-populations, these methods might not be suitable thus further studies are required. However, there are other techniques which might be used for EVs quantification, but NTA is accessible, easier and widely used by the ISEV committee.

1.4.2 Morphology

Microscopy techniques are used for evaluating the structural and morphological features of EVs. Due to the size of EVs, which is below the diffraction limit of visible light, EVs can be only resolved using method such as electron microscopy (EM) which has become a method of choice to investigate their structure and morphology. EM utilizes electrons, instead of photons, to create an image with a resolution in the nanometer scale. The most common EM techniques used, in the EVs field, are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). TEM-based imaging has an advantage due to its ability to include immuno-staining. This allows the detection of the presence of specific biomolecules on the outer surface of a vesicle, typically using gold-conjugated antibodies. This technology helps in identification of EVs sub-

populations that exhibit a pre-defined biomolecular identity and assigning this identity to a specific size and morphology. However, one drawback of TEM is that it cannot be used to quantify EVs, but it results in a 2D image of the EVs based on their transparency. Several studies have used TEM to characterise EVs isolated from bovine milk and human milk (Admyre et al., 2007; Zonneveld et al., 2014, 2021; Pisano et al., 2020) and milk from other species (Chen et al. 2014, Badawy et al. 2018). For instances, Admyre et al (2007) characterised human milk EVs by TEM, in conjunction with immunogold labelling. The outcome indicated that both colostrum EVs and mature milk EVs were positive for HLA-DR, Hsc70 and CD81. Another technique used to characterise EVs from milk is atomic force microscopy (AFM). The principle of AFM is based on the use of a sharp tip mounted at the end of a cantilever to monitor the interaction force between the tip and a sample deposited onto a surface whereby forces acting on the tip will lead to a deflection of the cantilever (Rupert et al. 2017, Szatanek et al. 2017). While AFM is a highly sensitive technique, and provides information about physical properties of the vesicles by measuring EVs to nanometre and sub-nanometre resolution, it is not commonly used. However, only some studies have applied this methodology for characterisation of milk EVs (Aqil et al. 2016, Aqil et al. 2017, Wang et al. 2017).

1.4.3 Surface markers

The MISEV2018 guidelines outline analysis of bulk EVs preparations to demonstrate the presence proteins associated to the category 1 (Transmembrane or GPI-anchored proteins); 2 (Cytosolic or periplasmic proteins) and assess their purity from common contaminants in category 3 (non-EVs structure related proteins), but no universal “negative controls” relevant to a particular subtype of EVs have been identified yet. These proteins are listed in Table 1.4. In milk-related studies, certain markers that belong to the above-mentioned category 1 or 2 are used more frequently than others. In fact Lasser et al (2011) separated breast milk EVs by DUC

to discern which subtypes exist. Immunostaining analysis of EVs revealed that breast milk EVs were positive for tetraspanins CD9, CD81, CD63 and immunoblotting analysis confirmed the presence of Hsc70 and CD81, but they were negative for the endoplasmic reticulum marker calnexin. Interestingly, MFGE8 protein, also known as lactacadherin, is frequently analysed in bovine milk EVs studies and is reported to be an exosomes-associated protein whereas lactoferrin, a non-EVs related milk protein, has been used in human milk EVs studies as an indication of contamination of separated EVs fractions with milk proteins (Lässer et al., 2011).

Protein marker	Exosomes	Ectosomes	Size (kDa)
TSG101(cytosolic protein)	x		49
CD63, CD81, CD9	x	x	25;26;25
CD82,CD53,CD37		x	30-90;26;40-52
CD45		x	220
Alix	x		96
Flotillin	x		47
Caveolin	x		46
HSC70	x		70
Sythenin	x	x	32
Calnexin		x	67
GRP94		x	94
Actinin 1-4		x	105

Table 1.4: EVs protein markers categorised per subtype protein size

1.4.4 Flow cytometry

Flow cytometry-based characterisation is also used for both quantitative and qualitative analysis of EVs and in case of milk EVs. Over time, a development was made to the technique where the EVs were fixed on large fluorophore-conjugated antibody labelled beads before analysing using a flow cytometer. This method of fixing the EVs on beads only provides information on the presence of surface proteins. Most flow cytometry approaches lack the high sensitivity to detect

smaller EVs, with limited literature present on validation and standardisation of this technology. In 2019 an article demonstrated the use of imaging flow cytometry in the phenotyping of EVs *in vitro* and plasma samples (Ricklefs et al. 2019). Based on that publication, our group has performed imaging flow cytometry analysis of EVs separated from commercially available bovine milk and infant milk formula by different separation techniques and analysed EVs specific markers CD9, CD81, CD63, ADAM10 and HLADR (Mukhopadhyaya et al., 2021) (Fig. 1.7). Although in the past Admyre et al (2007) used flow cytometry to confirm the distribution of HLA-DR a MHC class II cell surface receptor, CD81 and CD63, they did not apply the same technique as we used in our study. Therefore we were one of the first to perform flow cytometry on enriched milk EVs samples, including IMF EVs.

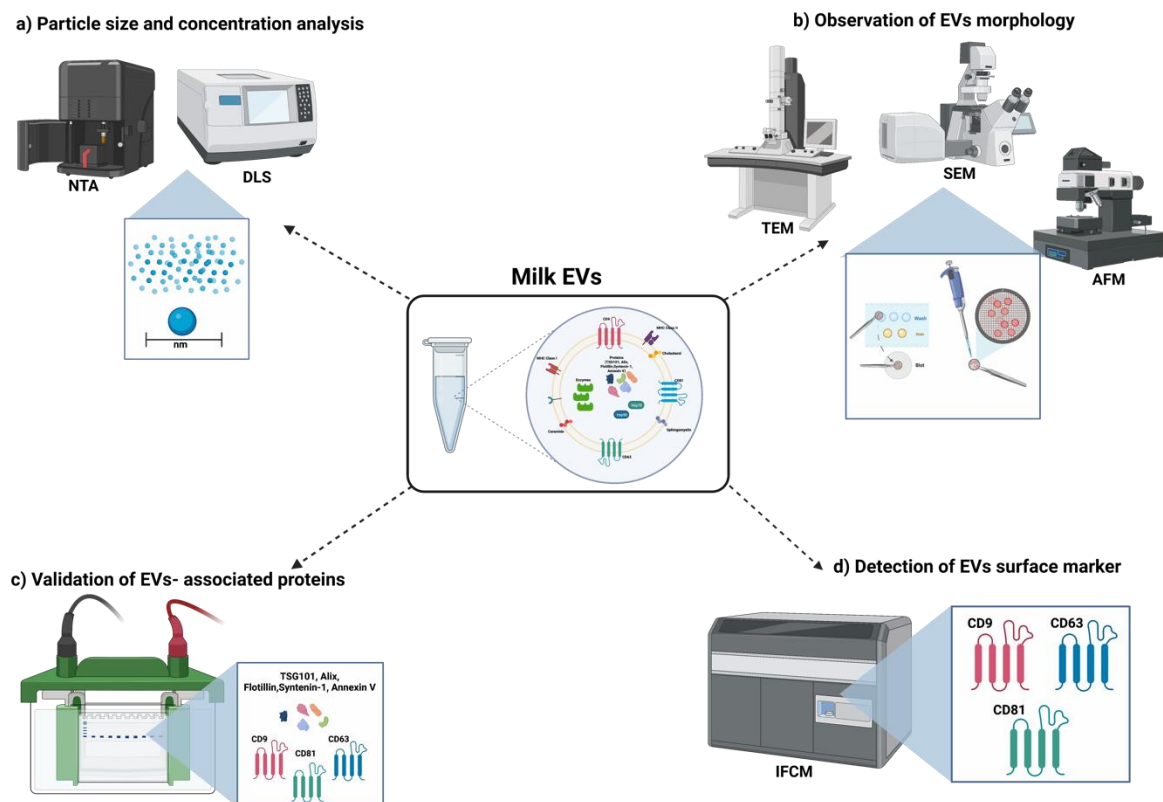


Figure 1.7: Milk EVs characterisation

a) Nanoparticle tracking analysis (NTA) or dynamic light scattering (DLS); b) transmission electron microscopy (TEM) or scanning electron microscopy (SEM) and atomic force microscopy (AFM); c) immunoblotting; d) imaging flow cytometric analysis (IFCM).

1.5 High throughput analysis of milk EVs cargo

1.5.1 Proteomics

In depth proteomic analysis of EVs may be performed by LC/MS-MS (Fig. 1.8). Particularly, in case of EVs separated from milk, we believe it is important to know what protein they carry. In fact, proteins are an invaluable component of milk because they provide nutrition for infants and also trigger the construction of the intestinal flora (Zhang et al., 2022). Considering infant nutrition, although IMF is used as common substitute of HM, it might lack functional benefits naturally carried by HM therefore there have been significant efforts to humanise IMF. Accordingly, peptides are one of the additional components which may be used to make IMF closer to HM. Regarding milk EVs, several attempts have been made to analyse and categorise the protein cargo of EVs. In fact different studies have reported newly discovered datasets of proteins identifies in milk EVs. For instances, Samuel et al (2017) using quantitative proteomics analysis showed that the proteomic cargo of EVs changes based on the lactation state of the cows. Specifically, mature milk EVs were enriched with proteins related to transport and apoptosis, whereas colostrum derived EVs were more enriched with proteins implicated in the immune response, inflammatory response, antimicrobial peptides, cell growth and complement activation, compared to mature milk EVs. Similarly Rahman et al. (2021) explored the proteomic profile of small EVs (sEVs) in late-stage lactating cows, where they identified 2225 proteins of which 429 were newly discovered. Among these, HSPA1B, RAB27B, and JCHAIN were highly enriched in milk sEVs from late-stage lactating cows and it was proposed that they may provide

nutrition that helps to prevent inflammatory disorders and promote health. The study concluded that the mammary gland undergoes major physiological alterations to adapt to the dry period while maintaining immune protection. A subsequent study investigated the bovine milk EVs proteome, where 20 proteins specific to the 35K fraction and 41 proteins (from the endosomal compartment, vacuole and extracellular region proteins) were specifically enriched in 100K fraction. Bioinformatics pathway analysis indicated that the 35K pellet contained EVs proteins involved in regulation, proliferation and cell survival, whereas the 100K EVs, likely to be small EVs, might be involved in metabolism, extracellular matrix turnover and immunity (Benmoussa et al., 2019).

1.5.2 Metabolomics

While proteins and nucleic acids that are cargo of EVs have been extensively studied, much less is known about metabolites carried by EVs. Metabolites are molecules of less than 1.5 kDa and metabolomics is a gold standard technique to identify chemical composition and quantify metabolites from biofluids driven by various biochemical processes (Lopez et al., 2020).

It is a vital branch of “-omics” science, together with proteomics and transcriptomics, focused on the study of the composition, relative abundance, dynamics, and interactions among metabolites in a particular organism. There are two different approaches in this field: targeted and untargeted metabolomic analysis. The first one quantifies *a priori* determined known metabolites across several metabolite classes, but targeted metabolomic studies are limited to the analysis of the specific set of quantified metabolites. Whereas, the untargeted approach uses complementary information to characterise potential truly novel insights of unknown metabolites (Phan et al., 2019). Additionally, high-field ^1H -NMR spectroscopy is highly efficient

for detecting several compounds in milk, including sugars, small organic acids, vitamins, nucleotides, and aromatic compounds; thus, representing one of the best tools for milk metabolomics research (Sundekilde et al., 2013; Rocchetti & O'Callaghan, 2021) (Fig. 1.8). HMOs (*e.g.* disialyllacto-N-tetraose) are abundant in human milk and much less likely to be associated with necrotizing enterocolitis, in comparison with preterm infants fed with formula. (H. Yu et al., 2014; Rasmussen et al., 2017). Moreover, knowledge about the composition and function of these metabolites are still accumulating and little is known about metabolites encapsulated in milk EVs. Thus, along with the protein content of milk EVs, we designated a new approach to analysed metabolite transported by milk EVs which will be discussed in Chapter 7.

1.5.3 Transcriptomics

Within the “-omics” techniques, the analysis of EVs transcriptome has gathered huge interest. Particularly, EVs transports miRNAs which are enclosed in their phospholipid bilayer and can potentially reach the designated cells after surviving to very harsh condition such as those of gastric digestion. Quantitative PCR (qPCR) is a widely accepted methodology used to analysing miRNAs contained in EVs, as summarised in Fig. 1.8. Specifically, milk EVs have been reported to be stable after passing through the GI tract. EVs collected from bovine milk while tested on intestinal cells determined an increased expression of miRNAs carried by milk EVs compared to levels observed for the cells exposed to skim milk (López de las Hazas et al., 2022). Similarly Samuel et al (2021) administrating milk EVs orally, proved the stability of bovine milk EVs in delivering their cargo. Interestingly in both cases the amount of miRNAs detected was consistent pre-digestion and post-digestion. Another study on human breast milk was performed by Zhou et al (2011) focused on multiple miRNAs transported by milk EVs which are reportedly involved in immune responses. It was shown that those miRNAs remain stable at harsh condition, such

as freeze-thaw cycles or prolonged room temperature incubation (Taganov et al., 2006; Eichner et al., 2010). Furthermore, Munagala et al (2016) studied bovine milk EVs as therapeutics during infancy. They reported the presence, in milk EVs, of several mammary gland and immune function related genes and miRNAs which are known to be involved in promoting thymic regulatory T cell maturation (Melnik et al., 2014). Zemleni et al (2017) catalogued exosome miRNA cargos in human milk and reported low concentrations of miRNAs carried by EVs in IMF compared to HM (Leiferman et al., 2019). Furthermore the miRNA content in milk is unique because of the high amounts of immune-related miRNAs which have been found also during the lactation period (Kosaka et al., 2010; Zhou et al., 2012). More importantly, relevant differences in cargo between human and bovine milk EVs and IMF EVs have not been established yet, suggesting that this field warrants further studies and accurate analysis on milk EVs cargo.

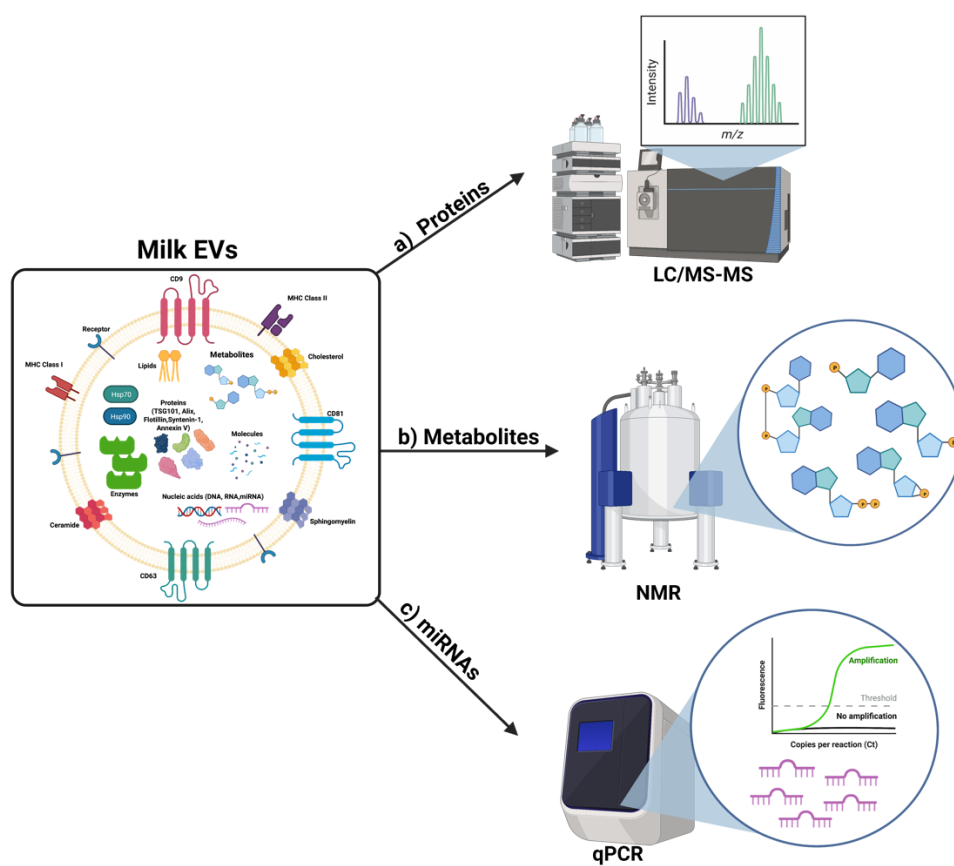


Figure 1.8: Multi-omics characterisation of milk EVs cargo

EVs cargo in depth analysis: a) Liquid chromatography–mass spectrometry (LC/MS-MS); b) NMR; c) qPCR.

Aims of project

There is still a vast debate about EVs similarities/discrepancies between IMF and HM, especially in terms of nutrition for infants and in bovine milk during the lactation period. Furthermore, the effects of IMF manufacturing on EVs has not been studied yet. Despite a separation of pure EVs is considered by the ISEV committee an “unrealistic goal”, we intended to establish an optimised methodology which could be applied for different milk EVs sources (human milk, bovine milk and infant milk formula) and subsequently evaluating several methodologies to characterise milk EVs

1. Our first aim was to compare the HCl or acetic acid treatment of milk (highly abundant in milk and of similar sizes to EVs) with milk without treatment for efficient removal of casein micelles and subsequently correlate differential or gradient ultracentrifugation methodologies for the separation of enriched milk EVs.
2. Afterwards, our methodology was applied to separate and characterise EVs from commercial skim milk and IMF
3. Considering that milk composition changes dramatically during the lactation period, we wanted to investigate similarities/differences of EVs between COL, FM and MM over the lactation curve in Holstein-Friesian cows

4. Industrial processing of milk might have detrimental consequences on EVs, therefore we aimed to evaluate the extent to which EVs survive during IMF manufacturing and observe at which stage of the process EVs were mainly affected.
5. Comparative analysis of EVs from IMF, the best alternative, and HM the “gold standard”. Our purpose was to understand the analogies and diversities amongst IMF EVs and HM EVs. We hypothesised that the harsh thermal treatments of bovine milk during the production of IMF leads to disruption of EVs, which might be extended also to IMF EVs cargo and could affect the quality of the final product used to feed infants.
6. Furthermore, we compared the EVs proteome using mass spectroscopy, EVs metabolites by ^1H -NMR and EVs miRNAs with qPCR in IMF and HM for in depth high throughput analysis.
7. Ultimately, we performed bioinformatics analysis of the data obtained by multi-omics characterisation of EVs cargo.

- 2 **Chapter 2: Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk**

2.1 Abstract

Many infants are fed IMF. However, IMF production from SM involves harsh treatment. So, we hypothesised that the quantity and/or quality of extracellular vesicles (EVs) in IMF may be reduced. Thus, firstly, we aimed to optimise separation of EVs from IMF and SM and, secondly, we aimed to compare the EVs isolates from these two sources. Prior to EVs separation, abundant casein micelles of similar sizes to EVs were removed by treating milk samples with either acetic acid or hydrochloric acid. Samples progressed to differential ultracentrifugation (DUC) or gradient ultracentrifugation (GUC). EVs characterisation included BCA, SDS-PAGE, nanoparticle tracking (NTA), electron microscopy (TEM), immunoblotting, and imaging flow cytometry (IFCM). Reduced EVs concentrations were found in IMF. SM-derived EVs were intact, while IMF contained disrupted EVs-like structure. EVs biomarkers were more abundant with isolates from SM, indicating EVs proteins in IMF are compromised. Altogether, a suitable method combining acid pre-treatment with GUC for EVs separation from milk products was developed. EVs appear to be substantially compromised in IMF compared to SM.

This chapter was published in *Food Chemistry* as research article (Appendix III).

“Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk.” Anindya

Mukhopadhyaya, **Jessie Santoro**, Barry Moran, Zivile Useckaite, Lorraine O'Driscoll. *Food Chemistry* Volume 353, 15 August 2021, 129309
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2.2 Introduction

Bovine milk is considered as a major source of nutrition for humans and consumed, across the globe, by all age groups. The proposed biological function of extracellular vesicles (EVs) in milk, including their role in cell growth and immune system modulation, is gathering increasing interest (Benmoussa & Provost, 2019). IMF, typically made from SM, is frequently used to fulfil the nutritional requirement of infants and toddlers. However, although the industrial processing stages of IMF manufacture from SM involves extensive thermal treatments steps -including sterilisation, evaporation and spray-drying that can involve temperatures of up to 280°C (Gathercole et al., 2017; Burke et al., 2018)- the influences of these harsh conditions on EVs within IMF has not been well investigated. This may be, at least in part, because milk and IMF are very complex fluids and a suitable method that is in keeping with MISEV2018 guidelines for separating and characterising EVs from both has not yet been reported. However, as the EU Commission Directive (2006/141/EC) specifies that IMF composition must be of sufficient biological quality, IMF and dairy ingredient industries needs to know how representative IMF is of SM. They need to know whether EVs are present and maintained as in SM or, alternatively, if efforts would be needed to retain/supplement EVs. Simply put, industry -and consumers- need to know what's in their IMF product. Substantial efforts have been invested in analysing EVs from bovine milk (Hata et al., 2010; Yamada et al., 2012; Reinhardt et al., 2013; Arntz et al., 2015; Wolf et al., 2015; Munagala et al., 2016b; Samuel et al., 2017; Vaswani et al., 2017; Somiya et al., 2018) and human milk (Admyre et al., 2007; Lässer et al., 2011; Zonneveld et al., 2014; Herwijnen et al., 2018). The removal of some “debris”, including casein, before proceeding to EVs separation from milk has been attempted with differential centrifugation, filtration, or treatment with EDTA or acids (Jensen et al., 2012). However, although extremely high concentrations *i.e.*, 10^{14} – 10^{16} /mL of casein exist in milk (O'Regan et al., 2009; Fox & Brodtkorb,

2008) which -because of their size (approx. 150 nm), fall well within the size range of EVs- a standardised pre-analytical protocol for casein micelles removal prior to EVs analysis is lacking. Furthermore, attempts to remove casein micelles from IMF prior to subsequent EVs analysis have yet to be reported. Based on a survey in 2016, DUC is the most commonly used EVs separation technique, with alternatives techniques such as GUC, SEC , and precipitation techniques used to a varying degree (Gardiner et al., 2016). Following a report by (Admyre et al., 2007) on the separation of EVs from breast milk using DUC, a number of subsequent studies used DUC-based approaches (Izumi et al., 2012; Reinhardt et al., 2013; Kusuma et al., 2016; Manca et al., 2018a). However, as reviewed by (Witwer et al., 2013) EVs samples separated by DUC methods are often quite impure as DUC does not take into consideration the density of EVs. GUC, especially the 'bottom-up' approach, was proposed to much more efficiently separate EVs from proteins and other such contents that have similar densities to EVs. Some subsequent milk EVs studies used GUC based approaches as their method of EVs separation (Sun et al., 2015; Benmoussa et al., 2016; van Herwijnen et al., 2016; Yamauchi et al., 2019). Of course, every research method has its pros and cons and so, ideally, such methods should be run in parallel on the same samples and critically compared. This is something that we addressed in this study where our main hypothesis was that industrial processing of SM to IMF leads to loss of EVs' quantity and/or quality. We also proposed that using ultracentrifugation alone may not be adequate to remove most casein micelles from either milk or IMF and so a pre-treatment would be needed to separate the most enriched EVs possible from these sources. So here, after investigating options of pre-treatment (acetic acid or isoelectric precipitation) to remove casein micelles - that would otherwise confuse EVs analysis- from SM and IMF, we compared SM EVs and IMF EVs that were separated by GUC or by DUC.

2.3 Material and Methods

2.3.1 Sample collection and infant milk formula reconstitution

Bovine skim milk (n=3 independent batches) and IMF, prepared as recommended by the manufacturer, (n=3 independent batches) were compared. Samples were commercial samples purchased from local vendors, as these are the sources that would be available to consumers to provide to infants/children. The preparation of IMF from its powder form was exactly as recommended by its manufacturer, in order to replicate the product that would be prepared to feed to infants. Specifically, 4.4 g of IMF powder was mixed with 30 mL of water that had been freshly boiled and then allowed to cool naturally. Crucially, a fresh batch of IMF was prepared for each biological repeat experiment. When prepared following the instruction on the container, the IMF was described by its manufacturer as a 'nutritionally complete' breast milk substitute with 0.7 mg per 100

mL feed iron content. Each independent batch of SM and IMF was divided into 3 equal aliquots. One was untreated (NT), and the others were pre-treated with either acetic acid or with HCl for isoelectric precipitation (IP) in an effort to remove abundant casein micelles and from the resultant whey, 38 mL was used for differential ultracentrifugation and 2.33 mL for gradient ultracentrifugation. All subsequent analyses detailed in the study were performed a minimum of 3 times.

2.3.2 Filtration and milk pre-treatment with HCl in SM and IMF

For the untreated samples, 50 mL of SM or IMF were centrifuged at 2,000 g for 20 min at 4 °C. The supernatant was carefully collected and the pellet containing any remaining fat globules or debris was discarded. This supernatant was filtered using Whatman™ Grade 1 filter paper

(diameter 150 mm) (GE Healthcare Life Sciences, Ireland). The filtrate obtained from untreated SM was designated as SM_NT and the filtrate obtained from untreated IMF was designated as IMF_NT. The AA treatment of SM and IMF (50 mL in both cases) was performed using a modification of a method previously reported for defatted bovine milk (Somiya et al., 2018). The modifications were that 6 N acetic acid was used (equivalent to the HCl) and additional filtration and ultracentrifugation steps were introduced in an effort to remove all casein protein aggregates and casein micelles, which reportedly was not achieved in the earlier study (Somiya et al., 2018). In brief, 6 N acetic acid (Sigma-Aldrich, Ireland) was used to adjust the SM or IMF to pH 4.6 and further incubated for 10 min at RT and centrifuged at 5,000 g for 30 min at 4°C. Furthermore, instead of performing only a 0.22-µm filtration and ultracentrifugation of the resulting whey at 210,000 g, a 3-step filtration was performed prior to subsequent ultracentrifugation steps. Specifically, the supernatant was passed through a Whatman™ Grade 1 filter paper (diameter 150 mm) (GE Healthcare Life Sciences, Ireland), followed by 0.45-µm membrane filtration and then 0.22-µm membrane filtration (Thermo Fisher Scientific Ltd, Waltham, MA, US). The filtrate from SM was designated as SM_AA and that from IMF was designated as IMF_AA. Isoelectric precipitation (IP) was performed using a modification of a method previously reported by Yamauchi et al (2019) for raw bovine milk. In brief, here the pH of SM or IMF were adjusted to pH 4.6 using 6 N HCl (Sigma-Aldrich, Ireland) to remove caseins. The pH adjusted SM or IMF was placed on ice for 10 min followed by centrifugation at 5,000 g for 30 min at 4°C. Resulting whey was subject to the 3-step filtration procedure. The filtrate from SM was designated as SM_IP and that from IMF was designated as IMF_IP. An overview of the methodology used here is represented in Fig. 2.1. While the protocol used by Yamauchi et al (2019) then proceeded to a sucrose gradient we, instead, used OptiPrep™ gradient and differential ultracentrifugation techniques, detailed in the following sections. Of note, neither Somiya et al (2018) nor Yamauchi et al (2019) analysed in EVs in IMF.

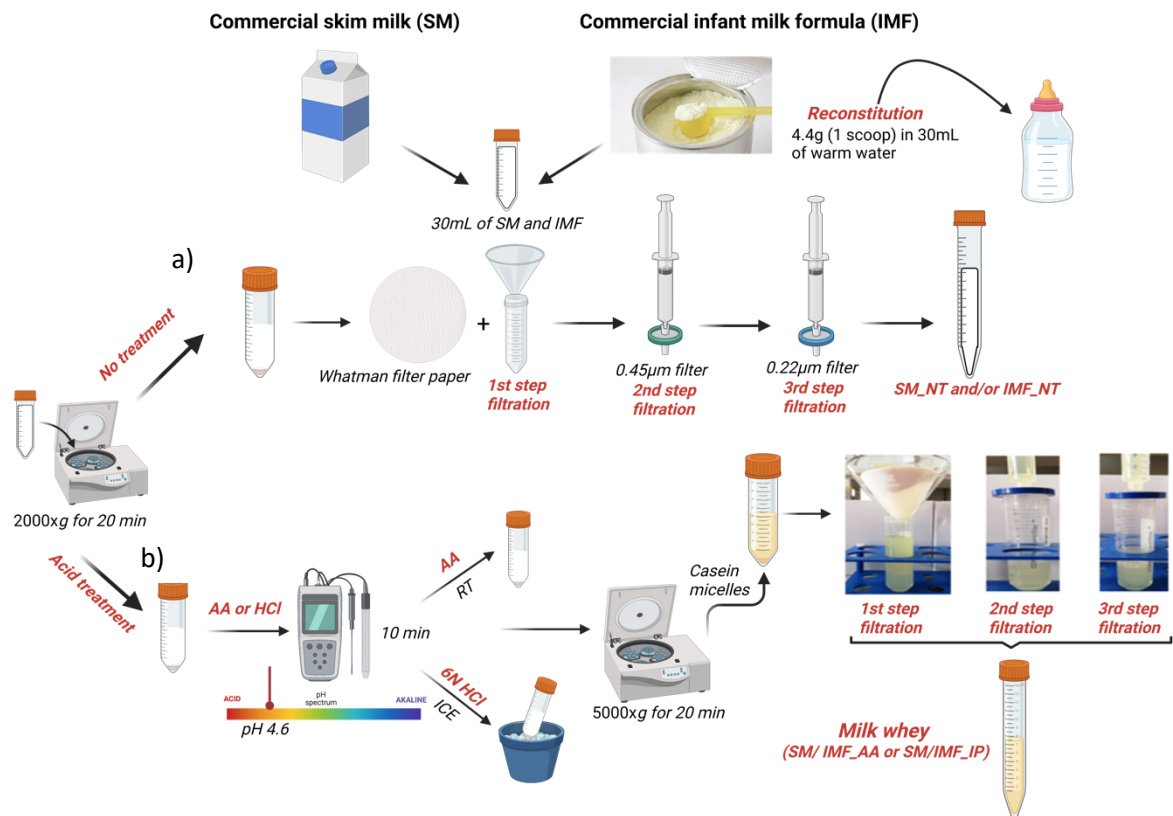


Figure 2.1: Summary of milk pre-treatment

SM and IMF pre-treatment before EVs separation. a) No treatment (NT): 30 mL of SM and IMF were centrifuged followed by three step filtrations. SM_NT (skim milk non treated); IMF_NT (infant milk formula non treated). b) Acid treatment: 30 mL of SM and IMF were mixed with AA (RT) or HCl (in ice) for 10 min, centrifuged followed by three step filtrations. AA (acetic acid); HCl (hydrochloric acid); SM_AA (skim milk acetic acid); IMF_AA (infant milk formula acetic acid); SM_IP (skim milk isoelectric precipitation); IMF_IP (infant milk formula isoelectric precipitation).

2.3.3 Separation of EVs by ultracentrifugation (UC) using either gradient UC or differential UC in SM and IMF

GUC was performed using iodixanol solution OptiPrep™ (60% (w/v)) (Sigma Aldrich, Ireland; Cat. #D1556) following the bottom-up technique (Kowal et al., 2016) (Fig. 2.2b). Briefly, a 40% (w/v) bottom layer was made by diluting 60% OptiPrep solution with each of the 6 sample types (4.66 mL OptiPrep + 2.33 mL sample = 7 mL). Further layering was done by a discontinuous gradient of 30% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol, made by diluting a stock solution of OptiPrep (60% (w/v)) with sterile PBS (Sigma Aldrich, Ireland). The gradient was

formed by adding 3 mL of the iodixanol solutions to a 17 mL polypropylene tube (Beckman Coulter, Brea, CA, US). Ultracentrifugation was performed using a SW Type 32.1 Ti rotor in Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 186,000 g for 18 h at 4°C. Post 18 h centrifugation, the supernatant was carefully pipetted out in 1 mL fractions. Their refractive indexes were recorded, and densities were calculated for all fractions. Based on previous studies (Colombo et al., 2014; Kowal et al., 2016) in this study fractions with densities of 1.05–1.19 g/mL were pooled for further analyses. Of note, as we aimed to separate all EVs present in SM and IMF and considering that (Kowal et al., 2016) observed positive signals for EVs proteins in fractions with densities of 1.08 g/mL, we chose 1.05–1.19 g/mL fractions. Post-pooling, the sample was washed in sterile PBS at 110,000 g for 90 min at 4°C before resuspending the pellet in 1 mL of sterile PBS. Differential ultracentrifugation (DUC) separation of EVs from the untreated or treated SM and IMF samples was performed based on protocol published by Benmoussa et al (2017) with addition, in this study, of a final step of ultracentrifugation at 200,000 g. A similar separation of EVs from urine (Gonzales et al., 2010; Musante et al., 2013) and milk (Somiya et al., 2018) at 200,000 g have been reported previously. In brief, samples were filled into 38 mL open-top polypropylene tubes and subjected to DUC using a SW Type 32 Ti swinging bucket rotor (Beckman Coulter, Brea, CA, US) in an Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA). Consecutive spins were at 12,000 g (12 K), 35,000 g (35 K), 75,000 g (75 K), 100,000 g (100 K) and 200,000 g (200 K) for 90 min each at 4°C. After each centrifugation step, the resulting pellets (12 K, 35 K, 75 K, 100 K or 200 K) were collected, resuspended in 1 mL filtered PBS and stored in -80°C for further analysis, while the supernatant was poured in a fresh tube and centrifuged at the next speed (Fig. 2.2a).

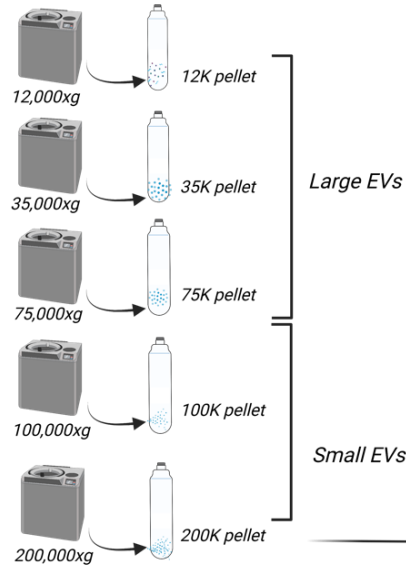
a)

Differential ultracentrifugation (DUC)

SM/IMF NT SM/IMF IP or AA



38mL of SM/IMF



b)

Density gradient ultracentrifugation (GUC)

SM/IMF NT SM/IMF IP or AA



2.33 mL of SM/IMF

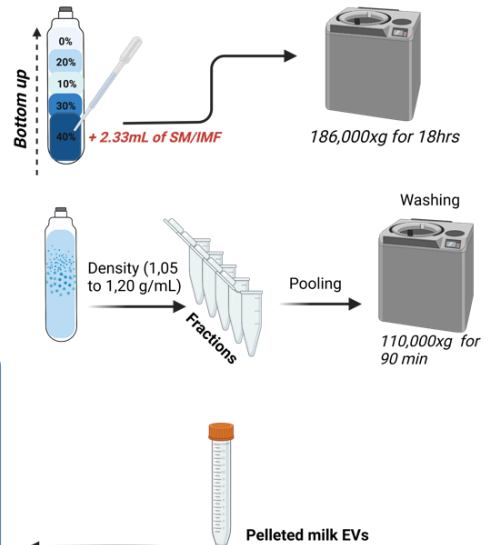


Figure 2.2: Differential ultracentrifugation (DUC) vs Density gradient ultracentrifugation (GUC) for milk EVs separation

Two EVs separation methods used simultaneously to separate EVs from SM and IMF. a) Differential ultracentrifugation using increasing speed. For each step the supernatant was used for the next spin, while the pellets was stored. b) Density gradient ultracentrifugation using the bottom up technique and Optiprep 60%. Separated EVs were collected in fractions for pooling and washing.

2.3.4 Determination of the Refractive Index (RI) and calculation of the densities

To measure the densities for each fraction post-GUC, a portable refractometer was used to measure the Refractive Index (RI) and densities were calculated based on the following formula:

Density= $(3.3411 \times \text{RI} - 3.4584)$. Prior to determine the RI, the portable refractometer was

calibrated at room temperature using 300 µL of ddH₂O. Once it was set at zero, 300 µL of each

fraction was carefully pipetted and the RI was recorded. This step was performed for all the six samples.

2.3.5 Bradford assay

Total EVs protein in all samples was measured by bicinchoninic acid (BCA) assay using reagent kit (Thermo Fisher Scientific, Waltham, MA, US; Cat. #10249133). Bovine serum albumin (BSA) standards (0- 800 ug/mL) (Sigma-Aldrich, Ireland) were used to generate a standard curve for interpolation of the protein quantities. Briefly, 10 µL of standards and sample was added to a 96-well plate in triplicate. Bio-Rad Dye reagent was diluted 1:5 in ddH₂O and 200µL added to each standard and sample. After 5 minutes incubation in the dark, absorbance was read at 570 nm using a FluoStar Optima microplate reader (Serial #: 08-100-241) and protein quantities were calculated from the standard curve.

2.3.6 SDS-PAGE for protein visualisation

The effects of AA or IP pre-treatment, compared to NT on milk proteins, prior to ultracentrifugation, was visualised a 10% polyacrylamide gel (Bio-Rad Laboratories). For this, 15 µg of protein samples were loaded on a 10% polyacrylamide gel, run for the first 30 min at 80V and then the voltage was increased to 120V. Once the run was finished, the gel was placed in a plastic container and covered with 3 volumes of fixing solution and leave for 2h at room temperature on a rocking platform. After 2 hr fixing solution was pour out and gel was covered with Coomassie Blue staining solution for 4 hr on a rocking platform. The staining solution was then removed, the gel was rinse with destaining solution, and left overnight on the rocking platform. Finally, once blue bands with a clear background were obtained, the separated proteins were visualised using a Chemidoc XRS system (Bio-Rad).

2.3.7 Lyses of EVs pellets

100µL EVs isolates from each separation methods was mixed with 1 volume of cell lysis buffer (250 mM Tris-HCL, pH 7.4; 2.5% SDS) (Invitrogen, Cat. #: FNN0011) and 1X proteinase inhibitor (Roche, Cat. #: 05892970001). EVs lysate was incubated on ice for 30 mins, vortexing every 10 mins for 10 seconds. After 30 minutes, the sample was centrifuged at 13,200 rpm at 4 °C for 10 minutes. The supernatant was transferred to a new Eppendorf and stored at -80 °C until further use. Protein quantification on lysed EVs was performed by BCA assay, as mentioned above.

2.3.8 Nanoparticle tracking analysis (NTA)

Particle concentration and sizes were estimated using NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK). EVs samples were automatically injected into the NTA system under constant flow conditions (flow rate = 50) and videos of the particles in motion were recorded and analysed using NTA 3.1.54 software.

2.3.9 RNA isolation and quantification

Total RNA isolation from the EVs isolates was performed using a previously published protocol (Pieters et al., 2015). Briefly, 1.0 mL TRI reagent (Invitrogen, Carlsbad, CA, US) was added with 200 µL chloroform (Sigma Aldrich, Ireland) to the pelleted EVs isolates and mixed vigorously for 15 s and incubated for 2 min at RT. The suspension was centrifuged at 12,000 g for 15 min at 4°C and the clear supernatant was transferred to a fresh tube and mixed with 500 µL isopropanol (Sigma Aldrich, Ireland) before storing at -80°C overnight. Following overnight incubation, the

mixture was centrifuged at 12,000 g for 30 min at 4°C followed by two washes with 75% ethanol. The ethanol was removed by centrifugation at 12,000 g for 5 min at 4°C and the pellet was air-dried at room temperature and resuspended in RNase-free water. RNA quantity was measured using the Qubit HS RNA Assay kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham, CA, US) on a Qubit fluorometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, CA, US). The Qubit HS RNA assay is described as highly selective for RNA and will not quantitate DNA, protein, or free nucleotides.

2.3.10 Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis following previously published protocol (Visnovitz et al., 2019). Specifically, 5 µL of sample was suspended in 5 µL of 0.22 µm-filtered PBS and placed onto carbon coated grids (Ted-Pella B 300 M, Mason Technology Ltd., Ireland; Cat. #: 01813-F) for 10 min at RT before fixing with 4% glutaraldehyde (Sigma Aldrich, Ireland) and contrasting with 2% phosphotungstic acid (Sigma Aldrich). The grids were examined at 100 kV using a JEOL JEM-2100 TEM (JOEL, Peabody, USA), as we previously described (O'Brien et al., 2013).

2.3.11 Immunoblotting analysis

Immunoblotting was performed as we previously described (O'Brien et al., 2013) except that 35 µg of lysed EVs protein was used here. For this, EVs were lysed using SDS lysis buffer (250 mM Tris-HCL, pH 7.4; 2.5% SDS), lysates (35 µg) were resolved on 10% SDS gels (Bio-Rad) and the protein was transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, US; Cat. #1620177). Blots were blocked with 5% (w/v) BSA in PBS containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies to anti-human TSG101 (1:1000; Abcam,

Cambridge, UK; Cat. # ab30871), anti-human CD63 (1:500; Abcam, Cambridge, UK; Cat. #: ab68418) or anti-human Actinin 4 (1/1000, Abcam, Cambridge, UK; Cat. #: ab108198). Secondary antibodies used were anti-mouse (1/1000 in 5% BSA/PBS-T, Cell Signalling, Danvers, MA, US; Cat. #: 7076) or anti-rabbit (1/1000 in 5% BSA/PBS-T, Cell Signalling, Danvers, MA, US; Cat. #: 7-74). Cell lysate from the Hs578Ts (i)8 human cell line was included in all gels as a positive control. SuperSignal West Femto Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Waltham, CA, US; Cat. # 11859290) was used for detection, imaging was performed using an automated Chemidoc exposure system (Bio-Rad Laboratories, Hercules, CA, US) and densitometric analysis was performed using ImageJ tool

2.3.12 Imaging flow cytometry

The imaging flow cytometry (IFCM) analysis of EVs separated from SM and IMF was performed following a protocol previously published (Ricklefs et al., 2019). Briefly, EVs surface antigens were exposed to antibodies diluted in 0.22 μ m-filtered PBS with 2% EVs-depleted-FBS supplemented with protease inhibitor and phosphatase inhibitor (IFCM buffer). The specific antibodies used were anti-human CD63 conjugated with FITC (CD63-FITC; 1:150, Cat. #: 353006, Biolegend, San Diego, CA, US); anti-human CD9-PE (1:1500, Cat. #: 312106, Biolegend, San Diego, CA, US); anti-human CD81-PE-Cy7 (1:150, Cat. #: 349,512 Biolegend, San Diego, CA, US); anti-human HLADR-BV421 (1:600, Cat. #: 307636, Biolegend, San Diego, CA, US) and anti-human ADAM10-APC (1:150, Cat. #: 352706, Biolegend, San Diego, CA, US). The selection of the antibodies was made based on the presence of surface markers on the EVs. Anti-CD9, anti-CD63 and anti-CD81 antibodies were selected as tetraspanins present on EVs surface, along with HLADR, an MHC II immunoregulatory molecule present on EVs surface. Additionally, ADAM10 was also selected as in a previous study, (Kowal et al., 2016) reported the presence of ADAM10

in EVs. The EVs were incubated with the antibodies for 45 min at RT in the dark, and washed using a 300 kDa filter (Nanosep, Pall Biotech, Ireland; Cat. #: 516-8531), resuspended in 50 μ L IFCM buffer and acquired within 2 h on the ImageStream X Mk II imaging flow cytometer (Amnis/ Luminex, Seattle, USA) at 60x magnification and low flow rate, as previously described (Ricklefs et al., 2019). EVs-free IFCM buffer, unstained EVs, single-stained controls and fluorescence minus one (FMO) controls were run in parallel. Fluorescence was within detection linear range in the following channels: FITC was measured in channel 2 (B/YG_480–560 nm), PE in channel 3 (B/YG_560–595 nm filter), PE-Cy7 in channel 6 (B/ YG_745-780 nm filter), BV421 in channel 7 (R/V_435-480 nm filter), APC in channel 11 (R/V_642–745 nm, filter), Brightfield in channel 1 and 9 (B/YG_435-480 and R/V_560-595 nm filter respectively) and Side Scatter Channel (SSC) in channel 12 (R/V_745-780 nm filter). Data analysis was performed using IDEAS software v6.2 (Amnis/ Luminex, Seattle, USA). EVs were gated as SSC-low vs fluorescence, then as nondetectable brightfield (fluorescence vs Raw Max Pixel Brightfield channel), gated EVs were confirmed in the IDEAS Image Gallery.

2.4 Statistical analysis

Using Graph Pad Prism 8 software, either (a) unpaired two-tailed Student's t-test was performed when comparing two groups or (b) one-way or two-way ANOVA was performed when comparing more than two groups. Data is presented as mean $\pm$ standard error of the mean (SEM) for at least three experiments (n=3). A value of $P < 0.05$ was considered statistically significant.

2.5 Results

2.5.1 Analysis of milk protein removal by acid treatment and ultracentrifugation

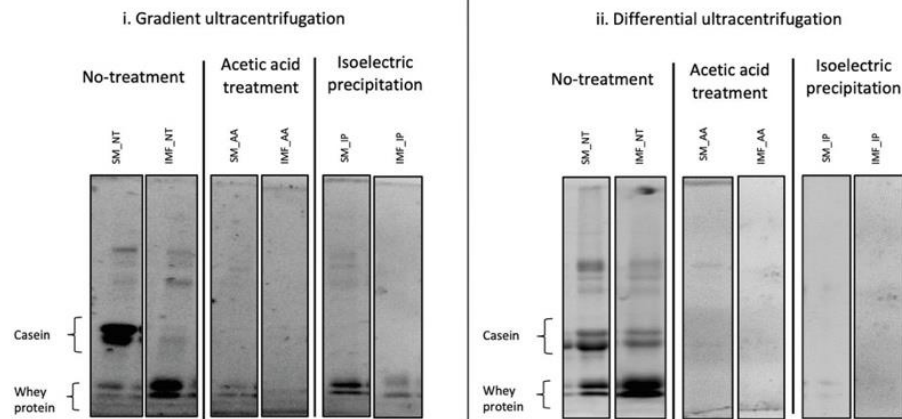
Total protein separation by polyacrylamide gel showed that the major groups of milk proteins, including caseins and whey proteins, were present post-GUC at high levels in the SM_NT samples and to a lesser extent in IMF_NT samples (Fig. 2.3a), while pre-treatment with AA or IP quite efficiently removed caseins. Post-DUC, the SM_NT and IMF_NT had strong bands representing caseins and whey proteins (Fig. 2.3a); while, again, AA and IP pre-treatment efficiently removed these proteins. While compared to NT, AA and IP have efficiently removed casein proteins, faint bands were still observed indicating seepage of contaminant proteins in the enriched EVs fractions. Due to the persistent presence of some casein even after AA or IP, the DUC generated 12 K, 35 K, 75 K and 100 K resuspended pellets or the crude EVs samples were not used for further extensive analysis, whereas the 200 K samples were further characterised. It is also important to note that in the untreated SM (SM_NT) and IMF (IMF_NT) samples, post-GUC or after 200,000 g DUC step, casein and whey proteins were still present to varying extents and so were present during further characterisation steps as these samples serve as control for further characterisation steps.

2.5.2 Protein concentration analysis of isolates from untreated or AA- or IP- treated SM and IMF

The total protein concentrations of EVs samples post-GUC and -DUC are presented in Fig. 2.3b. Considering the pooled fractions (1.05–1.19 g/mL), the total protein content of IMF_NT appeared to be significantly ($P < 0.01$) higher compared to all other treatment samples analysed

(Fig. 2.3b). The total protein contents on post-DUC samples are presented in Fig. 2.3b. The IMF_NT samples were associated with significantly higher total protein content compared to SM_NT samples ($P < 0.05$) and IMF_AA, SM_IP and IMF_IP samples ($P < 0.01$).

a. SDS-PAGE for protein visualisation



b. Protein concentration analysis

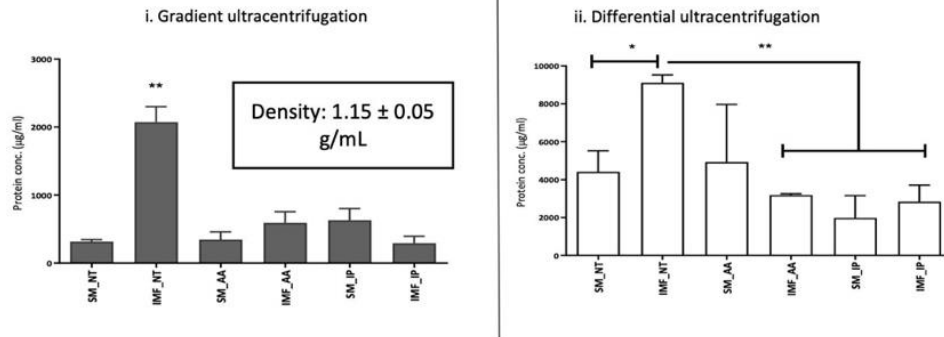


Figure 2.3: Protein and particle characterisation of skim milk and IMF EVs

a) Milk proteins were visualised on 10% acrylamide gels. Efforts to remove milk proteins were performed by AA and IP treatment. b) Isolates from skim milk (SM) and infant milk formula (IMF) post i) gradient ultracentrifugation (GUC) and ii) differential ultracentrifugation (DUC) were analysed for protein concentrations by BCA assay. Protein concentration (in $\mu\text{g/mL}$) of the three repeats were analysed using one-way ANOVA test and presented as mean bars $\pm$ SEM, * indicates a significance value of $P < 0.05$, ** indicates a significance value of $P < 0.01$.

2.5.3 Milk EVs concentration, size characterisation and RNA quantity

The sizes and concentrations of EVs separated from pre-treated or untreated SM and IMF, analysed by NTA are presented in Table 2.1. Considering size distribution, post-GUC particles from the SM samples were 156.1 ± 5.8 nm and from IMF samples were 159.9 ± 12.9 nm, while those post-DUC of SM_200K were 159.1 ± 5.2 nm and IMF_200K were 175.7 ± 8.4 nm. No significant differences were observed between these EVs sizes. Regarding the concentration of particles in each sample, significant differences were observed between SM and IMF samples. As presented in Table 2.1, post-GUC, the concentration of EVs in SM_NT samples was higher (7.1-fold; $P = 0.05$) compared with IMF_NT samples. Similarly, the EVs concentration of SM_IP samples was significantly higher (6.7- fold; $P = 0.01$) when compared with IMF_IP samples. In turn, the concentration in SM_AA samples was substantially higher (4.9-fold) than in IMF_AA, although not significantly different ($P = 0.26$) as a result of quite large differences in biological repeat SM_AA values (*i.e.* a large error bar). Similarly, post-DUC -as presented in Table 2.1(ii)- the EVs/particle concentration in SM_NT_200K samples was significantly higher (423.6- fold; $P = 0.04$) than in IMF_NT_200K. Likewise, the EVs/particle concentration in SM_AA_200K samples was 16.45-fold higher ($P = 0.03$) compared to that in IMF_AA_200K and the SM_IP_200K samples had 7.83-fold more particles ($P = 0.01$) when compared to the IMF_IP_200K samples.

Yields of EVs separated from skim milk (SM) and infant milk formula (IMF) by gradient ultracentrifugation (GUC) or differential ultracentrifugation (DUC), as estimated NTA.					
i) Gradient Ultracentrifugation					
	Skim milk		Infant milk formula		P value
Size (in nm)	Mean	Mode	Mean	Mode	
Non-treated (NT)	147.00 ± 8.39	113.60 ± 10.34	166.15 ± 2.75	140.45 ± 1.25	ns
Acetic acid treated (AA)	159.83 ± 5.55	135.46 ± 5.28	180.16 ± 16.77	133.53 ± 16.22	ns
Isoelectric precipitation (IP)	162.90 ± 13.51	141.03 ± 11.93	152.40 ± 45.10	109.50 ± 18.80	ns
Concentration (Particles/mL starting material)	Skim milk		Infant milk formula		
Non-treated (NT)	4.35 × 10 ¹⁰ ± 1.40 × 10 ¹⁰		6.13 × 10 ⁰⁹ ± 8.17 × 10 ⁰⁸		0.05
Acetic acid treated (AA)	1.39 × 10 ¹⁰ ± 8.25 × 10 ⁰⁹		2.82 × 10 ⁰⁹ ± 2.33 × 10 ⁰⁹		ns
Isoelectric precipitation (IP)	2.16 × 10 ¹⁰ ± 2.47 × 10 ⁰⁹		3.22 × 10 ⁰⁹ ± 1.45 × 10 ⁰⁹		0.01
ii) Differential Ultracentrifugation					
	Skim milk		Infant milk formula		P value
Size (in nm)	Mean	Mode	Mean	Mode	
Non-treated (NT)	173.60 ± 29.20	123.40 ± 60.00	180.40 ± 7.50	152.50 ± 50.10	ns
Acetic acid treated (AA)	150.10 ± 16.40	146.90 ± 47.33	149.28 ± 18.37	137.30 ± 68.22	ns
Isoelectric precipitation (IP)	149.23 ± 23.50	130.81 ± 46.11	184.19 ± 15.63	117.10 ± 67.70	ns
Concentration (Particles/mL of starting material)	Skim milk		Infant milk formula		
Non-treated (NT)	1.97 × 10 ¹⁰ ± 8.90 × 10 ⁰⁹		4.65 × 10 ⁰⁷ ± 2.50 × 10 ⁰⁷		0.04
Acetic acid treated (AA)	2.78 × 10 ⁰⁹ ± 1.00 × 10 ⁰⁹		1.69 × 10 ⁰⁸ ± 3.30 × 10 ⁰⁷		0.03
Isoelectric precipitation (IP)	3.65 × 10 ⁰⁹ ± 5.43 × 10 ⁰⁸		4.66 × 10 ⁰⁸ ± 2.02 × 10 ⁰⁸		0.01
ns = not significant.					

Table 2.1: Yield of EVs separated from SM and IMF by GUC or DUC

Directly comparing yields of EVs/particles from SM and IMF, respectively, following GUC compared to DUC, as illustrated in Table 2.2, the yields were always many folds higher with GUC compared to DUC. Within SM, the yields with GUC ranged from 2.2 to 5.9 folds higher than when compared to aliquots of the same samples separated by DUC. Similarly, with the IMF samples. Here the yields from IMF when using GUC ranged from 6.9 to 131.7 folds higher than when compared to aliquots of the same samples separated by DUC. In some, but not all cases, statistical significance was found. As can be observed from the SEM values, there were

differences between biological repeat samples. This variability when grouped together would have contributed to statistical significance not being reached in all cases.

i) Skim milk				
Concentration of particles/mL of starting material	GUC	DUC	Fold Higher Yield with GUC vs. DUC	P value
Non-treated (NT)	4.35 × 10 ¹⁰ ± 1.40 × 10 ¹⁰	1.97 × 10 ¹⁰ ± 8.90 × 10 ⁰⁹	2.2	0.06
Acetic acid treated (AA)	1.39 × 10 ¹⁰ ± 8.25 × 10 ⁰⁹	2.78 × 10 ⁰⁹ ± 1.00 × 10 ⁰⁹	5.02	ns
Isoelectric precipitation (IP)	2.16 × 10 ¹⁰ ± 2.47 × 10 ⁰⁹	3.65 × 10 ⁰⁹ ± 5.43 × 10 ⁰⁸	5.91	0.01
ii) IMF				
Concentration of particles/mL of starting material	GUC	DUC	Fold Higher Yield with GUC vs. DUC	P value
Non-treated (NT)	6.13 × 10 ⁰⁹ ± 8.17 × 10 ⁰⁸	4.65 × 10 ⁰⁷ ± 2.50 × 10 ⁰⁷	131.7	0.01
Acetic acid treated (AA)	2.82 × 10 ⁰⁹ ± 2.33 × 10 ⁰⁹	1.69 × 10 ⁰⁸ ± 3.30 × 10 ⁰⁷	16.7	ns
Isoelectric precipitation (IP)	3.22 × 10 ⁰⁹ ± 1.45 × 10 ⁰⁹	4.66 × 10 ⁰⁸ ± 2.02 × 10 ⁰⁸	6.92	ns

ns = not significant

Table 2.2: Comparison of concentration of particles/mL from SM and IMF by GUC versus DUC

As EVs typically carry RNA, but casein micelles do not, RNA was isolated from the SM and IMF isolated following GUC and DUC separation. The RNA concentration/mL of starting material obtained and RNA/particle post GUC and DUC are presented in Table 2.3. Following GUC, the total RNA and quantity of RNA/particle was always greater with SM compared to IMF. However,

statistically significant differences were not always observed, due to the relatively large difference between biological repeats.

a) GUC - RNA yield						
Samples Analysed	Skim milk		Infant milk formula		P value	
	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i.	ii.
Non-treated (NT)	93.00 ± 21.33	2.14 × 10 ⁻⁰⁹ ± 4.90 × 10 ⁻¹⁰	7.67 ± 1.87	1.25 × 10 ⁻⁰⁹ ± 2.15 × 10 ⁻¹⁰	0.01	ns
Acetic acid treated (AA)	68.67 ± 3.00	4.94 × 10 ⁻⁰⁹ ± 2.16 × 10 ⁻¹⁰	46.90 ± 6.10	1.66 × 10 ⁻⁰⁸ ± 1.77 × 10 ⁻⁰⁹	ns	ns
Isoelectric precipitation (IP)	90.33 ± 14.00	4.18 × 10 ⁻⁰⁹ ± 6.48 × 10 ⁻¹⁰	5.10 ± 1.90	1.58 × 10 ⁻⁰⁹ ± 5.90 × 10 ⁻¹⁰	0.01	ns
b) DUC - RNA yield						
Samples Analysed	Skim milk		Infant milk formula		P value	
	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i.	ii.
Non-treated (NT)	43.53 ± 1.67	2.21 × 10 ⁻⁰⁹ ± 8.46 × 10 ⁻¹¹	7.30 ± 2.97	1.57 × 10 ⁻⁰⁷ ± 6.38 × 10 ⁻⁰⁸	ns	ns
Acetic acid treated (AA)	6.60 ± 2.33	2.37 × 10 ⁻⁰⁹ ± 8.39 × 10 ⁻¹⁰	11.37 ± 1.17	6.73 × 10 ⁻⁰⁸ ± 6.90 × 10 ⁻⁰⁹	ns	ns
Isoelectric precipitation (IP)	51.00 ± 0.67	1.40 × 10 ⁻⁰⁸ ± 1.83 × 10 ⁻¹⁰	22.40 ± 19.20	4.81 × 10 ⁻⁰⁸ ± 4.12 × 10 ⁻⁰⁸	ns	ns
= comparing Total RNA yields (ng/mL); ii = comparing RNA concentration (ng/particle); ns = not significant.						

Table 2.3: Comparison of RNA (ng) total yield/mL and particle separated from SM versus IMF by a) GUC and b) DUC

Doing the same comparison but post-DUC, results were less consistent. When directly comparing ng RNA/particle following DUC *versus* GUC Table 2.4, with SM the amounts were typically great following GUC compared to DUC. However, the differences between repeat

biological experiment contributed to no statistical significance been reached. In the case of IMF, the amounts of RNA per particle was significantly greater ($P = 0.01$) following GUC compared to DUC.

i) Skim milk			
RNA conc. (ng)/ particle	GUC	DUC	P value
Non-treated (NT)	$2.14 \times 10^{-09} \pm 4.90$ $\times 10^{-10}$	$2.21 \times 10^{-09} \pm 8.46$ $\times 10^{-11}$	ns
Acetic acid treated (AA)	$4.94 \times 10^{-09} \pm 2.16$ $\times 10^{-10}$	$2.37 \times 10^{-09} \pm 8.39$ $\times 10^{-10}$	ns
Isoelectric precipitation (IP)	$4.18 \times 10^{-09} \pm 6.48$ $\times 10^{-10}$	$1.40 \times 10^{-08} \pm 1.83$ $\times 10^{-10}$	ns
ii) IMF			
RNA conc. (ng)/ particle	GUC	DUC	P value
Non-treated (NT)	$1.25 \times 10^{-09} \pm 2.15$ $\times 10^{-10}$	$1.57 \times 10^{-07} \pm 6.38$ $\times 10^{-08}$	0.01
Acetic acid treated (AA)	$1.66 \times 10^{-08} \pm 1.77$ $\times 10^{-09}$	$6.73 \times 10^{-08} \pm 6.90$ $\times 10^{-09}$	ns
Isoelectric precipitation (IP)	$1.58 \times 10^{-09} \pm 5.90$ $\times 10^{-10}$	$4.81 \times 10^{-08} \pm 4.12$ $\times 10^{-08}$	ns

ns = not significant.

Table 2.4: Comparison of RNA (ng)/particle separated from SM and IMF by GUC versus DUC

Complementary information was obtained by TEM and presented in Fig. 2.4. Following GUC separation, the SM EVs (SM_NT, SM_AA and SM_IP) appeared intact with limited background debris. IMF-derived EVs appeared more diffuse with less smooth surfaces, and with much more debris in the background (Fig. 2.4). Post-DUC, in SM EVs samples (SM_NT, SM_AA and SM_IP), multiple smaller vesicles were observed, although sometimes in clumps (arrows, Fig. 2.4). The IMF samples (IMF_NT, IMF_AA and IMF_IP) had very few EVs-like structures, but much more background debris.

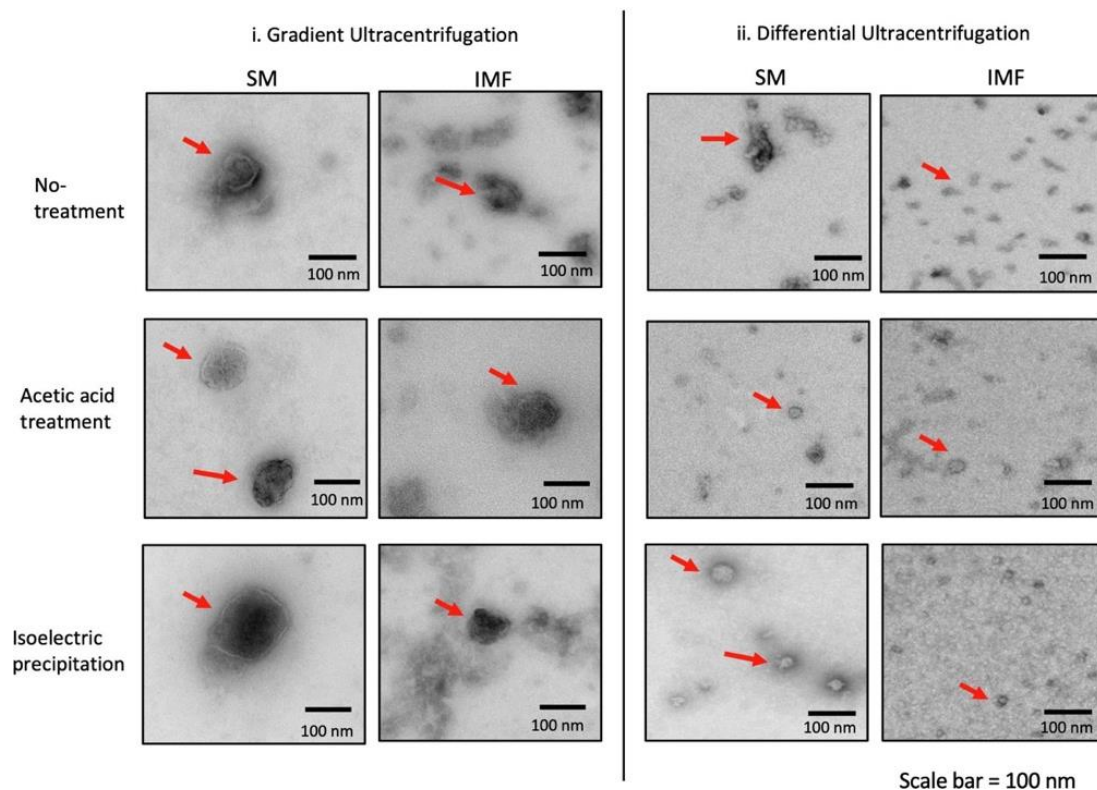


Figure 2.4: Transmission electron microscopy of EVs

Representative transmission electron microscope (TEM) images from i) gradient ultracentrifugation (GUC), and ii) differential ultracentrifugation (DUC) samples. Red arrows point towards vesicular structures in the sample

2.5.4 Immunoblotting analysis of EVs specific positive and negative markers

In line with MISEV2018 guidelines (Thery et al., 2018), immunoblotting was performed on lysed samples for two EVs positive markers, TSG101 and CD63, and a negative marker Actinin 4. The GUC generated SM_NT, SM_AA and SM_IP samples were typically positive for both TSG101 and CD63, but not Actinin 4 (Fig. 2.5a, with densitometric analysis of $n = 3$ presented in Fig. 2.5b). Although equal quantities of protein were loaded, TSG101 and CD63 were always at higher levels in SM-derived EVs compared to IMF-derived EVs samples and Actinin 4 was undetected in all

EVs samples (Fig. 2.5a). All the EVs/particles collected post-DUC of SM and IMF samples were also lysed and analysed for TSG101, CD63 and Actinin 4 (Fig. 2.5c), with densitometric analysis of $n = 3$ presented in Fig. 2.5d). Again, no signal for Actinin 4 was observed for either SM or IMF samples generated by DUC. Conversely, SM_NT, SM_AA and SM_IP samples were positive for both TSG101 and CD63. IMF_NT samples compared to SM_NT had faint TSG101 and CD63 signals. Unlike SM_AA and SM_IP samples, neither IMF_AA or IMF_IP samples showed bands for either TSG101 or CD63.

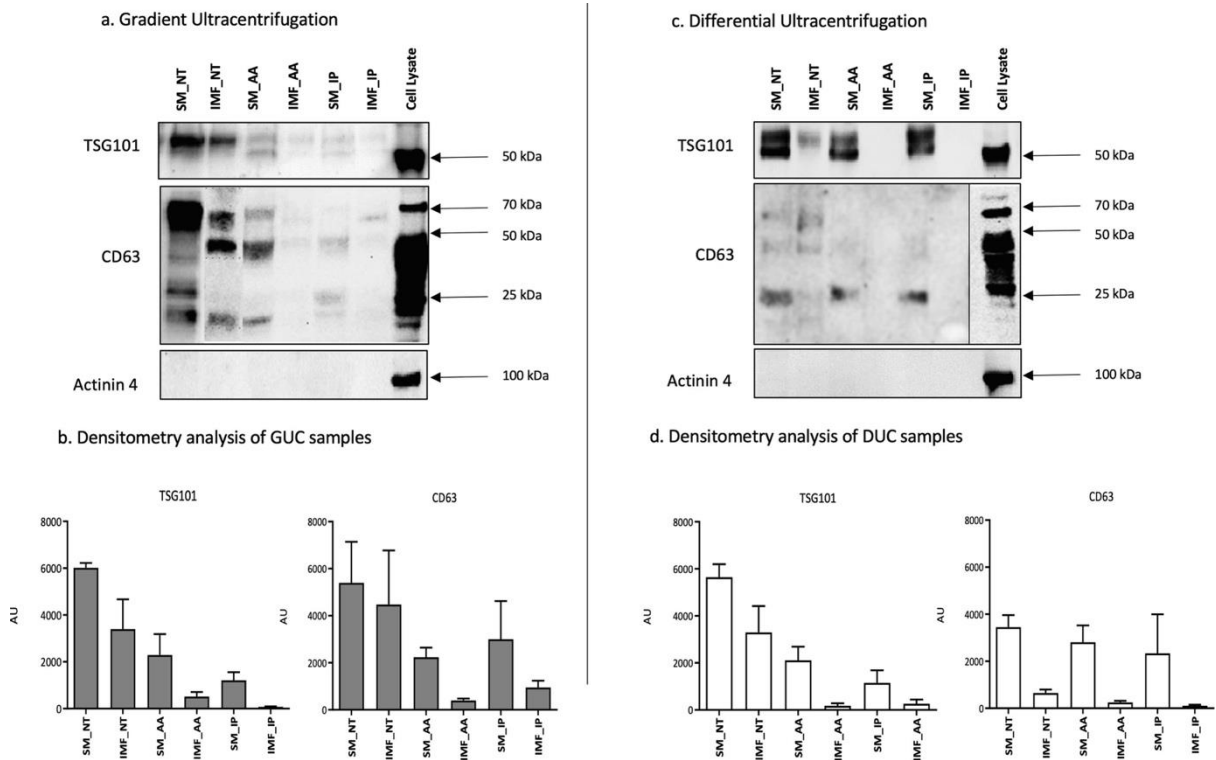


Figure 2.5: Immunoblotting of EV specific markers

Immunoblotting of SM and IMF EVs separated using gradient ultracentrifugation (GUC) and differential ultracentrifugation (DUC) and their densitometric analysis. Equal amount of protein (35 μ g) was loaded for all the samples and analysed for TSG101, CD63 and Actinin 4. Representative blots for GUC samples are presented in (a) and DUC samples are presented in (c), densitometric analysis of GUC samples are presented in (b) and those of DUC samples in (d). Each bar represents the mean of the densities of the signals obtained post immunoblotting (mean of $n = 3$ biological repeats $\pm$ SEM).

2.5.5 Detection of EVs using imaging flow cytometry analysis

Subsequently, analysis of intact EVs based on presence of EVs surface markers CD9, CD63, CD81, ADAM10 and HLADR was performed using imaging flow cytometry (IFCM). In all cases, post-GUC and post-DUC, higher quantities of EVs-like particles were separated from SM compared to IMF. Specifically, post- GUC (Fig. 2.6a), significant differences in the particles/mL of SM_AA vs. IMF_AA ($P < 0.01$) and SM_IP vs. IMF_IP ($P < 0.05$) samples were observed; SM_NT had higher quantities than IMF_NT, but statistical significance was not reached ($P = 0.08$). In post-DUC 200K samples, significant differences were observed in particles/mL of SM_NT vs. IMF_NT ($P < 0.01$) and SM_AA vs IMF_AA ($P < 0.05$); statistical significance was not obtained between SM_IP vs. IMF_IP samples (Fig. 2.6b). In keeping with this trend, and as illustrated in Fig. 2.6c, d, e, f and g that show the approximate number of + EVs/mL of sample that are positive for each of five individual EVs-specific markers (CD63, CD9, CD81, ADAM10 and HLADR), post-GUC and post-DUC -where there was a difference in surface marker detected- the levels were always higher with the SM EVs compared to the IMF EVs.

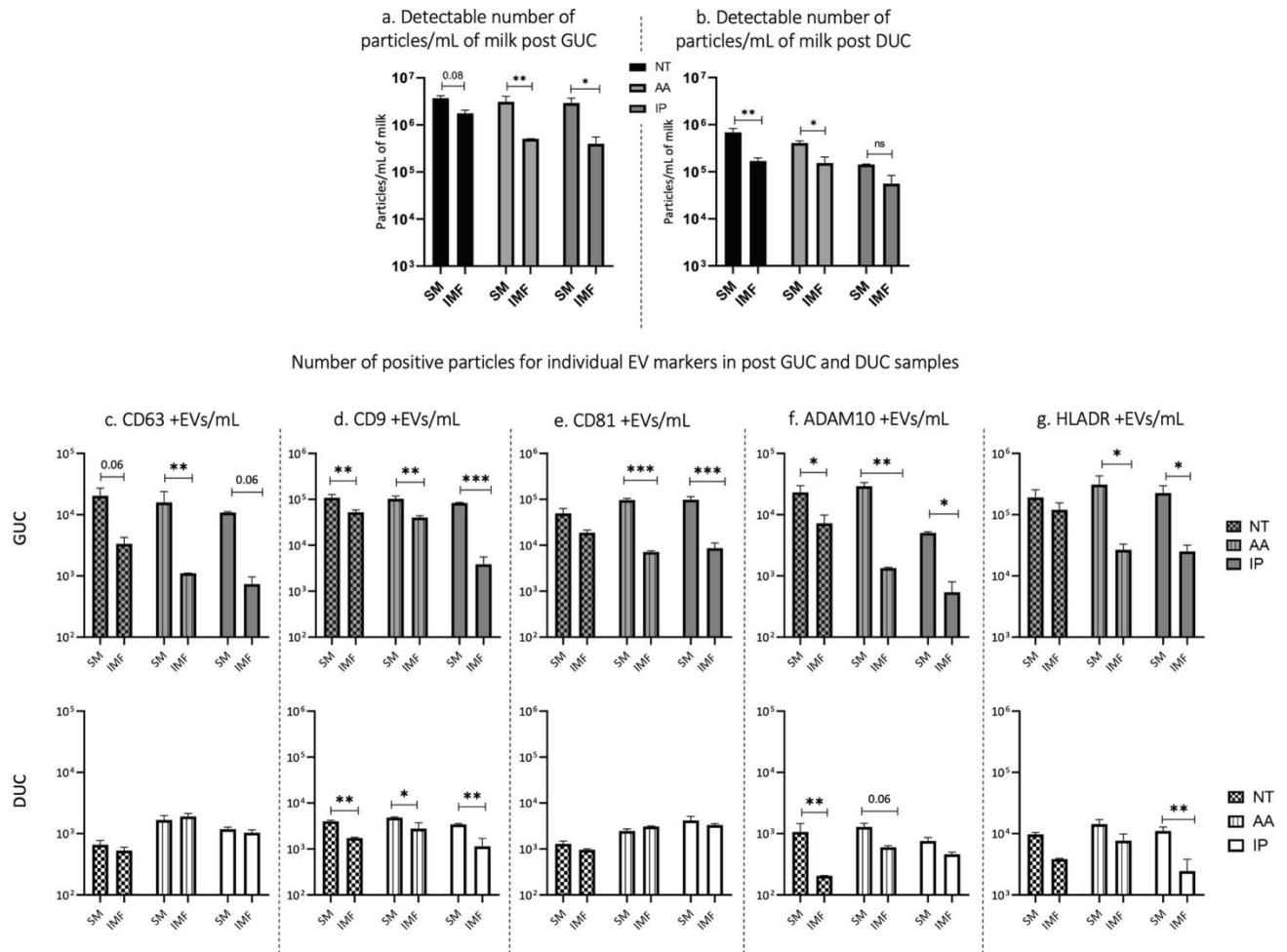


Figure 2.6: Imaging flow cytometry analysis of EV specific markers

Number of positive events (particles) in SM and IMF samples analysed by IFCM. The events were gated for low side scatter channel (SSC) signals and no signals on brightfield, based on EV size and events positive for these gating was acquired as objects (+_EVs) in low SSC. The total number of detectable particles in gradient ultracentrifugation (GUC) samples are presented in a) and those from differential ultracentrifugation (DUC) samples are presented in c), data presented as mean and individual values $\pm$ SEM. The number of positive CD9, CD63, CD81, ADAM10 and HLADR particles in GUC samples are presented in b) and DUC samples are presented in d). The IDEAS software was used to analyse the data, data presented as means and individual values $\pm$ SEM. A two-way ANOVA test was performed to compare differences in EVs in SM and IMF samples from each treatment groups; *indicates $P < 0.05$. **indicates $P < 0.01$ and ***indicates $P < 0.001$.

The findings from this study are summarised in Table 2.5 and it is evident that GUC is a laborious process, with approximately 18 h required just for ultracentrifugation added to the time for sample preparation.

Milk type	Pre-treatment	EV separation method	Vol. of sample used (mL)	Time to separate EV (h)	Immuno-blotting	EV size (nm)	EV concentration/mL of milk	TEM	IFCM
SM	NT	GUC	2.33	> 18	(✓)	147.0 ± 8.39	10 ¹⁰	+	10 ⁶
	AA	GUC	2.33	> 18	(✓)	159.8 ± 5.55	10 ¹⁰	+	10 ⁶
	IP	GUC	2.33	> 18	(✓)	162.90 ± 13.51	10 ¹⁰	+	10 ⁶
IMF	NT	GUC	2.33	> 18	(✓)	166.2 ± 2.75	10 ⁰⁹	+	10 ⁶
	AA	GUC	2.33	> 18	(✓)	180.2 ± 16.77	10 ⁰⁹	+	10 ⁵
	IP	GUC	2.33	> 18	(✓)	152.4 ± 45.10	10 ⁰⁹	+	10 ⁵
SM	NT	DUC	38	< 6.25	(✓)	173.6 ± 29.20	10 ¹⁰	+ (high debris)	10 ⁵
	AA	DUC	38	< 6.25	(✓)	150.1 ± 16.40	10 ⁰⁹	+ (high debris)	10 ⁵
	IP	DUC	38	< 6.25	(✓)	149.2 ± 23.50	10 ⁰⁹	+	10 ⁵
IMF	NT	DUC	38	< 6.25	(✓)	180.4 ± 7.50	10 ⁰⁷	+ (high debris)	10 ⁵
	AA	DUC	38	< 6.25	(✓)	149.28 ± 18.37	10 ⁰⁸	+ (high debris)	10 ⁵
	IP	DUC	38	< 6.25	(✓)	184.2 ± 15.63	10 ⁰⁸	+ (high debris)	10 ⁴

SM skim milk, IMF infant milk formula.

+ vesicle like structures detected.

(✓) samples could be analysed without technical limitations.

Table 2.5: Summary of the techniques used for separation of milk EVs and the characterisation of EVs

2.6 Discussion

2.7 Acid treatment and EVs separation

The aim of this study was to compare EVs present in SM and IMF. As IMF production involves harsh industrial processes, we hypothesised its EVs may be lost and/or compromised compared to those in SM. Comparisons were also made based on both milk pre-treatment and EVs separation methods to identify the optimal procedure to separate enriched EVs from SM and IMF. Our results indicate that irrespective of the treatment, the concentration, and the quality of EVs present in IMF are substantially compromised compared to SM. This suggests that in IMF, proteins carried by EVs that are present may be damaged or lost. Our results also suggest that both AA and IP pre-treatments remove casein micelles quite efficiently, and that the GUC technique is the optimum method for isolation of high yields of intact EVs without any effect on the EV morphology. To perform detailed characterisation of milk- or IMF- derived EVs, as pure/enriched EVs as possible need to be separated. However, using complex biofluids in conjunction with ultracentrifugation typically leads to contamination of EVs with proteins (Langevin et al., 2019; J. Li et al., 2019). Ultracentrifugation of milk is reported to lead to sedimentation of dense casein micelles (Fox, 2003). It has been reported that following milk fractionation by ultracentrifugation at 100,000 g, casein remnants exist in milk serum because ultracentrifugation alone does not break-up or remove casein micelles and the small casein micelles are difficult to sediment (H. B. Jensen et al., 2012). In agreement with this, by SDS-PAGE we observed strong casein -and to a lesser extent, whey- protein bands in samples that were collected after multiple ultracentrifugation steps of DUC in both SM and IMF samples, with similar observations made for SM and IMF samples that underwent GUC. Therefore, substantial consideration needs to be given towards removal of caseins micelle whose size and concentration overlapping with EVs, if claiming to study EVs. In industry, depending on the

subsequent use of the product, casein is sometimes removed by isoelectric precipitation (Fox & Brodkorb, 2008) using acids that generate 'acid-caseins' (Atamer et al., 2017). Acidification of milk reduces its pH to the isoelectric point of casein (pH 4.6) resulting in irreversible destruction of the casein micelle structure and further coagulation and precipitation of the casein complex that can be sedimented at low speed centrifugation (Chinprahast et al., 2015; Fox & Brodkorb, 2008). In fact, a recent study investigated acidification effects on defatted raw bovine milk and observed some morphological changes in EVs due to acid treatment. However, the use of acid to remove milk proteins and separate large scale EVs remains their preferred method (Rahman et al., 2019). In our study, a combination of acid treatment and ultracentrifugation was evaluated with both SM and IMF which, to the best of our understanding, has not previously been done. It is noteworthy that a recent study did compare IMF EVs to milk EVs and reported EVs to be undetectable in IMF (Leiferman et al., 2019). However, that study did not remove casein micelles, did not analyse samples by TEM, and only used DUC for EVs collections. So, while it is an important study, it is very different in many respects to the work we presented here. Further orthogonal steps were performed to analyse the EVs separated from SM and IMF and to explore the effects of pre-treatment on EVs morphology or protein composition.

2.8 Comparison of characterised EVs from SM and IMF

Characterisation was performed by NTA, a technique widely used to estimate number of particles as well as the size of particles in a medium (Szatanek et al., 2017; Théry et al., 2018). Interestingly and as we hypothesised, post-GUC and -DUC, the number of particles/mL of IMF samples (all treatment groups) were significantly lower than in the corresponding SM samples, while no differences in the particle sizes were observed. Additionally, to rule out this being because of milk pre-treatment, we included the non-treated SM and IMF in our characterisation

steps. Similar differences between non-treated SM and IMF samples were observed, where SM samples had higher concentration of EVs compared to IMF. However, the lack of significance in this difference may be due to a combined analysis of EVs and casein micelles confusing the results. Of note, regardless of whether SM or IMF were pre-treated or non-treated, when directly comparing GUC and DUC, GUC always yielded many folds more EVs/particles with GUC compared to DUC. Experimental variability between biological repeats, however, meant that statistical significance was not always reached. While NTA is a widely used technique in EVs research, it has certain limitations including its incapability to distinguish between particle sizes in a heterogeneous mixture being one (Witwer et al., 2013). Therefore, to further validate our observations, the EVs isolated from SM and IMF were imaged using TEM. The TEM analysis of GUC samples suggested that no negative effect of the pre-treatment or ultracentrifugation on the EVs separated from SM, all the EVs derived from SM samples had a very smooth outer surface, round shaped and evenly distributed on the grid. Compared to SM samples, however, GUC generated IMF samples were sparsely distributed across the grids and showed rough surfaces and shapes indicating to signs of disruption or disintegration. Interestingly in all the SM DUC samples, very small particles (approximately 30 nm) were detected that did not have resemblance to the EVs that were observed in GUC samples. In IMF samples, some EVs structures were presented but compromised, with high amount of debris present in all IMF samples. The presence of high amount of debris in IMF might be related to the industrial processing during IMF production. Emami et al (2018) reviewed the physicochemical degradation of protein formulations that undergo harsh spray drying, lyophilisation and heating steps. This possibly leads to high number of insoluble protein particles or aggregates that may pellet at the highest speed. Additionally, these harsh industrial conditions potentially also lead to disruption of EVs present in milk and so intact EVs-like structures were typically not observed in IMF samples. As EVs typically carry RNA, but casein micelles do not, the separation of enriched

EVs samples were evaluated quantifying RNA yield (and RNA amount per particle) from the isolates from GUC and DUC. Interestingly, SM_NT and SM_IP samples, post GUC, were associated with the highest total RNA yield compared to their corresponding IMF samples. Additionally, GUC isolates typically carried more RNA/particle. While statistical significance in these comparisons was not always reached, at least partly contributed to by the quite substantial differences between biological repeats of the experiment, overall, the data does support GUC rather than DUC producing the more EVs-enriched isolates. Further characterisation of the SM and IMF samples were done by immunoblotting for EVs markers. Two positive markers for EVs (tetraspanin CD63 and TSG101) and a negative milk EVs marker (Actinin 4) were evaluated based on MISEV2018 guidelines. In the GUC generated lysed samples, all SM samples, irrespective of their treatment, were positive for CD63 and TSG101 and negative for Actinin 4. Interestingly, IMF samples were associated with notably weaker signals indicating extremely low concentration of EVs present in the sample. Since the same amount of protein (35 µg) was loaded on the gel for each sample, lower density of bands indicates a very low EVs to protein ratio indicating absence of EVs in the sample. A similar observation was also made in DUC samples where all IMF samples were again associated with faint bands for both CD63 and TSG101 compared to SM samples. These results, again, support our hypothesis that due to the use of harsh processing steps on milk in an industrial setting, EVs in the starting material are negatively affected. Of note, the doublet observed in some samples for TSG101 may be due to internal initiation at MET¹⁰ (VerPlank et al., 2001) whereas, in case of CD63, due to multiple glycosylation sites, bands within the range of 25–70 kDa were observed which is consistent with published literature (Metzelaar et al., 1991; Van Deun et al., 2014). Subsequently, the EVs separated from SM and IMF samples were analysed by flow cytometer (Ricklefs et al., 2019). Only events that had very low SSC signal and no brightfield signals were considered as a positive EVs like particle and presented as particles/mL of milk. Following the observations from our

previous characterisation steps, we observed that all SM samples -generated both by GUC and DUC- have a higher EVs like particle counts compared to all IMF samples. Further, enumeration of surface markers on EVs that include tetraspanins CD9, CD81 and CD63, ADAM10 and HLADR were also carried out. In GUC generated samples, several significant differences were observed; all indicating that SM samples have higher EVs surface markers compared to IMF samples, indicating again to the lower number of EVs present in IMF samples. In contrast, the SM samples generated by DUC recorded a higher CD9, ADAM10 and HLADR + EVs/mL compared to IMF samples. This observation may indicate that isolates resulting from DUC, in comparison to GUC, - are not as enriched in EVs. While this study is focussed primarily on comparing the differences between SM and IMF samples and we clearly demonstrated that IMF has lower amounts of EVs/EVs-like particles, comparison between GUC and DUC were also made. However, the favourable aspect of this technique is the small volume of sample required compared to DUC. In fact, based on our NTA and IFCM results, GUC is associated with the highest yield of EVs compared to DUC; there is a log difference between the number of EVs detected in GUC compared to DUC. Additionally, the EVs samples generated post-GUC, can be used in a multiple range of characterisation technique and generates highly reproducible data, apart from immunoblotting. In contrast, DUC yields high amount of visible pellet/suspension at the end of each centrifugation step, however, this suspension contains a high number of contaminant proteins that interferes with the further EVs characterisation. The results from this study strongly indicate that a combination of AA/IP and GUC could be the best strategy to separate the most enriched EVs from milk. In fact, GUC technique is a well-accepted method for isolation of EVs, especially the bottom-up approach, as previously reviewed (Witwer et al., 2013). Thus, returning to our hypothesis that EVs in IMF may be compromised, we can clearly state that IMF contains some but significantly fewer EVs/EVs-like particles; many of which have damaged morphology and do not carry intact EVs protein markers. Additionally, we can also conclude that

our methodology that includes IP pre-treatment and then GUC takes longer times but requires comparatively less starting material and separated EVs can be successfully analysed by techniques such as NTA, TEM and IFCM.

2.9 Conclusion

Although we observed merit as well as challenges with both GUC and DUC, in our opinion GUC does seem to be somewhat more beneficial here even though it is certainly not perfect (*e.g.* as shown by some variability between biological repeat experiments, especially on EVs-RNA analysis); but both techniques are possible suitable options. We encourage other researchers to use the information included here to inform their own choices as to which they consider to be the better method. Future studies analysing samples taking through the industrial processing of IMF may help to identify ways of preventing or minimising the EVs loss from IMF. The outcome from this study suggests that a functional study to compare the effects of the SM and IMF EVs isolates is now warranted.

3 Chapter 3: An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve

3.1 Abstract

Extracellular vesicles (EVs) in milk have claimed benefits ranging from conveying immunological privilege to infants to being suitable as natural delivery vehicles for therapeutic drugs. However, a longitudinal study of bovine EVs quantities and characteristics in colostrum (COL), first milk (FM) and throughout the lactation curve of mature milk (MM) had never been performed and so was our aim. COL, FM, and 9 months of MM samples were collected. Caseins -overlapping size with EVs- were removed. EVs were collected by density gradient ultracentrifugation and characterised by SDS-PAGE, Bradford assay, nanoparticle tracking analysis, immunoblotting, imaging flow cytometry analysis, and transmission electron microscopy. COL and FM had substantially more EVs than MM, with COL enriched in small EVs. No significant differences were observed between months 1–9 of MM. Altogether, although COL and FM are particularly rich sources of EVs, mature milk throughout the lactation curve is also an abundant source of intact EVs.

This chapter was recently published in *Food Chemistry* as research article.

“An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve”. Jessie Santoro, Anindya Mukhopadhyay, Charlotte Oliver, Andre Brodkorb, Linda Giblin, Lorraine O'Driscoll, *Food Chemistry*, Volume 401, 2023, 134029, ISSN 0308-8146, <https://doi.org/10.1016/j.foodchem.2022.134029>.

3.2 Introduction

The milk secreted by mammary glands in the time immediately after birth is known as colostrum (COL). It is considered to be a very rich source of nutritional, growth and immunological factors, vitamins and minerals and bioactive compounds (VandeHaar & St-Pierre, 2006; O'Sullivan et al., 2019). Within cows, COL is gradually replaced by first milk (FM) from the 2nd to the 8th day post-calving (Mcgrath et al., 2016). Although FM continues to be a rich source of nourishment for infant growth, it has a substantial reduction in immunological compositions compared to COL (Sanwlani et al., 2020). Mature milk (MM) is then produced from approximately-one-month post-parturition. Its composition differs from COL and FM which are higher in fat and lactose (O'Sullivan et al., 2019). The lactation period ends around 9 months post-calving (Butler, 2014). The composition of total milk solids, milk proteins, and antibodies is reported to change substantially throughout this period *i.e.*, COL includes 23.9% total solids, 14% proteins, and 6% antibodies, compared to 16%, 6.8% and 3.3% in FM and 12%, 3.1% and 0.09% in mature milk, respectively (Foley & Otterby, 1978; Gu et al., 2012) (Fig. 3.1). Milk, the primary source of vital nutrients and bioactive components contributing to the growth and development of the newborn, is a source of extracellular vesicles (EVs) with biological process modulating capabilities (Admyre et al., 2007; Hata et al., 2010). EVs are membrane-surrounded vesicles that are released from cells into biofluids, such as milk. EVs, including those from milk, are considered to play a fundamental role in intercellular communication. This is highly relevant as EVs are abundant in milk and they carry quite extensive cargo including proteins, lipids, and nucleic acids that contribute to them being considered as bioactive components of milk (Manca et al., 2018b). The fact that milk EVs have been demonstrated to be stable under conditions of low pH *i.e.*, maintaining their integrity in challenging conditions representing the gastrointestinal tract, further supports their bioactive relevance (Sanwlani et al., 2020). Indeed, for example, characterisation of milk EVs' cargo (mRNA and miRNA) has highlighted their anti-inflammatory

and immunomodulatory potential for attenuating symptoms of inflammatory bowel diseases (Mecocci et al., 2021). Milk EVs are reported to transmit maternal information to the infant, helping to shape the adaptive immune system in newborns (Zempleni et al., 2017). Similarly, to human babies, the health of calves is believed to benefit substantially from the transfer of fats, proteins, immune cells, and apparently EVs, that nourish and offer protection against infection and inflammation (Wu et al., 2016; Arslan et al., 2021). Indeed, bovine milk is readily available, cheap, safe, and serves as a starting material for various dairy products including nutritional beverages such as infant milk formula (Zeng et al., 2019). However, although studies -by ourselves and others- of EVs from milk of several mammalian species, such as human, bovine, and porcine have been reported (Admyre et al., 2007; Gu et al., 2012; Zonneveld et al., 2014; Samuel et al., 2017; Somiya et al., 2018) knowledge of comparative EVs quantities and characteristics throughout the lactating stages are lacking. For the dairy industry, for consumers of milk/milk products and, indeed, for those investigating milk EVs as natural delivery vehicles for therapeutics, it is relevant to know if and how EVs change over the lactation curve. Hence, we hypothesised that as COL is considered a particular rich source of nutrients and antibodies that it contains most EVs. To investigate this, we collected bovine COL, FM, and MM; we separated EVs following our recently developed protocol (Mukhopadhyaya et al., 2021); and then performed comparative quantification and characterisation of the EVs.

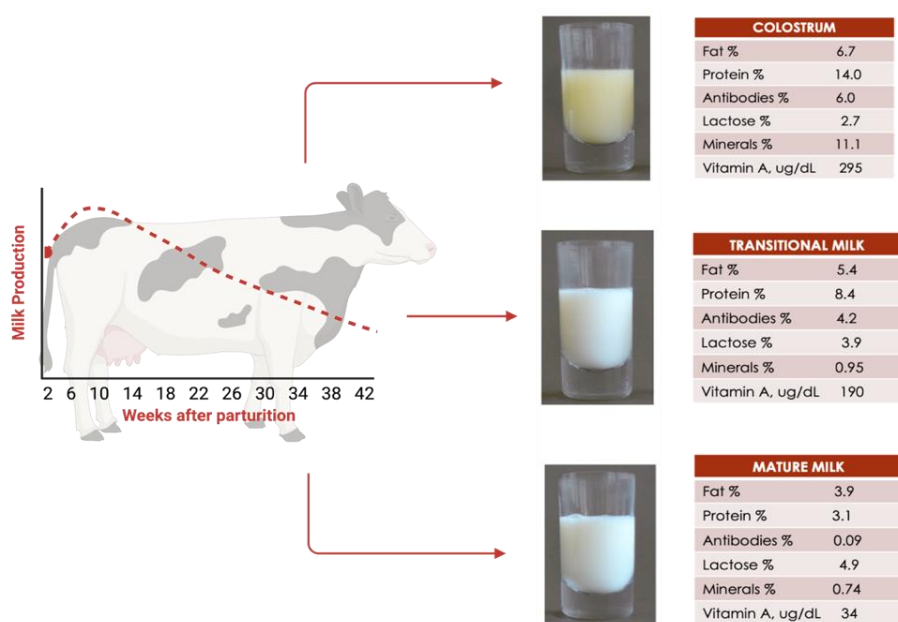


Figure 3.1: Milk production and composition at different stages of the lactation curve

Representative milk production graph from parturition and in the following weeks (left side). Variable composition of major nutrients in colostrum, first milk and mature milk, respectively (right side).

3.3 Materials and methods

3.3.1 Collection and defatting of milk samples

Bovine COL, FM and MM were collected from n=3 healthy Holstein-Friesian cows bred in the Dairygold farm Teagasc, Moorepark, Fermoy, Co. Cork, Ireland (for n=3 independent experiments). COL was collected within 24 h post-calving, FM after 7 days, and MM from months 1 to 9 post-calving. All subsequent analyses detailed in the study were performed a minimum of 3 separate times on each of the 3 sets of samples. All samples were defatted at 6,000 x g at 4°C for 10 minutes, the supernatant was filtrated through glass wool, and then immediately stored at -80 °C and shipped to Trinity College Dublin for the research to be performed.

3.3.2 Sample processing and EVs separation

EVs were separated from COL, FM and MM using a method that we recently developed (Mukhopadhyaya et al.; 2021) with slightly modifications. To note, isoelectric precipitation was performed in Teagasc before sample shipping. As represented in Fig. 3.2, to remove caseins from defatted COL, FM and MM, the pH was dropped to 4.6 using 5M HCl. Then, it was centrifuged at 8,000 x *g* at 20 °C for 30 min. Then 2.33 mL of whey was used for density gradient ultracentrifugation following the bottom-up technique using Optiprep iodixanol solution (60%). Ultracentrifugation used an SW Type 32.1 Ti rotor in Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 186,000 x *g* for 18 h at 4 °C. Pooled fractions (1.05-1.20 g/mL) were washed twice in sterile PBS at 120,000 x *g* for 2hrs at 4 °C. Each resulting pellet was resuspended in 500µL of 0.22 µm filtered PBS.

3.3.3 SDS-PAGE for protein visualisation

Removal of casein micelles from COL, FM and MM samples was confirmed by performing SDS-PAGE analysis and visualisation of protein bands on a 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Cat. #4561034). Specifically, 10 µg of EVs fractions were loaded on the gel, run at 120 V, stained with Coomassie blue (Sigma- Aldrich, Ireland) and visualised using a Chemidoc XRS system (Bio-Rad).

3.3.4 Protein quantification

Bradford assay was performed to determine total protein in samples using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Cat #500–0006). Bovine serum albumin (BSA) standards (0–800 µg/mL) (Sigma-Aldrich, Ireland) were used to generate a standard curve for interpolation of the protein quantities.

3.3.5 Nanoparticle Tracking Analysis

NTA was performed at the Laboratory for Biological Characterisation of Advance Materials (LBCAM), in Trinity College Dublin (TCD) as a core service.

The concentration and sizes of particles in the EVs fractions were estimated using NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK). Samples were automatically injected into the NTA system under constant flow conditions (flow rate =50) and a minimum of four × 60 second videos were recorded for each sample. The detection threshold during analysis was selected to ensure that only distinct nano-objects were analysed, and any artefacts were removed. Videos of the particles in motion were recorded and analysed using NTA 3.2 software.

3.3.6 Immunoblotting analysis

Immunoblotting was performed as we previously described (O’Brien et al., 2013) with some modifications. Specifically, EVs lysates (30 µg) were resolved on 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Cat. #4561034) and proteins were transferred to PDVF membranes (Bio Rad, Cat. #: 17001917) using Trans-Blot® Turbo™ Transfer System (Bio Rad, Cat. #: 1704150) at 25V for 7 min (Mixed MW). Blots were blocked with 5% (w/v) BSA in PBS

containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies against TSG101 (1:1000; Abcam, Cambridge, UK, Cat. #: ab30871), CD9 (1:1000; Abcam, Cat. #: ab92726), CD63 (1:500; Abcam, Cat. #: ab68418), ACTN4 (1:1000, Abcam, Cat. #: ab41525), BTN1A1 (Abcam, Cat. #: ab180605), Milk Fat Globule 1 (MFG1) (1:500, Abcam, Cat. #: ab200649), MUC-1 (Abcam, Cat. #: ab45167) and GRP94 (1:1000, Abcam, Cat. #: ab238126). Secondary antibodies used were anti-mouse (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7076) or anti-rabbit (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7-74). SuperSignal West Femto Chemiluminescent Substrate Kit (Fisher Scientific, Cat. # 11859290) was used for detection, imaging was performed using an automated Chemidoc exposure system (Bio-Rad Laboratories), and densitometric analysis was performed using ImageJ. Hs578T mammalian cell lysate (CL) and crude bovine milk were used as controls. Specifically, we used CL as control for all EVs markers including GRP94, whereas raw bovine milk was used as control for milk fat globule proteins BTN1A1, MFG1 and MUC1.

3.3.7 Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis as described in Chapter 1, Section 2.3.10.

3.3.8 Imaging flow cytometry analysis

The imaging flow cytometry (IFCM) analysis of intact/non-lysed EVs separated from COL, FM and MM samples was performed as described in Chapter 1, Section 2.3.12. Specifically, the specific antibodies used in this study were anti-CD63 conjugated with fluorescein isothiocyanate

(FITC) CD63- FITC, 1:150, Cat. #:353006, Biolegend) and CD9-PE (1:1500, Cat. #:312106, Biolegend). EVs were incubated with the antibodies for 45 min at RT in the dark and washed using a 300 kDa filter (Nanosep, Cat. #:516-8531). Samples were resuspended in 50 μ L IFCM buffer and acquired within 2hrs on the ImageStream X Mk II imaging flow cytometer (Amnis/Luminex, Seattle, USA) at 60x magnification and low flow rate and EVs-free IFCM buffer. Unstained EVs, single-stained controls, and fluorescence minus one (FMO) control were run in parallel. EVs were gated as SSC-low versus fluorescence, then as non-detectable brightfield (fluorescence vs Raw Max Pixel Brightfield channel). Gated EVs were confirmed in the IDEAS Image Gallery. Data analysis was performed using IDEAS software v6.2 (Amnis/Luminex, Seattle, USA). Fluorescence and channel used were reported as following: FITC was measured in channel 2 (B/YG_480–560 nm), PE in channel 3 (B/YG_560–595 nm filter), PE-Cy7 in channel 6 (B/YG_745-780 nm filter), BV421 in channel 7 (R/V_435-480 nm filter), APC in channel 11 (R/V_642–745 nm, filter), Brightfield in channel 1 and 9 (B/YG_435-480 and R/V_560-595 nm filter respectively) and Side Scatter Channel (SSC) in channel 12 (R/V_745-780 nm filter). Of note, a flowchart of the methodology used in this study was reported below (Fig. 3.2).

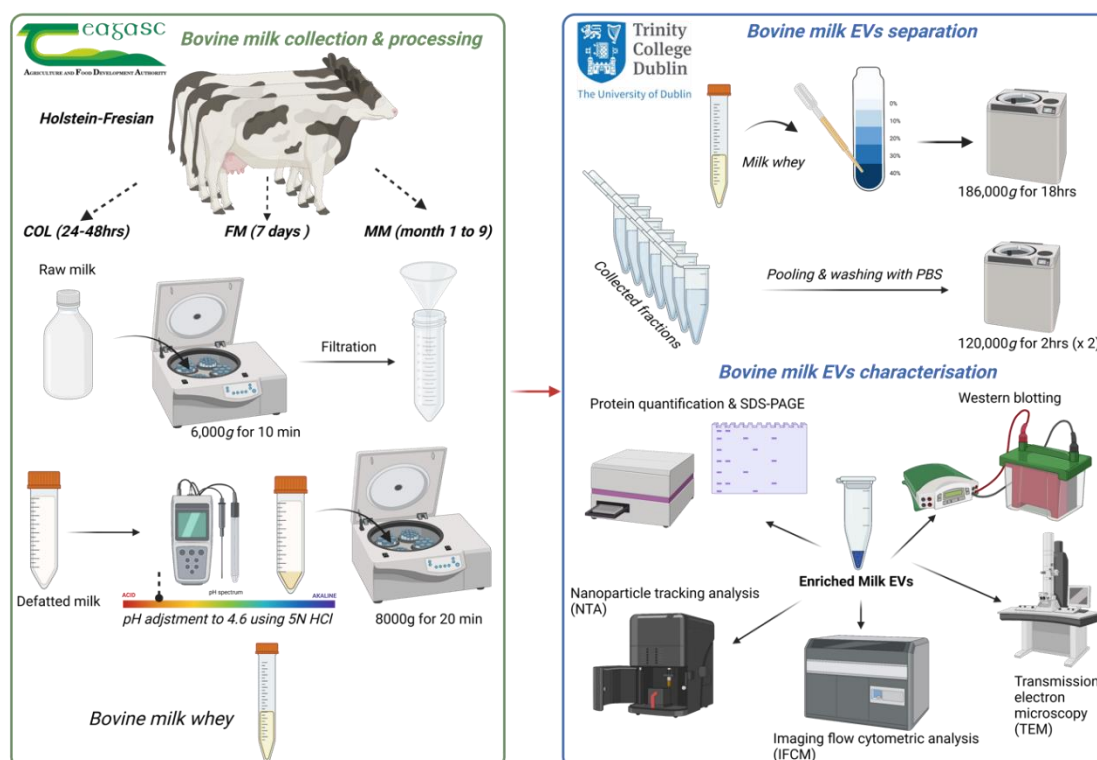


Figure 3.2: Summary of the methodology

Methodology including milk collection, pre-treatment (left side) and milk EVs separation and characterisation from bovine colostrum, first milk and mature milk (right side).

3.4 Data analysis

Graph Pad Prism 9 software was used to perform statistics analysis of all data. Unpaired two tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $p < 0.05$ were considered statistically significant. The Shapiro- Wilk test was done to evaluate the distribution of our data. All relevant Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments ($n=3$). EVs related information was submitted to the EV-TRACK knowledgebase and assigned EV-TRACK ID: EV220299 (Van Deun et al., 2017).

3.5 Results

3.5.1 Casein removal analysis by SDS-PAGE and protein quantification

Casein from COL, FM and MM was removed by isoelectric precipitation at pH 4.6. As shown in Fig. 3.3a we observed that acid treatment of milk efficiently removed most, if not all, of the casein, as evident when compared to untreated milk. The total protein quantification in the EVs samples, performed by Bradford assay (Fig. 3.3b), showed COL EVs samples had a very high protein concentration compared to FM and MM ($p < 0.001$).

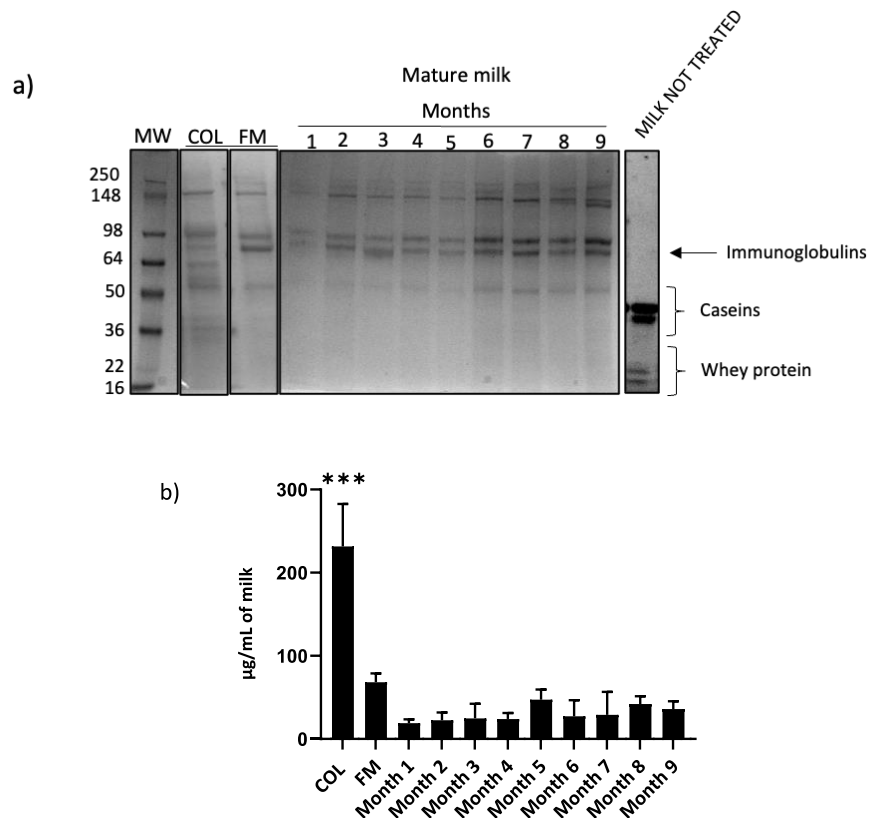


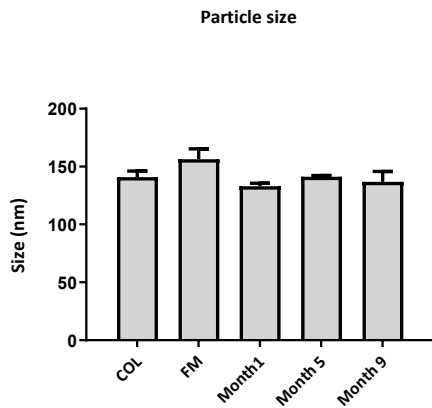
Figure 3.3: Milk protein characterisation of bovine colostrum, first milk and mature milk

a) Milk protein in EVs enriched fraction of COL, FM and MM were visualised by loading 10 µg of protein on 10% Mini-PROTEAN® TGX™ Precast Gel followed by Coomassie Blue staining. Molecular weights (MW) standard is reported to represent milk proteins presented in all milk EVs samples. b) Protein concentration was assessed by Bradford assay and reported in µg/mL of milk used. Each bar represents 3 biological repeats, statistical analysis using one-way ANOVA test was performed, error bars represent standard error of mean ($\pm$ SEM), *** indicates $p < 0.001$.

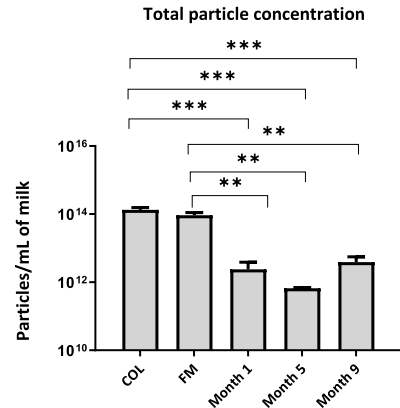
3.5.2 Particles concentration and size measurement by nanoparticle tracking analysis

NTA estimated particle sizes of COL EVs and FM EVs enriched fractions were 140 ± 5.1 nm and 156 ± 8.8 nm, respectively, while EVs in MM month 1 were 133 ± 2.6 nm, month 5 were 141 ± 1.0 nm and in month 9 were 136 ± 8.8 nm. Overall, no significant differences were observed in particle size among the samples analysed (Fig. 3.4a). Total particles/EVs concentration (Fig. 3.4b), as well as concentration of particles/EVs sized 30–150 nm (size range of small EVs/sEVs), and concentration of particles/EVs sized > 150 nm (size range representing medium and large EVs) from COL, FM and MM samples are presented in and Fig. 3.4c and Fig. 3.4d, respectively. Firstly considering the overall heterogenous pool, COL ($p < 0.001$) and FM ($p < 0.01$) had significantly greater amounts of EVs when compared to MM. Specifically, COL had $1.3 \times 10^{14} \pm 2.2 \times 10^{13}$ particles/mL and FM had $9.3 \times 10^{13} \pm 1.8 \times 10^{13}$ particles/mL, whereas the corresponding values for MM particle concentration month 1 was $2.3 \times 10^{12} \pm 1.5 \times 10^{12}$ particles/mL, month 5 was $6.6 \times 10^{11} \pm 3.0 \times 10^{10}$ particles/mL and month 9 was $3.9 \times 10^{12} \pm 1.6 \times 10^{12}$ particles/mL (Fig. 3.4b). Then considering the EVs based on size distribution, a similar trend was observed in that COL had significantly higher concentration of sEVs (Fig. 3.4c) compared to FM ($p < 0.05$) and MM ($p < 0.001$). Likewise, FM had significantly ($p < 0.01$) higher concentration of particles compared to MM. For those sized > 150 nm Fig. 3.4d, COL had a significantly ($p < 0.01$) higher concentration of particles compared to MM and FM had significantly ($p < 0.05$) higher concentration compared to MM. However, no significant differences were observed between COL and FM. Overall, COL and FM had similar amounts of particles/EVs with COL typically having highest concentrations of particles considered to represent EVs. Thus, both COL and FM were substantially more enriched in particles/EVs compared to MM. Once MM was being produced, however, the particle/EVs concentrations did not differ significantly over the 9 months of the lactation curve.

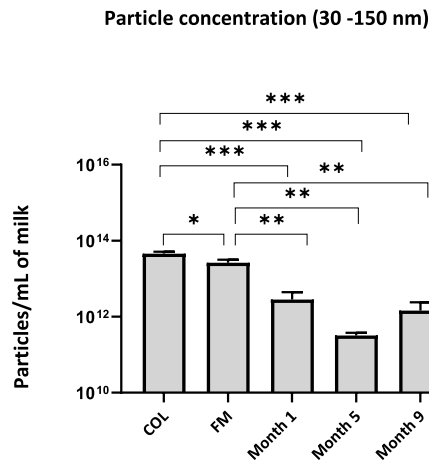
a)



b)



c)



d)

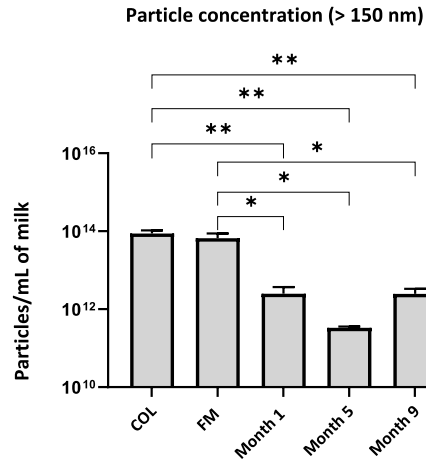


Figure 3.4: Particle sizes and concentration from colostrum, first milk and mature milk EVs

a) Comparison of particle size in colostrum, first milk and mature milk. b) Total particle concentration comparison of colostrum, first milk and mature milk EV represented by months 1, 5 and 9; c) Comparison of particle concentration within 30–150 nm of colostrum, first milk and mature milk EV samples. d) Comparison of particle concentration over 150 nm of colostrum, first milk and mature milk EV samples. Three replicates of each sample were analysed by NTA independently, data analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

An additional comparison of bovine milk EVs throughout the lactation curve was done by comparing particles/EVs separated from a pool of many cows this known as bulk milk. As illustrated and detailed in Fig. 3.5, the particle/EVs concentrations in bulk milk for the pool of

particles were similar to mature milk and were significantly ($p < 0.0001$) fewer than COL and ($p < 0.001$) FM (Fig. 3.5a). Likewise, the concentration of subsets of 30-150nm (Fig. 3.5b) and >150 nm (Fig. 3.5c).

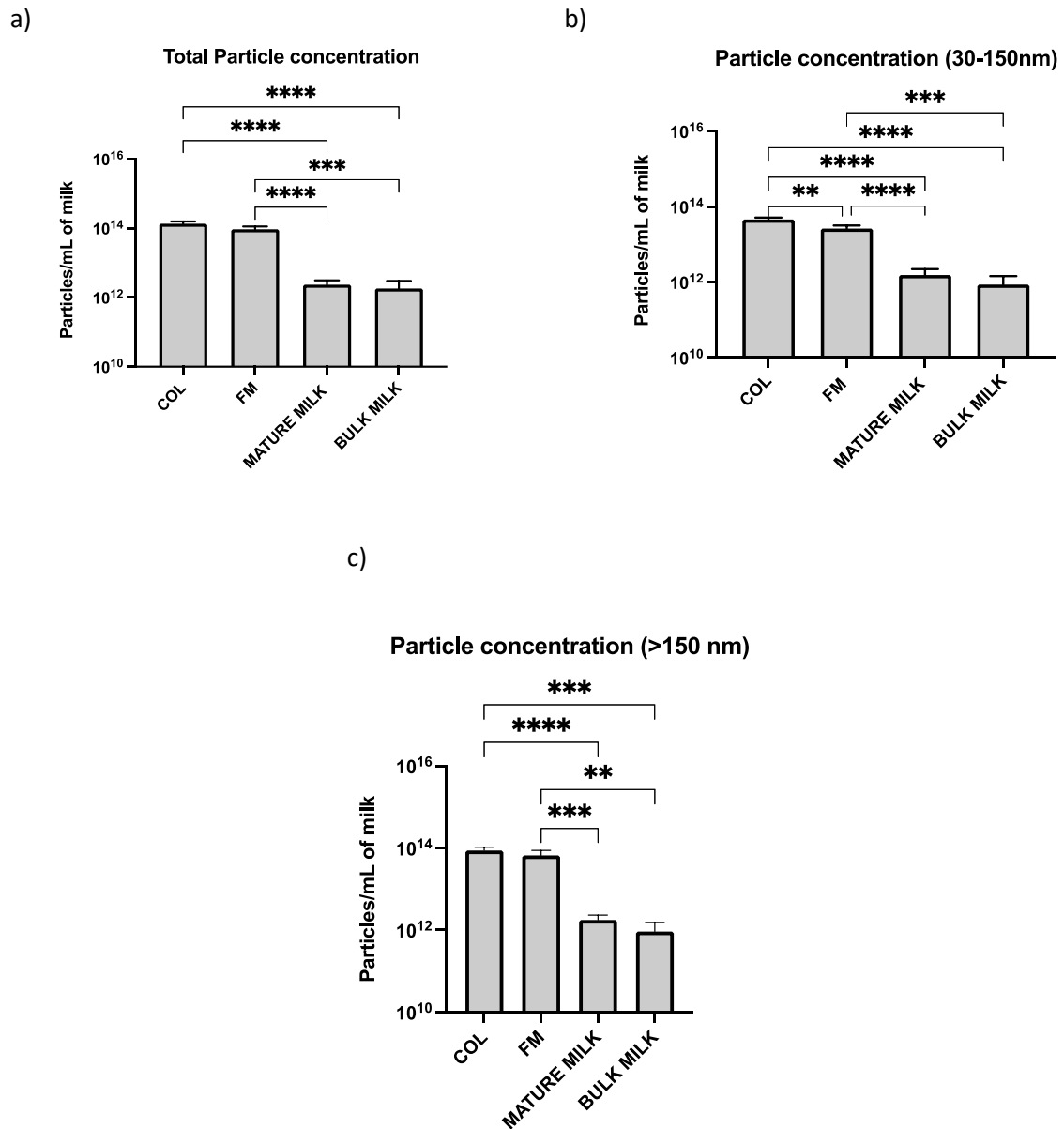


Figure 3.5: Particle concentration from colostrum, first milk, mature milk, and bulk milk EVs

a) Comparison of total particle concentration in COL, FM, MM, and bulk milk. b) Comparison of particle concentration within 30-150 nm in COL, FM, MM, and bulk milk. c) Comparison of particle concentration mean over 150 nm in COL, FM, MM, and bulk milk. The mean of three cows of all months for each sample were analysed by NTA and data analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** $p < 0.0001$.

3.5.3 Immunoblotting analysis using protein markers associated with EVs

To confirm the presence of EVs in COL, FM and MM, immunoblotting analysis was performed on lysed samples for three EVs markers (TSG101, CD9 and CD63) and one negative marker (ACTN4). All samples were found to be positive for TSG101, CD9 and CD63, whereas no signal was detected for ACTN4 in any samples (Fig. 3.6a). Likewise, GRP94, known to be associated to large vesicles (Théry et al., 2018), was not detected in any of the samples analysed. Additionally, we investigated markers associated to milk fat globules, BTN1A1, MFG1 and MUC-1. These were not detected in any of the milk EVs samples, confirming the enrichment of EVs in our samples and the absence of other vesicles and MFGs (Fig. 3.6b). Overall, what was observed by immunoblotting, where 30 µg of protein was loaded for all samples, was that all 3 EVs-positive markers evaluated were detected, and the amounts detected were quite reproducible between separate experiments. Although immunoblotting analysis of lysed EVs is qualitative not quantitative, no substantial differences were evident between COL, FM, and MM. Thus, from this point onwards, months 1, 5 and 9 were selected to represent the MM throughout the lactation period.

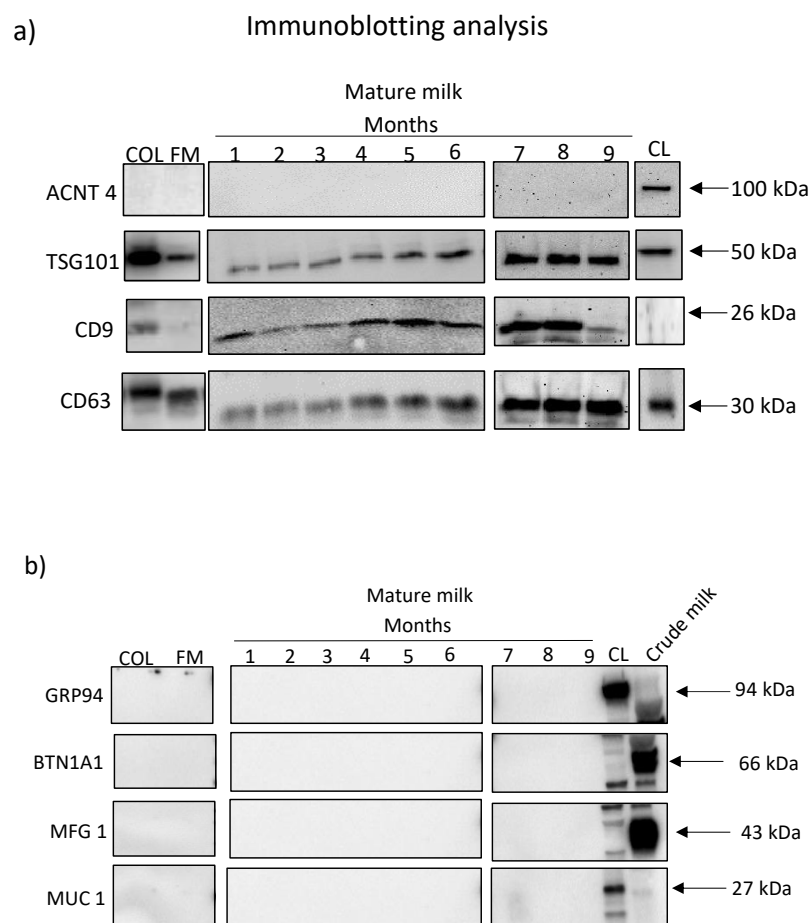


Figure 3.6: Detection of EVs markers by immunoblotting

Immunoblotting analysis of EVs separated from colostrum, first milk, and mature milk was performed by loading 30 µg of protein for all the EVs samples and CL (cell lysate). a) Analysis of EVs-associated positive markers TSG101, CD63, CD9 and negative marker ACTN4. b) Analysis of three milk fat globules markers BTN1A1, MUC1, MFG1 and one microvesicles marker GRP94.

3.5.4 Presence of EVs surface markers detected by imaging flow cytometric analysis

Quantitative characterisation of single intact COL, FM and MM EVs samples were performed by IFCM. Here, EVs were analysed based on the presence of EVs surface markers CD9 and CD63. COL was found to have significantly higher amounts of CD9+ and CD63+ particles compared to

FM and MM (Fig. 3.7a) (Fig 3.7b). Throughout the remainder of the lactation curve no significant differences were evident.

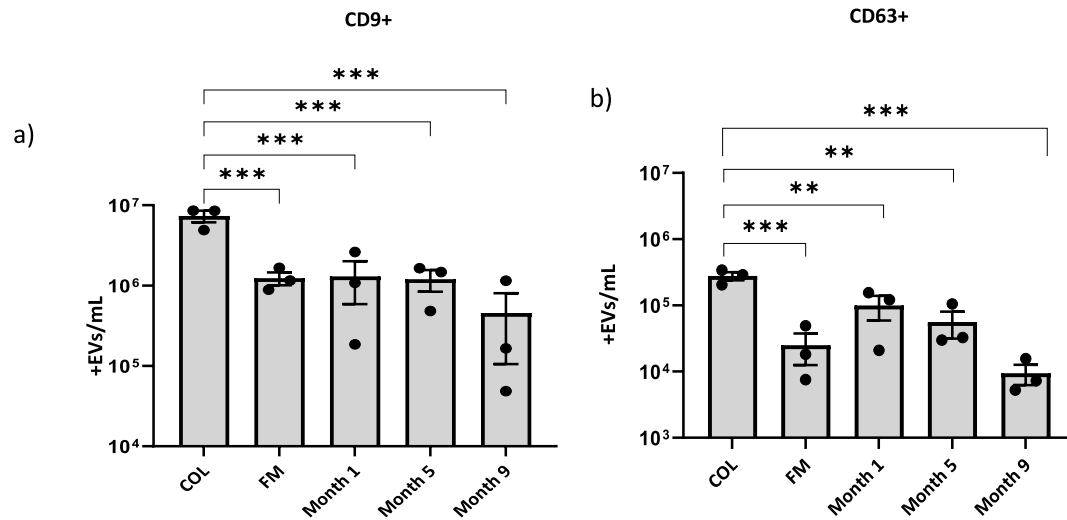


Figure 3.7: Imaging flow cytometry analysis of EVs surface marker

Number of positive events (particles) in colostrum, first milk and mature milk analysed by imaging flow cytometry (IFCM). The number of positive b) CD9 and c) CD63 in colostrum, first milk and mature milk. The IDEAS software was used to analyse the data. Data presented as mean and individual values $\pm$ SEM. One-way ANOVA tests were performed, and significance is indicated as ** p < 0.01, *** p < 0.001.

3.5.5 Transmission electron microscopy analysis of bovine milk EVs

The structure of EVs separated from COL, FM, and MM, and as analysed by TEM, were of vesicular morphology with intact membranes, with no substantial differences observed between sample preparations (representative micrographs presented in (Fig. 3.8)).

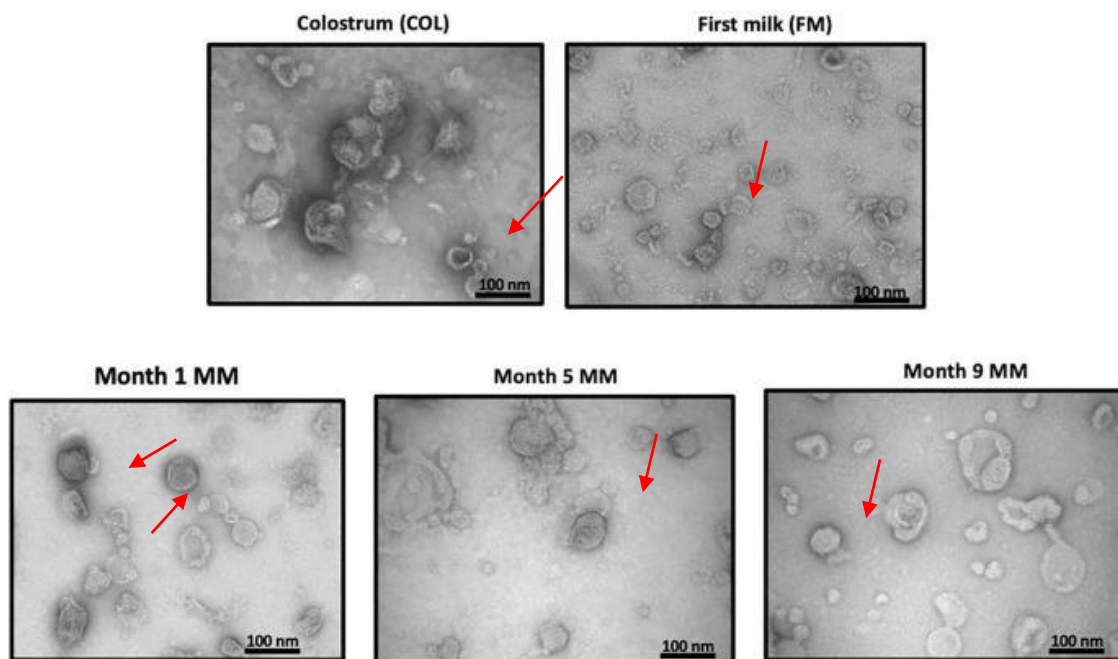


Figure 3.8. Transmission electron microscopy analysis of EVs morphology

Representative transmission electron microscopy analysis of colostrum, first milk and mature milk EVs samples. Scale bar = 100nm.

3.6 Discussion

In the dairy industry, milk yield is one of the most important factors to determine the profitability of dairy cows (VandeHaar & St-Pierre, 2006; Hutjens, 2016). Although, in Chapter 2 we presented some information regarding milk EVs from commercially available sources, we further extended our interest to explore bovine milk EVs and characterise them

throughout the entire lactation cycle, as this had never been done before. Therefore, we aimed to collect and characterise EVs from bovine COL, FM, and MM, to investigate their similarities and differences over the lactation curve. Of note, some previous studies have explored milk EVs from different species at distinct stage of lactation, but EVs separation and characterisation throughout the entire lactation cycle was lacking. For instances, Ferreira et al (2021) separated porcine milk vesicles, that they termed 'exosomes', from colostrum at day 0 and mature milk but at days 7–14 post-partum only. Similarly, Lin et al (2020) separated vesicles that they termed 'exosomes' from porcine and bovine milk, but only within a few days of parturition. Furthermore, in both studies the fundamental characterisation of the "exosomes" was very limited. Thus, while arguably all studies have their merits, it is our understanding that the research we present here is the first longitudinal study of EVs from colostrum, first milk, and mature milk spanning the whole of the lactation curve from the same donors. Furthermore, these EVs were collected by a method accepted to remove most non-EVs components (*i.e.*, ultracentrifugation on a density gradient) and there were subsequently extensively characterised by orthogonal methods, in keeping with MISEV2018 guidelines (Théry et al., 2018). Taking this approach, here we observed that although abundant intact EVs are present at all the stages of the lactation curve, the EVs concentration is significantly higher in COL and FM, decreasing in month 1 of MM to a level that is then maintained throughout the remainder of the 9 months mature milk period. COL was particularly enriched in small extracellular vesicles (sEVs). Separation of EVs from milk is challenging due to the biological complexity of this matrix, thus previously we developed a combination of acid pre-treatment before performing EVs separation to remove casein and then separated relatively pure EVs that are suitable for characterisation (Mukhopadhyaya et al., 2021). Thus, based on that success here we followed the same casein removal and EVs separation methods for COL, FM, and MM from months 1 to 9, to represents the entire bovine lactation curve. Following separation of EV enriched fractions, SDS-

PAGE analysis indicated efficient casein removal. This is important as many milk EVs studies do not remove the very highly abundant casein micelles which have a size of approximately 150 nm (falling well within the size range of EVs) and so they substantially confuse the interpretation of milk EVs data. In fact, to our knowledge, this is the first ever study in which isoelectric precipitation was performed to remove casein micelles from bovine COL (as well as FM and MM across the lactation curve) and separate pure EVs-enriched fractions post ultracentrifugation. As indicated in the Results Section, NTA analysis suggested significantly lower particles/EVs concentration in MM, compared to COL and FM. While Hata et al (2010) and Samuel et al (2017) reported abundant EVs in both bovine COL and MM, in those studies no acid pre-treatment was performed prior to remove casein micelles. Also, those studies did not extend to comparing each of COL EVs and FM EVs and MM EVs, as we report here. Here, in addition to establishing that both COL and FM are enriched in EVs compared to MM, we discovered that this then remains steady in the following months in MM throughout the lactation curve. Further characterisation of COL, FM and MM samples was performed by immunoblotting for EVs-specific protein markers. In line with the MISEV2018 guidelines (Théry et al., 2018) three positive EVs markers (TSG101 and two tetraspanins CD9 and CD63), one negative marker (ACTN4) and one microvesicles marker (GRP94) were evaluated. COL, FM, and MM EVs were positive for TSG101, CD9 and CD63, and all were negative for ACNT4 and GRP94, which supports the presence of EVs in all samples. In the context of work by others, Yun et al (2020) and Ross et al (2021) reported the presence of EVs in bovine COL. However, again no pre-treatment was used to remove casein micelles which makes EVs quantification and characterisation challenging and potentially more unreliable. Additionally, here we also used markers for milk fat globules (MFGs) such as BTN1A1, MUC- 1 and MFG1 which were not detected in any of our milk EVs samples. Therefore, following our optimised protocol, when applied in this study we successfully removed most of the non-EVs components to achieve higher purity during EV separation. Imaging flow cytometry

technology was used to quantify EVs surface markers CD9 and CD63 on intact EVs. Supporting what was observed by the cruder NTA analysis, COL had highest quantities of particles/EVs [CD9+ and CD63+] compared to FM and MM, confirming that bovine COL is highly enriched in EVs. Additionally, CD9+ and CD63+ EVs did not differ significantly between the MM months. Furthermore, the presence of EVs in all milk samples was further validated by qualitative TEM analysis. All COL, FM, and MM samples analysed had typical vesicular morphology *i.e.*, round with intact membranes and were heterogenous in size.

3.7 Conclusion

In this chapter we further demonstrated the success of our optimised method for EVs separation; not only from commercially available milk, but also from raw bovine milk. This first longitudinal study of EVs in COL, FM and MM showed all sources to be rich in vesicles carrying typical EVs markers, intact membranes, and with expected heterogeneity in sizes. As we hypothesised, COL has substantially more EVs than MM. It is particularly enriched in small EVs. No significant differences were observed between EVs in months 1–9 of MM. Altogether, although COL is a particularly rich sources of EVs, mature milk throughout the lactation curve is also an abundant source of intact EVs. Thus, if a very rich source of EVs is required, we would suggest COL as an optimal source. However, for scalable sources -such as would be required if EVs are to be used as natural, cheap, safe drug delivery vehicles- there is reason to be optimistic that milk collected at any stage of the lactation curve would be suitable for this purpose.

4 Chapter 4: Characterisation of EVs from different stages of IMF manufacturing at industry level

4.1 Abstract

Ultra-high treatment is a methodology used worldwide to make IMF by the dairy industry. The use of high heat temperature is necessary for safety and quality purposes, but it can lead to protein denaturation and disturb many bioactive components in milk; our studies indicate that EVs may be affected. However, which steps of industrial manufacturing of (IMF) have potentially negative effects on EVs is unknown. Therefore, here we separated and characterised EVs from multiple stages of IMF production, to unravel which steps, if any, might have significant impact on EVs quality and quantity.

4.2 Introduction

While breastfeeding is highly recommended for infants, in some cases it is not possible, unsuitable, or is inadequate. Therefore, an alternative option to feed infants is required. Bovine milk-based formula is the most commercial type of IMF available, because bovine milk is one of the first foods introduced into an infant's diet (Koletzko, 2018). IMF can be used to replace HM, because of its invaluable buffet of 'biologically active' components, such as α -lactalbumin, milk proteins with anti-microbial and immune-modulating properties such as lactoferrin and milk fat globules (MFGs) (Sandström et al., 2008; Lönnerdal, 2009). Thus, IMF is developed, but bovine milk is subjected to severe changes during its preparation. Safe and palatable product are the two most important aims to obtain IMF final composition. As described in Chapter 1, Section 1.2.3, for IMF manufacturing, during the skimming procedure, concentrated milk must be processed at very high temperature (~ 160 - 205 °C). As proteins are sensitive to heat, it is essential that the evaporators should maintain an optimal temperature that will not cause heat damage and denaturation of the milk proteins while the desired effect is obtained (Moejes & van Boxtel, 2017). Indeed, at various stages of IMF manufacturing, additional components are supplemented to simulate HM composition. Generally, IMF is made in large scale using ultrahigh treatment (UHT), employed worldwide and the goal is to produce infant milk formula and protect most, if not all, the nutritional ingredients. Substantial efforts are made to make IMF similar to HM. However, as the UHT manufacture of IMF includes skimming, homogenisation, high heat treatment, evaporation and spray drying, these steps may affect significantly bioactive components, naturally present in milk, such as EVs. In Chapter 2, we demonstrated that there is a robust loss of EVs in IMF compared to commercial skim milk (Mukhopadhyaya et al., 2021). During the process, the composition of the starting material may be compromised because of these harsh treatments; however, we do not know in which steps of the IMF manufacturing EVs

are lost remarkably (Fig. 4.1). Hereby we collected samples at different stages of IMF UHT, we explored the EVs quantity and quality in order to better understand when loss occurred, and this potentially identify a way to improve IMF.

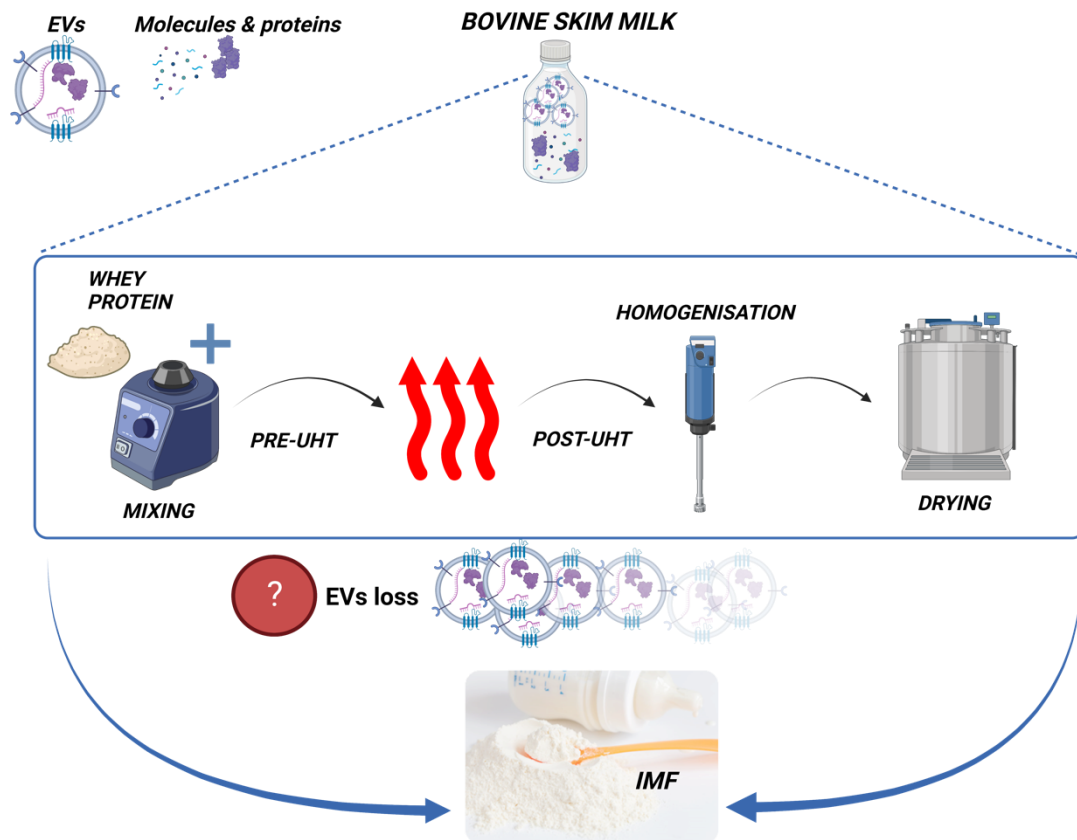


Figure 4.1: Schematic representation of IMF manufacturing and potential EVs loss during the preparation

4.3 Material and methods

4.3.1 Collection of IMF manufacturing ingredients resulting from Ultra High Temperature (UHT) processing

Samples from six stages of IMF UHT processing were collected at the pilot plant at Teagasc, Moorepark, Co. Cork, these included skim milk (SM), whey protein isolate (WPI), skim milk +

wey protein isolate (SM+WPI), pre ultra-high temperature (PRE-UHT), post ultra-high temperature (POST-UHT), spray dried formula, summarised in detail in Fig. 4.2. SM and WPI were mixed at 4°C while additional fat, vegetable oils and mineral blend were separately mixed at 80 °C and added to SM+WPI mix. UHT process was performed for 3s at 125°C and homogenisation was followed at 150+50 Bar. Finally spray drying to obtain the powder formula (final product) was done with an inlet of 185°C and output of 80°C.

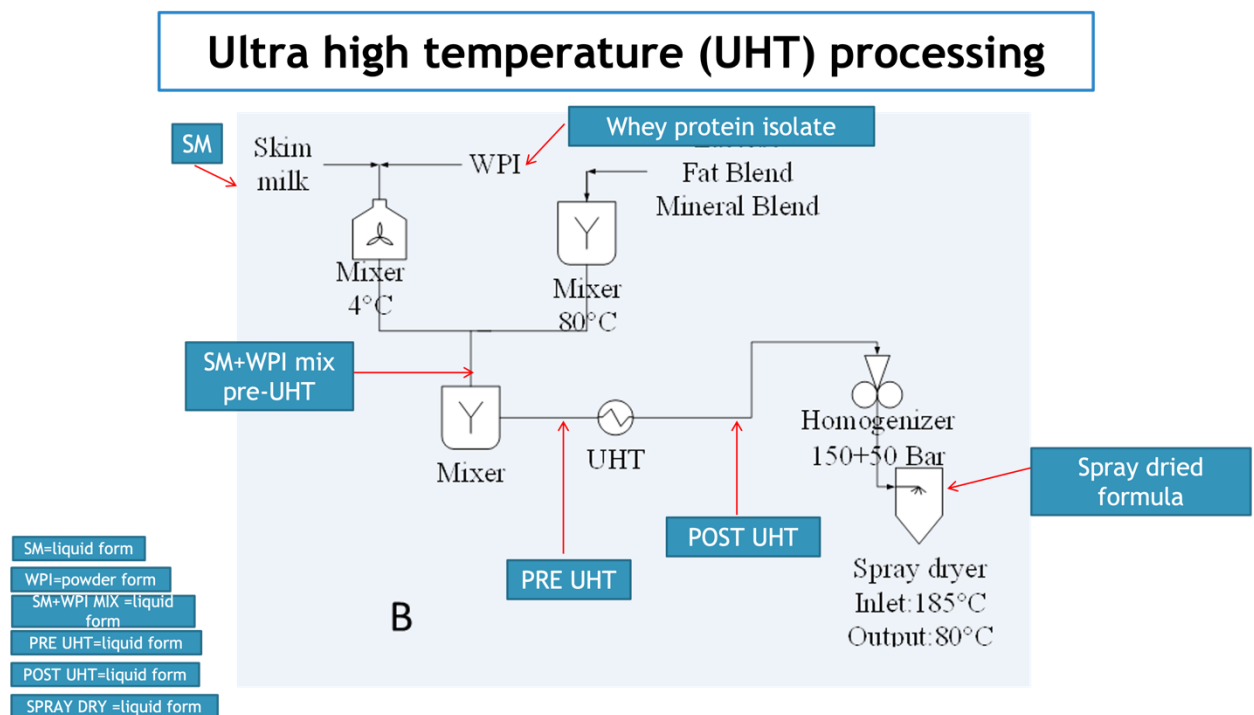


Figure 4.2: Stages of IMF manufacturing using UHT processing

Description of IMF UHT manufacturing. Selected ingredients collected for this study are indicated by the red arrows. SM= skim milk; WPI = whey protein isolate; SM+WPI= skim milk + whey protein isolate; PRE-UHT= pre-ultra-high treatment; POST-UHT= post-ultra-high treatment.

4.3.2 Sample processing and acid pre-treatment

IMF UHT ingredients were processed before EVs separation, as described in Chapter 2, Section 2.3.2. Here, powder samples were reconstituted as recommended upon collection at the pilot plant, subsequently 20 mL of all samples were used for acid pre-treatment.

4.3.3 EVs separation using density gradient ultracentrifugation

After sample pre-processing, EVs separation from IMF UHT ingredients was performed using 2.33 mL of the filtrated whey following the same procedure outlined in Chapter 3, Section 3.3.2.

4.3.4 Total protein quantification of EVs pellet using Micro BCA™ Protein assay kit

Total protein of EVs pellet was estimated using Micro BCA™ protein assay kit (Thermo Scientific, Cat. #: 23235), which compared to Bradford assay uses concentrated reagents and a protocol with an extended incubation time (approx. 2hrs). Standards were in PBS prepared from a bovine serum albumin (BSA) standard ampule into several clean vials which were used to generate a linear working range of 0.5-40 µg/mL, as reported in Table 4.1. Total volume of Working Reagent (WR) was prepared by mixing 25 parts of Micro BCA Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1). Next, 10 µL of EVs were diluted with 140µL of PBS (*i.e.*, 1 in 15 dilution) and 150 µL of standards (2-40 µg/mL) were added into a 96-well plate. 150 µL of the WR was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered using a Sealing Tape for 96-well plate (Thermo Scientific, Cat. #: 15036) and incubate at 37 °C for 2 hours, after which it was left cooling at room temperature. Absorbances were measured at 562nm on a FluoStar Optima microplate reader (Serial #: 08-100-241). Finally, protein quantification was determined using the standard curve.

Tubes	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	4.5 mL	0.5 mL of Stock	200 µg/mL
B	8.0 mL	2.0 mL of vial A dilution	40 µg/mL
C	4.0 mL	4.0 mL of vial C dilution	20 µg/mL
D	4.0 mL	4.0 mL of vial C dilution	10 µg/mL
E	4.0 mL	4.0 mL of vial D dilution	5 µg/mL
F	4.0 mL	4.0 mL of vial E dilution	2.5 µg/mL
G	4.0 mL	4.0 mL of vial F dilution	1 µg/mL
H	4.0 mL	4.0 mL of vial G dilution	0.5 µg/mL
I	8.0 mL	0	0 µg/mL= BLANK

Table 4.1: Preparation of Diluted Albumin (BSA) Standards

4.3.5 SDS-PAGE for EVs protein characterisation

SDS-PAGE analysis was employed for visualisation of protein bands in all EVs sample. The methodology used was performed following the procedure in Chapter 3, Section 3.3.3.

4.3.6 Nanoparticle tracking analysis

Particle concentration and sizes in UHT EVs samples were analysed using Nanoparticle Tracking Analysis (NTA) as described in Chapter 3, Section 3.3.5.

4.3.7 Immunoblotting analysis

Immunoblotting for all the samples was performed following the methodology in Chapter 3, Section 3.3.6, with some modifications. Here, 10 µg of EVs lysates were resolved on 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Cat. #4561034) and separated proteins were

transferred to PDVF membranes (Bio Rad, Cat. #: 17001917) using Trans-Blot® Turbo™ Transfer System (Bio Rad, Cat. #: 1704150) at 25V for 7 min. Next, blots were blocked with 5% (w/v) BSA in PBS containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies against TSG101 (1:1000; Abcam, Cambridge, UK, Cat. #: ab30871), CD63 (1:500; Abcam, Cat. #: ab68418), syntenin (1:1000, Abcam, Cat. #: ab900682) and calnexin as negative control (1:1000, Abcam, Cat. #: ab133615). To note, Hs578T mammalian cell lysate (CL) and HM EVs were used as control. Detection and imaging were performed as described in Chapter 3, Section 3.3.6.

4.3.8 Imaging flow cytometry analysis (IFCM)

Imaging flow cytometry analysis (IFCM) of intact/non-lysed EVs separated from IMF UHT ingredients were performed as previously described in Chapter 2, Section 2.3.12, with slight modifications. In this study we used CellMask™ Deep Red Plasma Membrane Stain (Invitrogen, Cat. #:C10046) to further estimate EVs concentration by uniform labelling of the plasma membrane. CellMask was used at 4X concentration, thus 10 µL were added to 10 µL of EVs followed by staining at 37°C in a thermomixer for 30 min. Additionally, anti-human CD81-PE-Cy7 (Cat. #: 349512 Biolegend, San Diego, CA, US), anti-human CD63 Brilliant Violet 421™ (Cat. #: 355030 Biolegend, San Diego, CA, US) and CD9-PE anti-human (Cat. #: 312105 Biolegend, San Diego, CA, US) were probed as EVs surface marker. Specifically, EVs were stained with the antibodies (1:10 dilution with 0.22 µm filtered PBS) for 1hr at RT in the dark. Any unbound EVs and/or antibodies were washed using a 300 kDa filter (Nanosep, Cat. #:516-8531) using 0.22 µm filtered PBS, to achieve a final volume of 100 µL. Subsequently, stained EVs were acquired within 2h for 5 minutes on the ImageStream X Mk II imaging flow cytometer (Amnis/Luminex, Seattle, USA) at 60x magnification, high gain mode, low flow rate and EVs- free filtered PBS,

unstained EVs, single-stained controls and fluorescence minus one (FMO) control were run in parallel.

4.3.9 Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis following procedure performed in Chapter 3, Section 3.3.7.

4.4 Data analysis

Statistical analysis of the presented data in this study were performed using GraphPad Prism 9 software. Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments (n=3). Thus, unpaired two-tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Comparison within samples and with P value < 0.05 were considered statistically significant.

4.5 Results

4.5.1 Protein characterisation of EVs pellet in IMF UHT ingredients

Total protein in EVs isolates was quantified using MicroBCA™, illustrated in Fig. 4.3. We observed that the SM EVs sample had significantly ($p < 0.01$) higher protein concentration compared to pre-UHT, post UHT and spray dry ($p < 0.05$) respectively (Fig. 4.3a). Similarly, SM+WPI was as well significantly ($p < 0.01$) higher in protein compared to ones quantified in pre/post-UHT and spray dried formula. In Fig. 4.3b, we analysed EVs samples using SDS-PAGE for casein removal. In all

EVs sample from UHT ingredients caseins were almost absent compared to the stronger bands observed in the representative crude milk, by means that the acid treatment successfully removed casein micelles (Fig. 4.3b). Interestingly in SM, WPI and SM+WPI EVs samples several bands were still observed, particularly in WPI and SM+WPI samples where the whey proteins were clearly detected. Instead, fainter bands were visualised in pre/post-UHT and even less in the final product, confirming that heat treatment of IMF manufacturing affect sensibly the protein content and EVs in the last stages, as reported here.

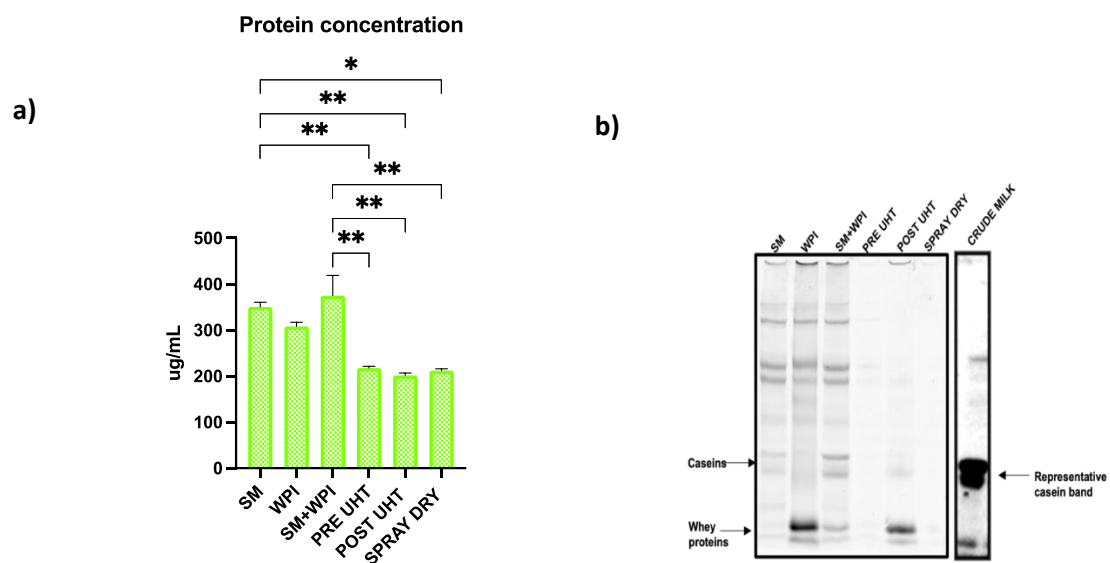


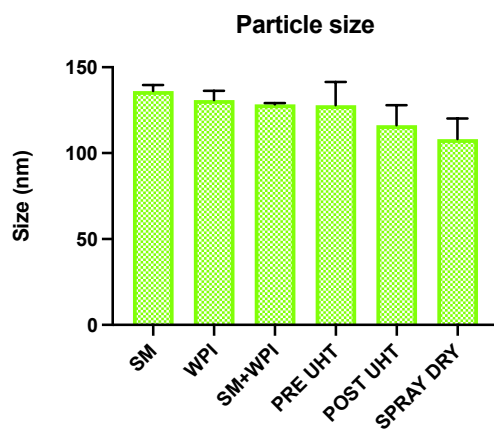
Figure 4.3: Protein characterisation of EVs pellet in IMF UHT ingredients

a) Total protein concentration in IMF UHT EVs ingredients estimated by MicroBCA™ assay and reported in $\mu\text{g/mL}$ of milk used. Each bar represents 3 biological repeats, statistical analysis using one-way ANOVA test was performed, error bars represent standard error of mean ($\pm$ SEM), * indicates $p < 0.05$; ** indicates $p < 0.01$. b) Proteins in the EVs pellet of IMF UHT EVs were visualised by loading 10 μg of protein on 10% Mini-PROTEAN® TGX™ Precast Gel followed by Coomassie Blue staining.

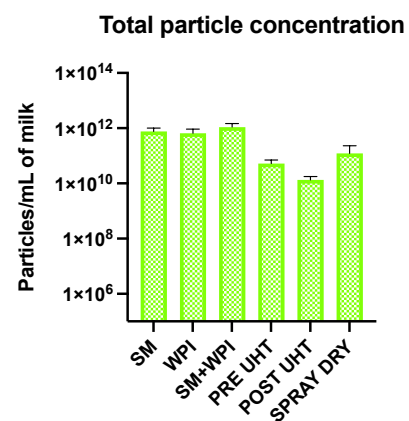
4.5.2 Particle concentration and size of EVs in IMF UHT ingredients using NTA

Particle size and concentration of EVs isolates in IMF UHT ingredients was analysed by NTA (Fig. 4.4). Regarding particle size, no significant differences were observed in all the samples as they were generally homogenous (Fig. 4.4a). Likewise, for the total particle concentration (Fig. 4.4b), particle between 30-150nm (Fig. 4.4c) and particle >150nm (Fig. 4.4d), we did not observe any significant differences in all the comparison. However, we observed that number of particles between each stages differs, therefore a table was reported with associated data (Table 4.2). Although statistical significance was not achieved, particle concentration was slightly increased in the first EVs preparation, SM, WPI, SM+WPI, respectively, compared to the last stage of IMF production.

a)

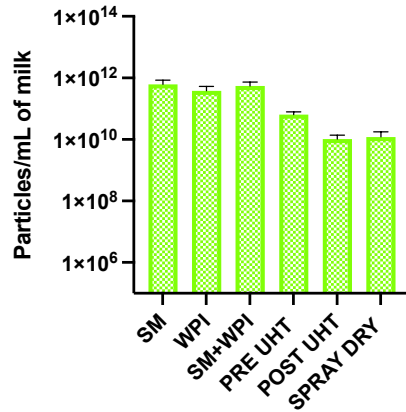


b)



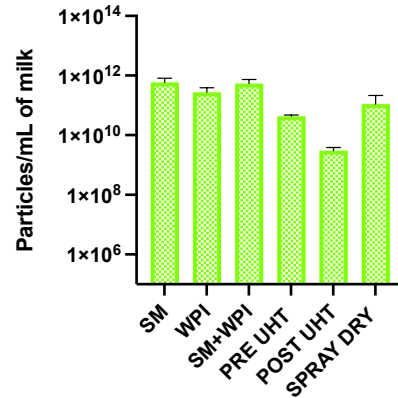
c)

Particle concentration (30-150nm)



d)

Particle concentration (> 150nm)

**Figure 4.4: Particle concentration and size of EVs in IMF UHT ingredients**

NTA analysis of particle sizes and concentration performed a minimum of four x 60-second videos recorded for each sample. a) Comparison of particle sizes of IMF UHT samples. b) Comparison of total particle concentration of IMF UHT samples. c) Comparison of particle concentration between 30-150nm IMF UHT samples. d) Comparison of particle size > 150nm of IMF UHT samples. Three replicates of each sample were analysed by NTA independently, data analysed by one-way ANOVA test. No significant differences were observed.

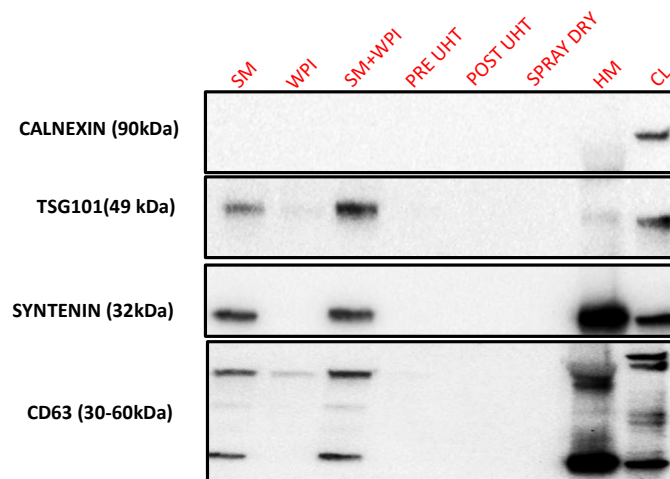
Samples	Particle size	Total particle concentration	Particle concentration (30-150nm)	Particle concentration > 150nm
SM	136.2 ± 3.5	$7.6 \times 10^{11} \pm 2.4 \times 10^{11}$	$6.1 \times 10^{11} \pm 2.2 \times 10^{11}$	$5.9 \times 10^{11} \pm 2.3 \times 10^{11}$
WPI	130.9 ± 5.8	$6.5 \times 10^{11} \pm 2.6 \times 10^{11}$	$3.8 \times 10^{11} \pm 1.4 \times 10^{11}$	$2.7 \times 10^{11} \pm 1.1 \times 10^{11}$
SM+WPI	128.4 ± 0.7	$1 \times 10^{12} \pm 3.8 \times 10^{11}$	$5.4 \times 10^{11} \pm 9 \times 10^{11}$	$5.4 \times 10^{10} \pm 1.9 \times 10^{11}$
PRE UHT	127.9 ± 13.5	$7.1 \times 10^{10} \pm 2.6 \times 10^{10}$	$6.4 \times 10^{10} \pm 1.5 \times 10^{10}$	$4.3 \times 10^{10} \pm 4.1 \times 10^9$
POST UHT	125.6 ± 12.1	$5.8 \times 10^{10} \pm 4.53 \times 10^{10}$	$1 \times 10^{10} \pm 3.6 \times 10^9$	$3 \times 10^9 \pm 8.1 \times 10^8$
SPRAY DRY	117.6 ± 12.7	$2.1 \times 10^{11} \pm 1.1 \times 10^{11}$	$1.2 \times 10^{10} \pm 5.5 \times 10^9$	$1.09 \times 10^{11} \pm 1 \times 10^{11}$

Table 4.2: Summary of particle sizes and concentration in IMF UHT ingredients

4.5.3 Analysis of EVs marker in IMF UHT ingredients by western blot

We investigate the presence of EVs marker in all IMF UHT isolates immunoblotting analysis. As recommended by MISEV2018 guidelines (Théry et al., 2018), three positive EVs marker, TSG101, syntenin and one tetraspanin CD63, were used. However, to demonstrate the high quality of our EVs preparation, we used also calnexin which is a non-EVs associated protein. Although the NTA data did not highlight any significant differences between the EVs samples, here we observed instead that the three positive markers were clearly detected in SM and the mix SM+WPI, while very fainter bands were associated to WPI (Fig. 4.5a). Interestingly, lower detection of these marker was obtained in the last stages, pre/post UHT and spray dried formula. Moreover, in all the IMF UHT EVs samples, calnexin was absent, as showed in Fig. 4.5a. In fig. 4.5b, we reported the densitometric analysis of the identified bands, which reflected higher intensities of the bands for SM and SM+WPI which consistently decreased in the rest of the samples.

a)



b)

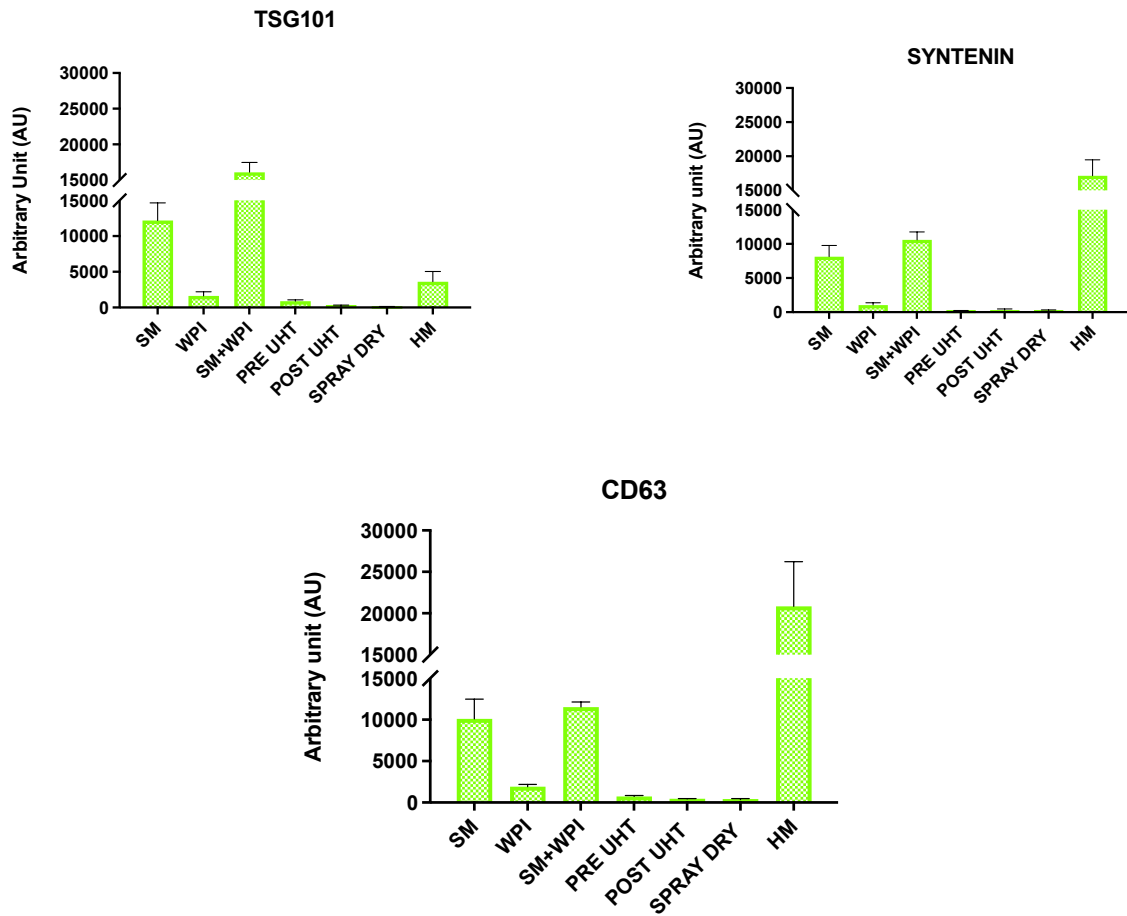


Figure 4.5: EVs marker analysis in IMF UHT ingredients

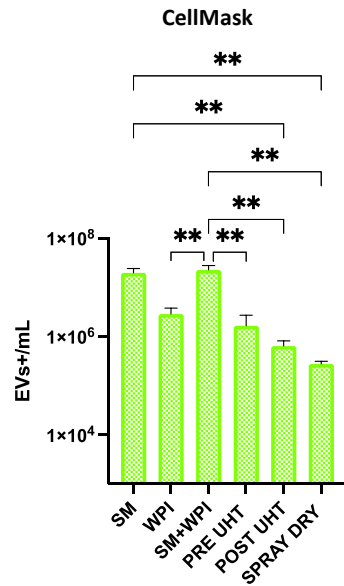
a) Immunoblotting analysis of EVs marker TSG101, syntenin, CD63 and one negative marker calnexin from IMF UHT EVs. b) Densitometric analysis of each marker analysed. Bars represents the mean of the densities of the signals obtained after immunoblotting ($n = 3 \pm \text{SEM}$).

4.5.4 Detection of EVs marker using imaging flow cytometric analysis (IFCM) of IMF UHT EVs ingredients

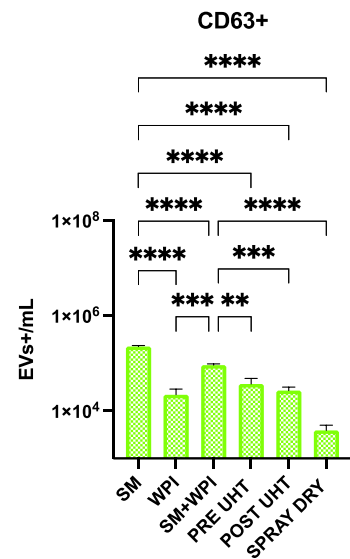
Additionally, IFCM was carried out for all the samples to further characterised the EVs preparation using CellMask, CD63 and CD81. In the NTA analysis we did not detect any significant differences within the samples which was not the case for IFCM. In fact, for CellMask: we observed that SM had significantly ($p < 0.01$) higher number of positive particles compared to POST-UHT and SPRAY DRY samples; while SM+WPI had significantly ($p < 0.01$) higher number of

particles compared to WPI, PRE-UHT, POST-UHT and SPRAY DRY formula, as showed in Fig. 4.6a. CD63+: SM had significantly ($p < 0.0001$) higher number of positive particles compared to the other EVs preparation, similarly SM+WPI had significantly higher number of positive events compared to WPI ($p < 0.001$), PRE-UHT ($p < 0.01$), POST-UHT ($p < 0.001$) and SPRAY DRY formula ($p < 0.0001$), represented in Fig. 4.6b. As expected, same observation was carried out for CD81+: SM had the highest number of positive particles ($p < 0.001$) compared to WPI, PRE-UHT, POST-UHT, SPRAY DRY and ($p < 0.01$) compared to SM+WPI (Fig. 4.6c). Therefore, the trend captured by immunoblotting and IFCM matched, which confirmed an important loss of EVs in the last stages of IMF manufacturing.

a)



b)



c)

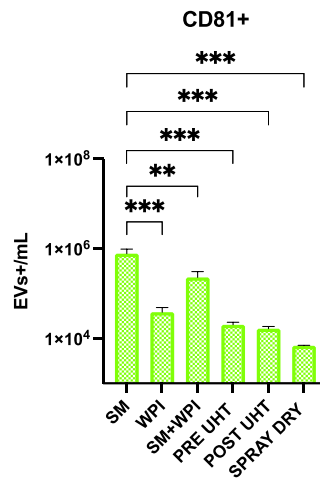


Figure 4.6: IFCM analysis of EVs surface marker in IMF UHT ingredients

Number of positive particles in EVs from UHT samples. a) Cell Mask; b) CD63+; c) CD81+. Events were gated for low side scatter channel (SSC) signals and no signals on brightfield. Data were acquired as objects (+EVs) in low SSC. The IDEAS software was used to analyse the data as mean $\pm$ SEM. Statistical analysis was done by One-way ANOVA tests. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

4.5.5 Analysis of EVs morphology in IMF UHT ingredients using transmission electron microscopy (TEM)

Finally, the structure of the separated EVs from IMF UHT ingredients was observed by TEM (Fig. 4.7). Interestingly, before the ultra-high temperature treatment, in SM and SM+WPI the particles were intact with rounded shape and likely associate to EVs structure, whereas for the WPI EVs preparation, the particles were atypical and slightly distorted. However, while in PRE-UHT some EVs like particles were still detected, we observed a drastic change in the last two stages, in POST-UHT samples the EVs/particles morphology was lost and their membrane were damaged. Most important for our study, we could not observe any EVs structure or particles in the spray dried formula (empty grid) in agreement with immunoblotting and IFCM data were the loss of EVs was mainly detected in PRE-UHT, POST-UHT and spray dried EVs preparation.

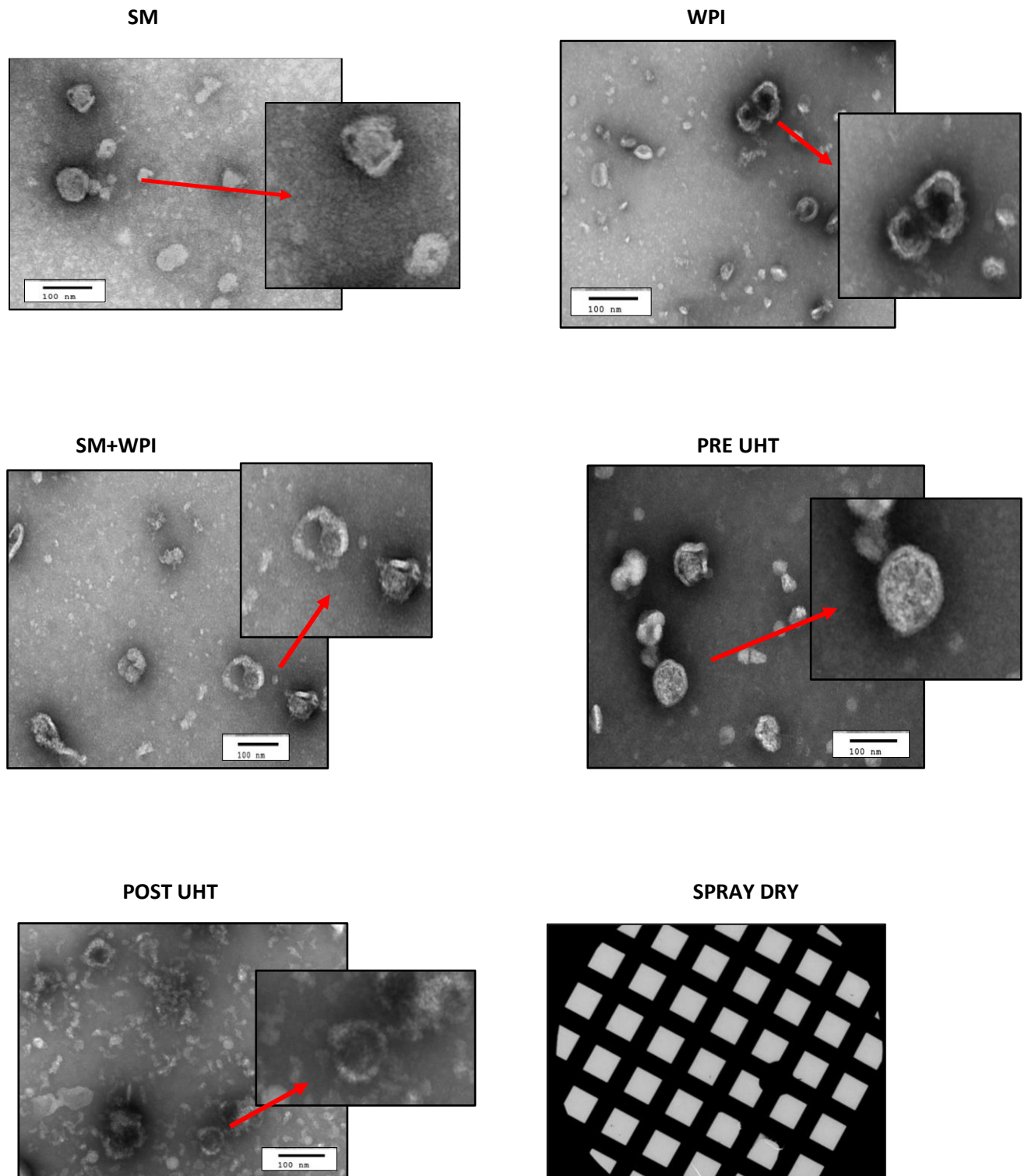


Figure 4.7: Transmission electron microscopy of EVs in IMF UHT ingredients
Representative transmission electron microscopy analysis of IMF UHT EVs sample. Scale bar=100nm

4.6 Discussion

UHT treatment of milk produces sterile milk with long-shelf life which helps to guarantee the safety of the product for the infants. Besides, treatment at higher temperature as for UHT causes little protein denaturation, but ensures sterility of the product throughout the IMF manufacturing (Lönnerdal & Hernell, 1998). However, this process even for shorter period, can have negative effect on EVs yield and morphology. We analysed different stages throughout IMF UHT manufacturing to investigate which step might have higher impact on the EVs.

In the 6 samples collected from the pilot plant (SM, WPI, SM+WPI, PRE-UHT, POST-UHT and spray dried formula), we demonstrated that caseins were absent in all of them, compared to non-treated milk. However, we observed significant higher amount of protein from the isolates in the first stages (SM and SM+WPI) compared to pre/post UHT and the spray dried formula, also very fainter bands were observed in the SDS-page for the same samples. Hence, these results might be correlated to protein denaturation in the EVs isolates from the latest stages of the IMF manufacturing where the higher temperature is applied constantly. Similar results were observed by Kleinjan (2021) where they separated EVs from raw, pasteurised, homogenised and UHT milk, using density gradient without acid pre-treatment. There they performed EVs protein analysis using SDS-page. In all samples caseins were still detected with abundance in the UHT EVs sample. Conversely, in our results we demonstrated that acid pre-treatment efficiently removed most if not all the caseins. Although EVs concentration was higher in SM, WPI and SM+WPI compared to the UHT EVs samples, we did not observe any significant differences either for particles concentration and sizes by NTA. However, the trend obtained by NTA might be associated to the product storage, because aggregates of proteins and milk fat could settle down at the bottom (Akkerman et al., 2021). Besides NTA is limited and it cannot discriminate within particles, either EVs or protein aggregates. Therefore, we performed immunoblotting using EVs

markers, (TSG101, syntenin and CD63) where only in SM EVs and the SM+WPI EVs the selected proteins were readily detected, whereas lower detection was observed in the rest of the samples. Furthermore, all the EVs sample were negative for calnexin. To further support our hypothesis, we quantified the number of positive particles in all the samples using CellMask, CD63+, CD9+ and CD81+ by IFCM. We reported a different trend compared to NTA but in agreement with immunoblotting data. SM EVs samples had highest number of positive events for all the marker, compared to pre/post UHT and spray dry formula, enforcing the significant loss of EVs throughout the IMF preparation. EVs morphology analysed by TEM showed severe changes throughout the IMF manufacturing. In fact, as expected, SM, WPI and SM+WPI EVs preparation presented particles resembling EVs morphology, with intact edges and typical rounded shape. While PRE-UHT and POST UHT EVs electron micrographs showed disrupted surfaces, greater number of debris and particles less resembling EVs structure. Interestingly, in the spray dried formula we could not detect any EVs/particles except of an empty grid. Thus, even though fewer particles were detected by NTA and IFCM in the spray dry EVs samples, it is arguably they are associated with EVs as further supported by the lower amounts of EVs markers in the immunoblotting. Indeed it is more plausible to be a potential formation of sediment in UHT processed milk (Akkerman et al., 2021). Although Kleinjan et al (2021) and Zhu et al (2022) used different methodology for EVs separation, they also showed that high-temperature heating greatly affect the morphology and destroy the integrity of EVs. Furthermore, the UHT samples hardly contained any spherical membrane-enclosed vesicular structures. Although, we did not observe any significant decrease while analysing the particle concentration in all samples, we demonstrated that after ultra-high treatment EVs were critically lost and damaged compared to the starting material SM.

4.7 Conclusion

Ultra-high treatment guarantees a safe and palatable product with a long shelf life, but we demonstrated that EVs yield is substantially decreasing towards the last stages of UHT manufacturing. Furthermore, EVs in the starting materials are intact and of high quality in respective of the ones collected after high temperature treatment. Therefore, publication of the results, attained from this section of the study, should be useful for future improvements to IMF manufacturing.

5 Chapter 5: Comparison of EVs quantity and quality from IMF and the “gold standard” HM

5.1 Abstract

Human milk, source of nutrition for infants, contains EVs. Breast-feeding is highly recommended, but IMF is used as an alternative to HM. We hypothesised that IMF manufacturing may have detrimental consequences on EVs thus we aimed to separate EVs from IMF and HM and compare their EVs' quantity and quality. EVs/particle concentration in IMF was significantly lower compared to HM suggesting loss of EVs in IMF. Therefore, IMF has significantly fewer EVs compared to HM. EVs marker were detected at lower amounts from IMF isolates in comparison with HM isolates. Altogether, EVs in IMF are substantially compromised compared to that in the "gold standard" HM.

5.2 Introduction

Human milk is sufficient to provide fundamental nutrients for infant growth and development, its composition is unique and extremely enriched in immunological factors crucial for the newborn. HM is the finest form of nourishment for babies. However, breastfeeding is not always possible or sometimes contraindicated for certain medical conditions, therefore its best alternative is IMF. The formulation of IMF is various, in fact there are three forms: ready to feed, concentrated liquid and powder. Even though IMF is widely used in all its formulation, it has been reported that formula-fed children are known to have a higher possibility to encounter in several diseases: necrotising enterocolitis (NEC), gastroenteritis, otitis media, respiratory diseases, as well as obesity, inflammatory bowel disease, and diabetes compared to breast-fed infants. Hence, breastfeeding is strikingly recommended at least for the first six months of life (Kim et al., 2020; van Sadelhoff et al., 2020). Consequently, there have been many attempts to produce IMF that resembles HM as closely as possible as well as compelling advancements in the dairy industry, for instances supplementation with probiotics and prebiotics, but replicating the functionality of HM has not been completely rewarding (Miqdady et al., 2020). Investigating IMF composition in relation to infant nutrition is highly demanding to provide further improvements. In chapter 4, we proved that EVs during IMF procedure were consistently compromised, the yield was decreasing significantly and the EVs morphology was disrupted towards the final preparation. The importance of studying EVs from HM and IMF is associated to their potential activity in intestinal development which is crucial for the infants. Indeed by mimicking pathological condition in vitro, it has been reported that HM EVs reduced cell inflammation injury, while decreasing apoptosis and promoting cell proliferation, as well as down-regulation of p53 and supporting tight junction formation essential for cell survival (Pisano et al., 2020; Yu et al., 2017; Xie et al., 2020). Likewise, in case of mucosal injury, treatment with

HM EVs stimulated a much higher expression of mucin-2 by goblet cells nourished by human oligosaccharides (HMOs) which are known to be absent in IMF but naturally present in HM (Wu et al., 2019). However, as knowledges on IMF EVs are scarce, we wondered how much alike are EVs from IMF and HM. Therefore, high throughput analysis of EVs from HM and IMF using comprehensive proteome characterisation by LC-MS/MS, high-resolution ¹H-NMR spectroscopy, and RT-qPCR to investigate miRNAs will be described in the following chapters.

5.3 Materials and methods

5.3.1 Human breast milk samples collection

Milk samples from 20 mothers who gave informed consent were included in this study. The criteria were: healthy women, non-smokers, and no alcohol consumption. Mothers with major morbidities were excluded. Breast milk was collected using an electric pump, and the milk was immediately centrifuged at 2000 x *g* to remove fat and cell debris and stored in -80°C until further use.

5.3.2 Infant milk formula samples reconstitution

Six IMF powder and six ready to drink formulas from three brands were purchased locally for this study. Powder formulas were reconstituted following the manufacturer instruction, *i.e.*, 4.6 g was dissolved in 30 mL of warm distilled water. Liquid formulas were used within 2 days of purchase and before their expiration date. Subsequently, milk samples were processed for defatting and milk pre-treatment.

5.3.3 Defatting and acid pre-treatment for casein removal

20 mL of HM and IMF samples was defatted, and acid-treatment was performed as described in Chapter 2, Section 2.3.2.

5.3.4 Separation of EVs using density gradient ultracentrifugation

EVs were separated from HM and IMF using similar procedure performed in Chapter 2, Section 2.3.3 with slight modifications. 2.33 mL of whey (HM and both IMF) was used for density gradient ultracentrifugation following the bottom-up technique using Optiprep™ iodixanol solution (60%) (Fig. 5.1). Ultracentrifugation was performed using an SW Type 32.1 Ti rotor in Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 186,000 x g for 18h at 4°C. Pooled fractions were washed twice in sterile PBS with centrifugation at 120,000 x g for 2h at 4°C. Afterwards, enriched milk EVs pellets were resuspended in 200 µL of 0.22 µm filtered PBS and stored at -80°C or immediately processed for further analysis.

5.3.5 Protein quantification using Micro BCA™ Protein assay kit

Total protein in EVs isolates from HM and IMF samples was estimated as described in Chapter 3, Section 3.3.4.

5.3.6 SDS-PAGE for protein visualisation

SDS-PAGE was performed using 10 µg of EVs isolates applying the same procedure performed in Chapter 3, Section 3.3.3.

5.3.7 Nanoparticle tracking analysis

Particle concentration and sizes in HM and IMF samples were analysed by NTA as explained in Chapter 3, Section 3.3.5.

5.3.8 Immunoblotting analysis

Immunoblotting was performed as described in Chapter 3, Section 3.3.6, with some modifications. In brief EVs lysates (10 µg) were resolved on 10% Mini- PROTEAN^R TGXTM Precast Gel (Bio-Rad Laboratories, Cat. #4561034) and proteins were transferred to PDVF membranes (Bio Rad, Cat. #: 17001917) using Trans-Blot[®] TurboTM Transfer System (Bio Rad, Cat. #: 1704150) at 25V for 7 min. Blots were blocked with 5% (w/v) BSA in PBS containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies against TSG101 (1:1000; Abcam, Cambridge, UK, Cat. #: ab30871), CD9 (1:1000; Abcam, Cat. #: ab92726), CD63 (1:500; Abcam, Cat. #: ab68418), syntenin (1:1000, Abcam, Cat. #: ab900682) and calnexin as negative control (1:1000, Abcam, Cat. #: ab133615). Secondary antibodies used were anti-mouse (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7076) or anti-rabbit (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7-74). SuperSignal West Femto Chemiluminescent Substrate Kit (Fisher Scientific, Cat. # 11859290) was used for detection, imaging was performed using an automated Chemidoc exposure system (Bio-Rad Laboratories) and densitometric analysis was performed using ImageJ. Hs578T mammalian cell lysate (CL) was used as control.

5.3.9 Transmission electron microscopy

10 µL of EVs samples was prepared for transmission electron microscopy (TEM) following the procedure in Chapter 3, Section 3.3.7.

5.3.10 Imaging flow cytometry analysis of Cell Mask™ and EVs surface marker

Detection of EVs marker CellMask™ Deep Red Plasma Membrane Stain 4X (Invitrogen, Cat. #:C10046), anti-CD81-PE-Cy7 (Cat. #: 349512 Biolegend, San Diego, CA, US), anti-CD63 Brilliant Violet 421™ (Cat. #: 355030 Biolegend, San Diego, CA, US) and anti-CD9-PE (Cat. #: 312105 Biolegend, San Diego, CA, US) was performed by imaging flow cytometry (IFCM) analysis as in Chapter 4, Section 4.3.8, with slight modifications. Specifically, 10 µL of non lysed EVs from HM and IMF samples were used; CellMask (4X) was added to all samples and staining allowed at 37°C for 30 min. Subsequently, milk EVs samples were incubated with the antibodies for 1hr at RT in the dark. 300 kDa filter (Nanosep, Cat. #:516-8531) were used for washing of unbounded EVs and antibodies with 0.22 µm-filtered PBS, to achieve final volume of 100 µL. Within 2hr preparation stained EVs were acquired for 5 minutes on the ImageStream X Mk II imaging flow cytometer (Amnis/Luminex, Seattle, USA) at 60x magnification, high gain mode, low flow rate. EVs-free filtered PBS, unstained EVs, single-stained controls and fluorescence minus one (FMO) control were run in parallel. EVs were gated as SSC-low vs fluorescence, then as non-detectable brightfield (fluorescence vs Raw Max Pixel Brightfield channel), gated EVs were confirmed in the IDEAS Image Gallery. Data analysis was performed using IDEAS software v6.2 (Amnis/Luminex, Seattle, USA).

A summary of the methodology used in this study is illustrated in Fig. 5.1

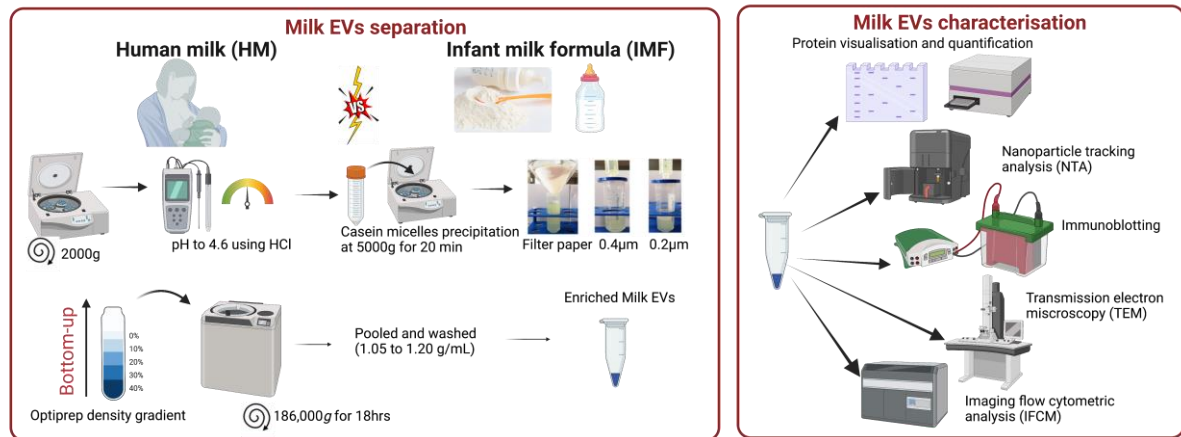


Figure 5.1: Methodology used for EVs separation and characterisation

5.3.11 Data analysis

Descriptive statistics of all reported data was performed using Graph Pad Prism 9 software.

Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments ($n=3$). Unpaired two-tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $P < 0.05$ were considered statistically significant.

5.4 Results

5.4.1 Quantification of protein concentration in HM EVs and IMF powder and liquid EVs and visualisation of separated milk proteins

EVs isolates protein was quantified using Micro™ BCA assay. Protein concentration in IMF EVs powder ($244.3 \pm 6.8 \mu\text{g/mL}$) and liquid ($261.2 \pm 12.1 \mu\text{g/mL}$) formulae, was significantly lower

($p < 0.0001$) compared to that of HM EVs ($1078.0 \pm 22.8 \mu\text{g/mL}$), as reported in Fig. 5.2a. Separated milk proteins were visualised by SDS-PAGE electrophoresis. Most of the caseins and whey proteins had been removed either in HM EVs and IMF EVs compared to non-treated samples (Fig. 5.2b).

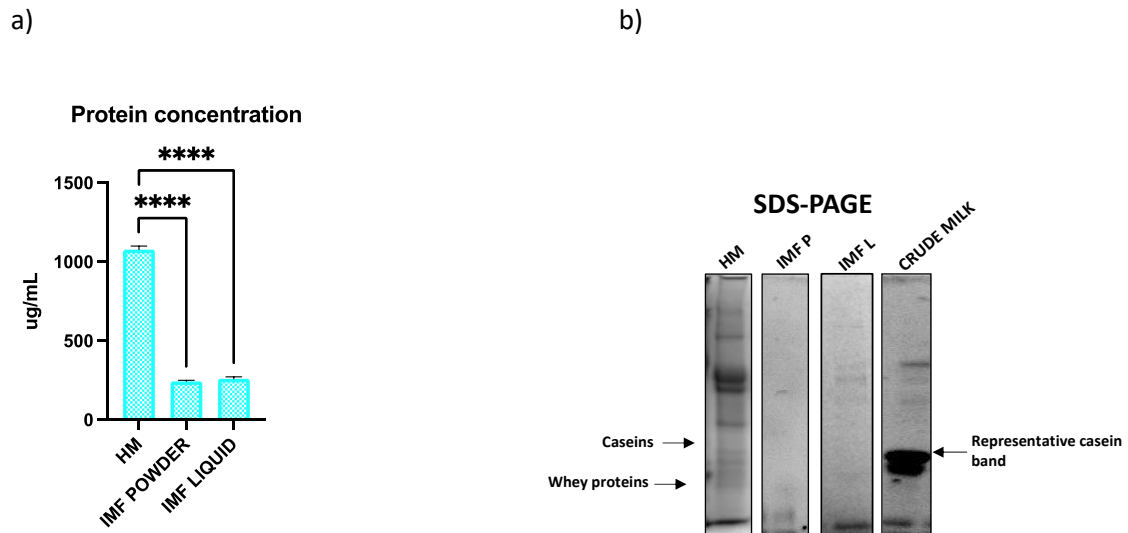


Figure 5.2: Protein concentration in EVs pellet in HM and IMF

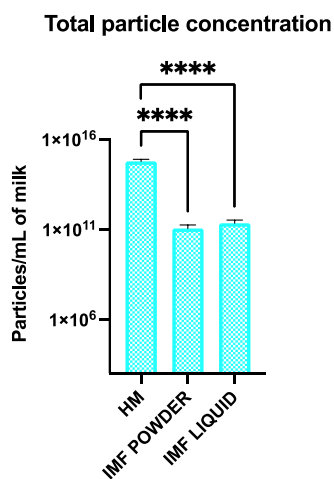
a) Total protein quantity in IMF EVs and HM EVs powder and liquid was estimated by MicroBCA assay. Each bar represents 3 biological repeats, statistical analysis was performed using one-way ANOVA test, error bars represent standard error of mean ($\pm$ SEM), **** indicates $p < 0.0001$. b) Protein in the EVs enriched fraction of HM and IMF were visualised by loading $10 \mu\text{g}$ of protein on 10% Mini-PROTEAN® TGX™ Precast Gel followed by Coomassie Blue staining. IMF P= IMF powder; IMF L= IMF liquid

5.4.2 Particle concentration and size analysis of HM EVs and IMF EVs using NTA

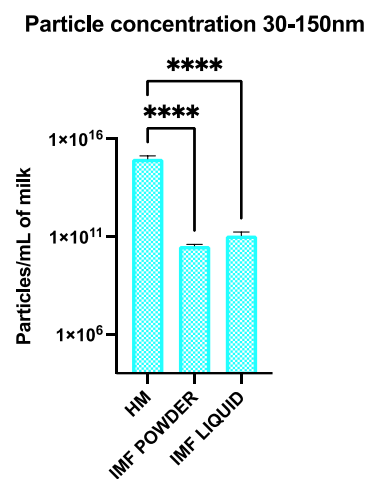
NTA analysis was performed for all the samples to quantify and estimate EVs concentration and size, (Fig. 5.3). Particle concentration in IMF EVs powder and liquid formula was $1.1 \times 10^{11} \pm 6.4 \times 10^{10}$ and $2.1 \times 10^{11} \pm 6.4 \times 10^{11}$ particles/mL, respectively, which was significantly lower ($p < 0.0001$) lower compared to HM EVs samples of $5.9 \times 10^{14} \pm 1.8 \times 10^{14}$ particles/mL (Fig. 5.3a). Considering small versus medium/large EVs, the concentration of particles between 30-150nm

was significantly ($p < 0.0001$) lower with IMF EVs powder ($3.4 \times 10^{10} \pm 6.2 \times 10^9$) and IMF EVs liquid ($1.1 \times 10^{11} \pm 5.6 \times 10^{10}$) compared to HM EVs samples, which was $9.6 \times 10^{14} \pm 3.9 \times 10^{14}$ (Fig. 5.3b). Likewise, the concentration of particles over 150nm was significantly ($p < 0.0001$) lower in IMF powder ($7.1 \times 10^{10} \pm 6.1 \times 10^{10}$) and IMF EVs liquid ($9.9 \times 10^{10} \pm 5.4 \times 10^{10}$) compared to HM EVs samples ($4.1 \times 10^{14} \pm 6.6 \times 10^{13}$) (Fig. 5.3c). Furthermore, particle sizes from IMF powder and liquid were 96.4 ± 3.3 and 106.2 ± 4.7 nm, respectively (Fig. 5.3d) which were significantly ($p < 0.0001$) smaller compared to HM EVs samples of 201.1 ± 14.0 nm.

a)

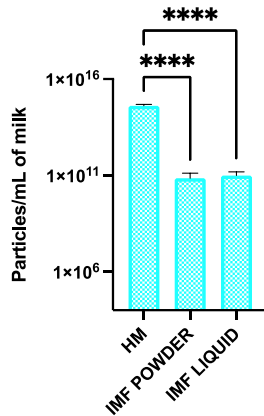


b)



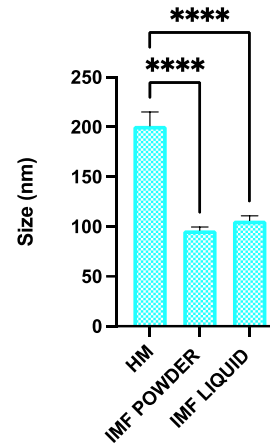
c)

Particle concentration > 150nm



d)

Particle size

**Figure 5.3: Particles sizes and concentration of HM EVs and IMF EVs**

a) Total particle concentration comparison; b) Comparison of particle concentration within 30-150 nm.; c) Comparison of particle concentration over 150 nm; d) Comparison of particle size. Three replicates of each sample were analysed by NTA independently, data analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; **** indicates $p < 0.0001$.

5.4.3 Detection of EVs marker in HM EVs and IMF EVs using Imaging Flow Cytometric analysis

EVs from HM and IMF samples were stained for EVs surface markers and analysed by flow cytometry, Fig. 5.4. Specifically, using cell membrane stain CellMask, both IMF powder and liquid isolates had significantly ($p < 0.001$) fewer positive particles compared to HM samples (Fig. 5.4a), which is in line with the NTA data. Then, considering specific EVs-associated markers CD81, CD63 and CD9, IFCM showed that both powder and liquid formulae IMF had significantly ($p < 0.0001$) fewer CD81+ particles (Fig. 5.4b), ($p < 0.01$) CD63+ particles (Fig. 5.4c) and ($p < 0.05$) CD9+ particles, compared to HM EVs, (Fig. 5.4d).

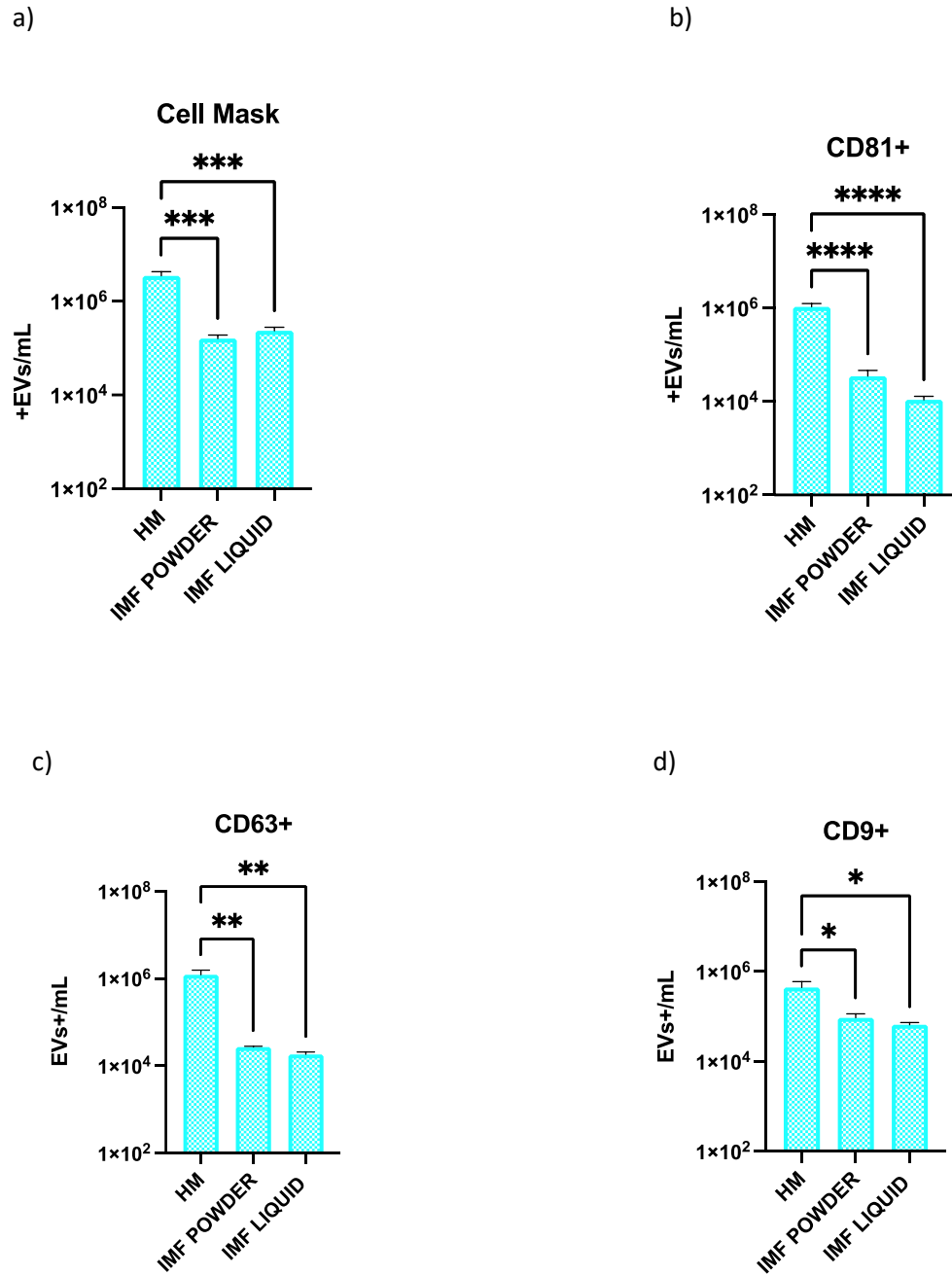


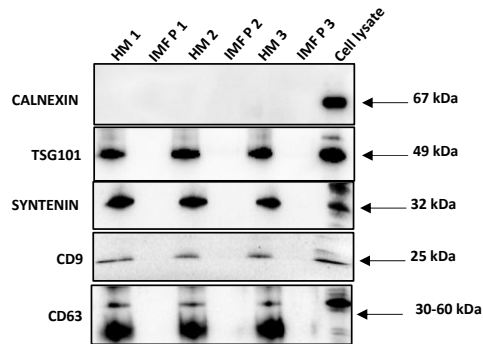
Figure 5.4: Imaging flow cytometry analysis of EVs surface marker

Number of positive events (particles) in HM and IMF powder and liquid formula analysed by imaging flow cytometry (IFCM). Events were gated for low side scatter channel (SSC) signals and no signals on brightfield, based on EVs size and these were as objects (+EVs) in low SSC. Data presented as mean and individual values $\pm$ SEM. The number of positive a) Cell Mask; b) CD81; c) CD63 and d) CD9 in HM and IMF powder and liquid formula. The IDEAS software was used to analyse the data. One-way ANOVA tests were performed, and significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

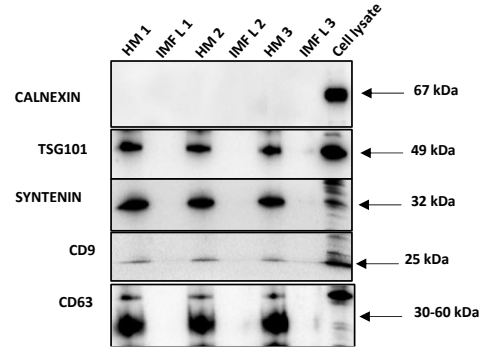
5.4.4 Analysis of EVs marker in HM EVs and IMF EVs powder and liquid formula using immunoblotting

In accordance with MISEV2018 guidelines (Théry et al., 2018) and applying an orthogonal approach, immunoblotting was also used to validate the presence of EVs (Fig. 5.5). Specifically, four EVs-associated proteins including two cytosolic TSG101 and syntenin, two tetraspanins CD63 and CD9; and an endoplasmic reticulum, non-EVs associated marker, calnexin, were analysed. TSG101, syntenin, CD63 and CD9, detected in HM EVs isolates and were very low in IMF powder (IMF P1-3; Fig. 5.5a) and liquid formula (IMF L1-3; Fig. 5.5b) isolates, in agreement with the flow cytometry observations. Of note, all EVs samples were negative for calnexin. The densitometric analysis, in Fig. 5.5c, showed same trend where IMF EVs band intensities in powder and liquid formulae were significantly ($p < 0.0001$) lower compared to HM EVs samples (Fig. 5.5c).

a)



b)



c)

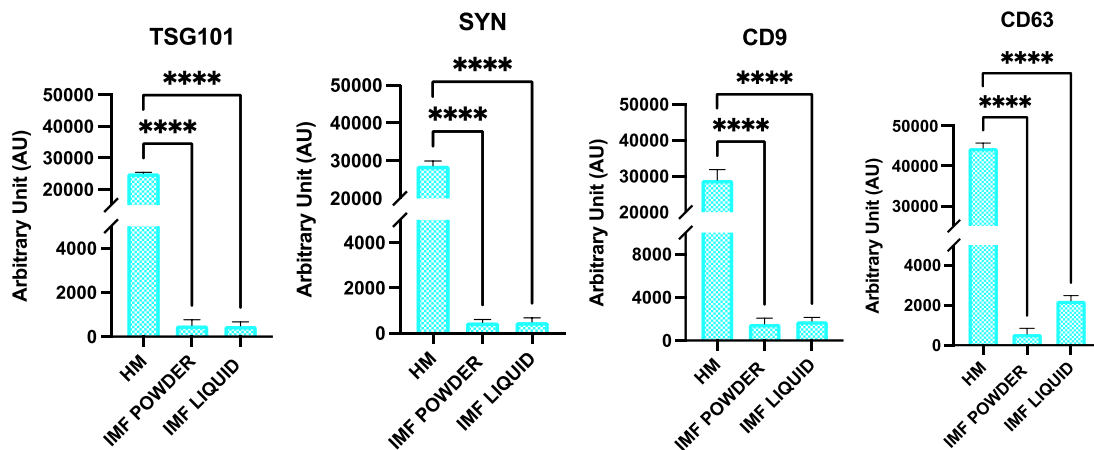


Figure 5.5: EVs marker analysed by immunoblotting

Analysis of EVs-associated markers TSG101, syntenin, CD9, CD63 and calnexin. a) Immunoblotting analysis of a) HM EVs vs IMF EVs powder; b) HM EVs vs IMF EVs liquid performed by loading 10 µg of protein for EVs and CL (cell lysate). c) Densitometric analysis of each marker analysed. Bars represents the mean of the densities of the signals (n = 3 ± SEM). One-way ANOVA tests were performed, and significance is indicated as **** p < 0.0001.

5.4.5 EVs structure in HM and IMF powder and liquid formula analysed by transmission electron microscopy

To further investigate the presence of EVs in all the samples, we performed transmission electron microscopy to analyse the EVs morphology and their integrity, as shown in Fig. 5.6. HM EVs (Fig. 5.6a) appeared intact, with a smooth surface and had defined EVs like structure. Conversely, structures from IMF powder and liquid (Fig. 5.6b) & (Fig. 5.6c) were broken or at least EVs were distorted in both sample types. Overall, IMF had relatively fewer structures of EVs like morphology with substantial debris visible in the background.

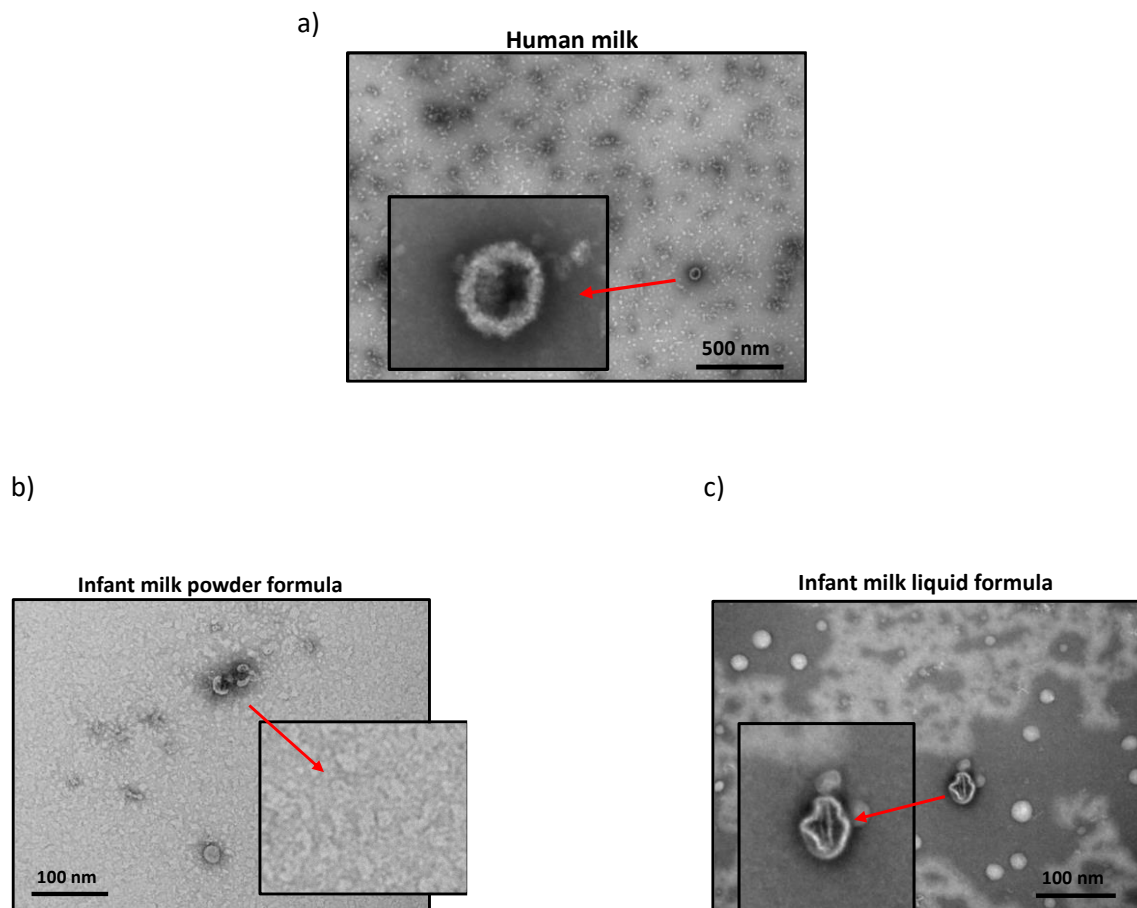


Figure 5.6: Transmission electron microscopy analysis of EVs morphology

Representative transmission electron microscopy analysis of a) HM EVs, b) IMF EVs POWDER, c) IMF EVs LIQUID. Particle morphology is marked by the red arrows. Scale bar = 100-500nm

5.5 Discussion

It is established that breast-fed infants harbour better immune defences against several types of infections and exhibit lower frequencies of inflammatory diseases than formula-fed infants (Quigley et al., 2019). Human breast milk is the optimal source of nutrition for the infant and provides a multitude of bioactive compounds, including EVs. In this chapter, we separated and characterised EVs from HM and IMF, which are the two main sources of nutrient for the newborn. We reported high quality for our EVs preparation, following MISEV2018 guidelines (Théry et al., 2018). Regardless numerous studies on milk EVs till now, to date there is only one study which have studied HM EVs and IMF EVs in form of comparison study, where EVs characterisation was partially covered compared to ours and only few donors were picked (Leiferman et al., 2019). Here, we separated EVs from multiple donors of HM and two type of feeding formula, powder and liquid. Milk samples were enriched in EVs while most of the potential contaminants were almost completely removed. However, this methodology was not employed by Leiferman et al (2019), in fact they used differential ultracentrifugation, with higher chance to carry some contaminants while pelleting EVs, whereas combining acid pre-treatment with gradient ultracentrifugation ensure a high standard EVs preparation. Particles concentration in HM and IMF was evaluated by NTA analysis where IMF had significantly lower amount of EVs (between 30-150nm and over 150nm) compared to HM. Moreover, due to NTA limitation to distinguish between particles of any kind and EVs, we also performed flow cytometric analysis. Samples were stained with CellMask, CD81, CD63 and CD9. IMF EVs had significantly lower number of positive particles for all the markers compared to HM EVs, remarking the lower concentration of EVs from IMF compared to HM and in agreement with NTA data. Additionally, to further validate our data, EVs markers such as TSG101, CD9, CD63 and syntenin were analysed by immunoblotting analysis and all the above were lower detected from

IMF EVs in comparison with HM EVs samples, in accordance with IFCM analysis. Finally, EVs morphology was observed by TEM which confirmed the presence of EVs structure in all the samples. However, the fewer EVs detected in IMF powder and liquid had disrupted surfaces and less defined edges with consistent number of debris. Instead, HM EVs were rounded and more resembling EVs morphology.

5.6 Conclusion

Although it is required for safety and product quality of IMF (and milk derivatives) to be processed at high temperature, bioactive components, in milk, as EVs are subjected to severe damages which lead to a significant loss of EVs compared to HM. Even though IMF, with numerous efforts, is a valuable substitute of HM, the lack of EVs (quantity and quality) remain a compelling gap between IMF and HM. Therefore, we believe it is essential to gather more attention in this matter and focus on future application of HM EVs as step forward for the advancement of IMF formulation.

6 Chapter 6: Global profiling of EVs proteome: discovering the EVs protein content in infant milk formula compared to human milk

6.1 Abstract

A comprehensive analysis of the EVs proteome in HM and IMF is warranted considering that they are both a source of nutrients for infants. Therefore, here, EVs from HM and IMF were processed for LC-MS/MS analysis to investigate, in what we understand to be the first study of its kind, the EVs proteome of HM and IMF from both liquid and powder sources. We discovered IMF EVs carry only 15 detectable proteins compared to 660 found in HM EVs. Crucially, the IMF EVs proteome registered lower amounts of proteins associated with growth, intestinal absorption and regulation and maturation of the immune system compared to HM EVs. The data here provides new knowledge on IMF and provides sound scientific criteria, for the industry, to improve IMF.

6.2 Introduction

Proteins in milk have been studied for decades, and proteomics is the main approach deployed to decipher the proteome of human milk (Dekker et al., 2022). However, milk EVs are known to be essential for transmission of nutrients, and immune capabilities, particularly from human milk (Admyre et al., 2007). Despite the fact that the composition of the human milk proteome is well established (Dayon et al., 2021), very little is known about milk EVs proteome, specifically in IMF. Milk EVs are known to be vehicles supporting intracellular communication by delivering biologically active cargoes, including proteins (Hu et al., 2021). Although, proteomic analysis has been used to explore EVs content in milk, including milk from human and bovine (van Herwijnen et al., 2016; Samuel et al., 2017; Kirchner et al., 2020; Babaker et al., 2022), a global proteomic profile of IMF EVs and a comparison with HM EVs is still missing. Therefore, we aimed to provide a comprehensive proteomic characterisation of milk EVs considering milk from multiple human donor and liquid and powder sources of IMF from multiple suppliers in order to identify any significant gaps of the proteomic content from IMF and the gold standard HM. Thus, EVs from IMF and HM were collected, fundamentally characterised their proteome using LC-MS/MS, and key proteins differentially detected then validated by immunoblots.

6.3 Material and methods

6.3.1 EVs lysed using urea in ammonium bicarbonate (ABC) and protein quantification

Lysed EVs isolates (50µL each) using lyses buffer prepared with 6M urea (Sigma, Cat. #: U5378) in 50mM ammonium bicarbonate (Honeywell, Cat. #: 1066-33-7) dissolved in mass spectrometry water (Sigma, Cat #: 90682-1L). Thus, 50 µL of ammonium bicarbonate was added to the samples, vortexed and incubate at RT for 30 min followed by sonication on ice for 20s (pulse on time 5 sec, pulse off time 5 sec). After sonication, samples were centrifuged at 16,000 x *g* for 10 minutes at 4°C. Protein in lysates was estimated by Bradford assay. 1:10 dilution of 2mg/mL BSA (Sigma-Aldrich, Ireland) was used to generate standards curve (0 – 50 µg/mL) (Table 6.1), protein quantification was finalised by adding 40 µL of Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Cat #500-0006) followed by an incubation time of 20 min. Absorbances were read at 595 nm using a FluoStar Optima microplate reader (BMG Labtech).

0.2 (µL)	mg/mL	6M UREA in 50mM ABC- Sample buffer (µL)	MS grade water	Protein concentration in 5 µL of sample
0		5	155	0
5		5	150	1
10		5	145	2
15		5	140	3
20		5	135	4
25		5	130	5

Table 6.1: Volume of reagents used for Bradford assay of Urea lysed milk EVs samples

6.3.2 Trypsin digestion of lysates EVs

Fresh 100mM dithiothreitol (DTT) (Sigma, Cat #: D06232- 1G) was added to each 50 µg of EVs samples to achieve a final concentration of 5 mM. Samples were vortexed and incubate at 60°C for 30 min to ensure all disulphide bonds were successfully removed with brief centrifugation. Alkylation was performed using 200 mM iodoacetamide solution (Sigma, Cat #: I1149- 5G) added to each sample and mixed vigorously and samples were incubated at RT in dark for 30 min. Next, 50mM ABC solution was added to each sample to achieve the desired concentration. 50 µL of reduced and alkylated EVs samples was transfer to a vial of trypsin singles kit (Sigma, Cat #: T7575), vortexed and placed overnight in a thermomixer at 350rpm at 37°C. After 18hrs, the trypsin digestion was stopped by adding 1% acetic acid (Sigma, Cat. #: 338826) to the samples. EVs were first centrifuged 10,000 rpm for 5 minutes and desalting of digested protein was done using C18 ZipTips (Merck Millipore, Cat. #: ZTC18S096), which were carefully equilibrated. Binding and washing of the samples were done with 0.1% trifluoroacetic acid (Sigma, Cat. #: T0699) and samples were then eluted from the ZipTip and dispensed in 10 µL of 50% acetonitrile (Sigma, Cat. #: 494445) in 0.1% trifluoroacetic acid without introducing air. Samples were concentrated and acetonitrile evaporated by Speedvac vacuum. 6 µL of samples were resuspended in 25 µL volumes of mass spectrometry buffer (2.5% acetonitrile, 0.5% acetic acid in MS grade water). Lastly, samples were centrifuged at 15,000 rpm at 4°C for 5 min and stored in -80°C.

6.3.3 Peptide quantification in EVs' lysates

Peptides were quantified by Quantitative Colorimetric Peptide Assay Kit (Thermo Fisher, Cat #: 23275), following manufacturer instruction. Briefly, a standard curve (0- 1000µg/mL) was

generate from the Peptide Digest Assay Standard (Table 6.2). Next, 1:5 dilution of EVs samples and standards were pipetted in a 96-well plate. 180 μL of the working reagent was added to each well, thus the plate contents were mixed thoroughly on a plate shaker for 30 sec and incubated at 37°C for 15 minutes. After cooling down, absorbances were measured at 562 nm and 40 ng/ μL peptides of each sample collected for LC-MS/MS

Tubes	MS grade water (μL)	Volume of Digest (μL)	Final Concentration of Digest (μL)
A	0	150 of Stock	1000
B	75	75 of Vial A	500
C	75	75 of Vial B dilution	250
D	75	75 of Vial C dilution	125
E	75	75 of Vial D dilution	62.5
F	75	75 of Vial E dilution	31.3
G	75	75 of Vial F dilution	15.6
Blank	-	Use water for blank	0

Table 6.2: Preparation of diluted Peptide Digest Assay Standards

6.3.4 Mass spectrometry analysis of HM EVs and IMF EVs

MS analysis was performed by Eugene Dillon at the UCD Conway Mass Spectrometry Core, University College Dublin (UCD).

Samples were run on a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to a nanoElute (Bruker Daltonics, Bremen, Germany) ultra-high pressure nanoflow chromatography system (UHPnLC). The peptides were separated on a reversed-phase C18 Aurora column (25cm x 75 μm ID, C18, 1.6 μm ; IonOpticks, Australia) at a constant flow rate of 250 nL/min and an increasing acetonitrile gradient. Mobile phases were 0.1% (v/v) formic acid in water (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). The peptides were

separated by a gradient starting from 2% of mobile phase B and increased linearly to 32% for 60 minutes. This was stepped up to 95% of mobile phase B where it was maintained for 7 minutes. The injection volume was 5 μ L, equivalent to a loading of 250ng per sample. The timsTOF Pro mass spectrometer was operated in positive ion polarity with Trapped Ion Mobility Spectrometry (TIMS) and Parallel Accumulation Serial Fragmentation (PASEF) modes enabled. The accumulation and ramp times for the TIMS were both set to 100 ms, with an ion mobility ($1/k_0$) range from 0.62 to 1.46 Vs/cm. Spectra were recorded in the mass range from 100 to 1,700 m/z. The precursor (MS) Intensity Threshold was set to 2,500 and the precursor Target Intensity set to 20,000. Each PASEF cycle consisted of one MS ramp for precursor detection followed by 10 PASEF MS/MS ramps, with a total cycle time of 1.16 s. Raw data from the Q-Exactive was processed using the MaxQuant (version 1.6.4.0) incorporating the Andromeda search engine. To identify peptides and proteins, MS/MS spectra were matched against Uniprot bovine and human database which generate two data sets. All searches were performed using the default setting of MaxQuant, with trypsin as specified enzyme allowing two missed cleavages and a false discovery rate of 1% on the peptide and protein level. The database searches were performed with carbamidomethyl (C) as fixed modification and acetylation (protein N terminus) and oxidation (M) as variable modifications. For the generation of label free quantitative (LFQ) ion intensities for protein profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant in a maximum time window of 1 min.

6.3.5 Statistical analysis of LFQ intensities of identified proteins in HM EVs and IMF EVs using Perseus

HM EVs' and IMF EVs' protein dataset, processed by MaxQuant, were uploaded into Perseus software version 1.6.15.0 (Max-Planck-Institute of Biochemistry, Martinsried, Germany).

General matrix was obtained by filtering the data for two categories: potential contaminant and reverse hits, followed by transformation to Log2x. Subsequently, categorical annotation of the rows, identification of the groups and filtration for a minimum of 2 valid values of the identified proteins from the HM and IMF were performed simultaneously.

6.3.6 Validation by immunoblotting of the key proteins found in HM EVs vs IMF EVs

Selected key proteins were validated by immunoblotting as described in Chapter 5, Section 5.3.8. Primary antibodies used were anti-CD36 (1:1000; Cell Signalling; Cat #: 14347), anti-SLC34A2 (1:1000; Cell Signaling; Cat #: 66445), anti-FABP3 (1:1000, Cell Signalling; Cat #: 14780) and anti-BTN1A1 (1:10000; Abcam; Cat #: ab180605). The primary antibodies used for the validation of unique proteins in HM EVs samples were anti-aldolase (1:1000; Abcam, Cat #: ab252953), anti-CHRLD2 (1:500; R&D system, Cat #: MAB24481), anti-CFL1 (1:1000; Cell Signaling; Cat #: 5175), anti-EEF2 (1:10000, Abcam; Cat #: ab75748) and anti-YWHAE (1:100, Abcam; Cat #: ab92311). Secondary antibodies were anti-mouse (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7076) or anti-rabbit (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7- 74) (Table 6.3). Of note, bovine milk EVs (BM EVs) and raw HM were used as positive control.

Key protein in HM EVs and IMF EVs proteome	Molecular weight (MW)	Dilution	Specifics
Anti-CD36 (D8L9T) (Rabbit mAb)	70-110 kDa	1:1000	Cell signaling; Cat #: 14347
Anti-SLC34A2 (D3V3I) (Rabbit mAb)	73,90-130 kDa	1:1000	Cell signaling; Cat #: 66445
Anti-FABP3 (A7) (Mouse mAb)	14 kDa	1:1000	Cell signaling; Cat #: 14780
Anti-BTN1A1 (Rabbit mAb)	66 kDa	1:10000	Abcam; Cat #: ab180605
HM EVs unique protein	Molecular weight (MW)	Dilution	Specifics
Anti-Aldolase (Rabbit mAb)	39 kDa	1:1000	Abcam; Cat #: ab252953
Anti-CHRD2 (Mouse mAb)	49.5 kDa	1:500	R&D system; Cat #: MAB24481
Anti-CFL1 (D3F9) XP ^R (Rabbit mAb)	19 kDa	1:1000	Cell Signaling; Cat #:5175
Anti-EEF2 (Rabbit mAb)	95 kDa	1:10000	Abcam; Cat #: ab75748
Anti- YWHA (Rabbit mAb)	29 kDa	1:1000	Abcam; Cat #: ab92311

Table 6.3: Antibodies used to validate key proteins in HM EVs and IMF EVs obtained from MS analysis

6.4 Data analysis

Descriptive statistics of all reported data was performed using Graph Pad Prism 9 software.

Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments (n=3). Unpaired two-tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $P < 0.05$ were considered statistically significant.

6.5 Results

6.5.1 EVs proteomic analysis of HM and IMF samples

We identified 15 proteins for IMF EVs and 660 proteins for HM EVs (Fig. 6.1) and heatmaps of both EVs sample are illustrated in Fig. 6.2a and 6.2b.

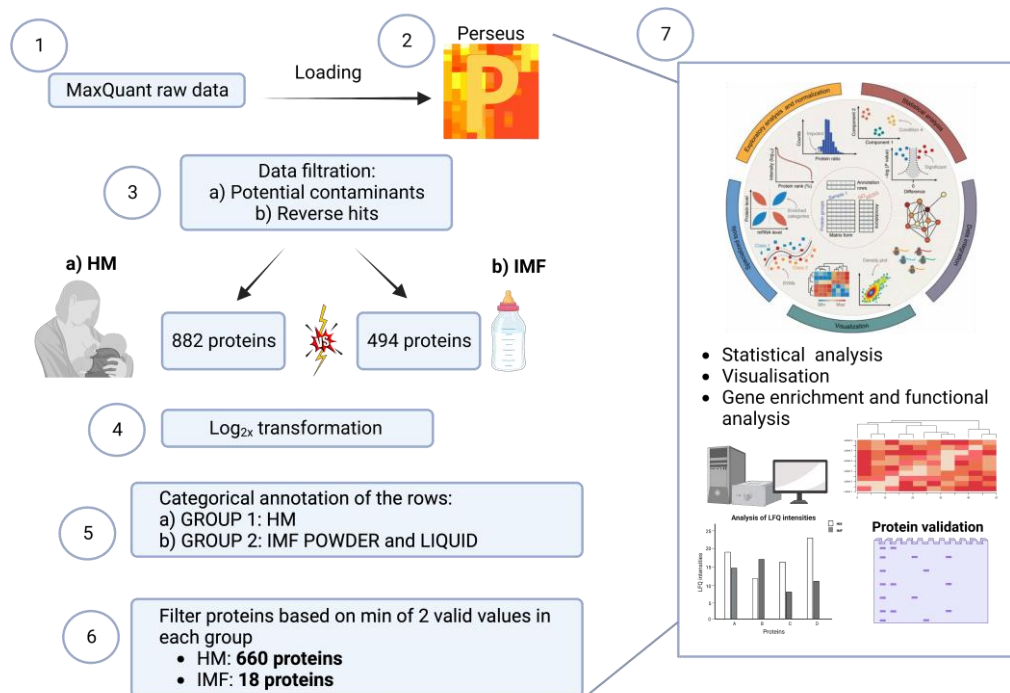
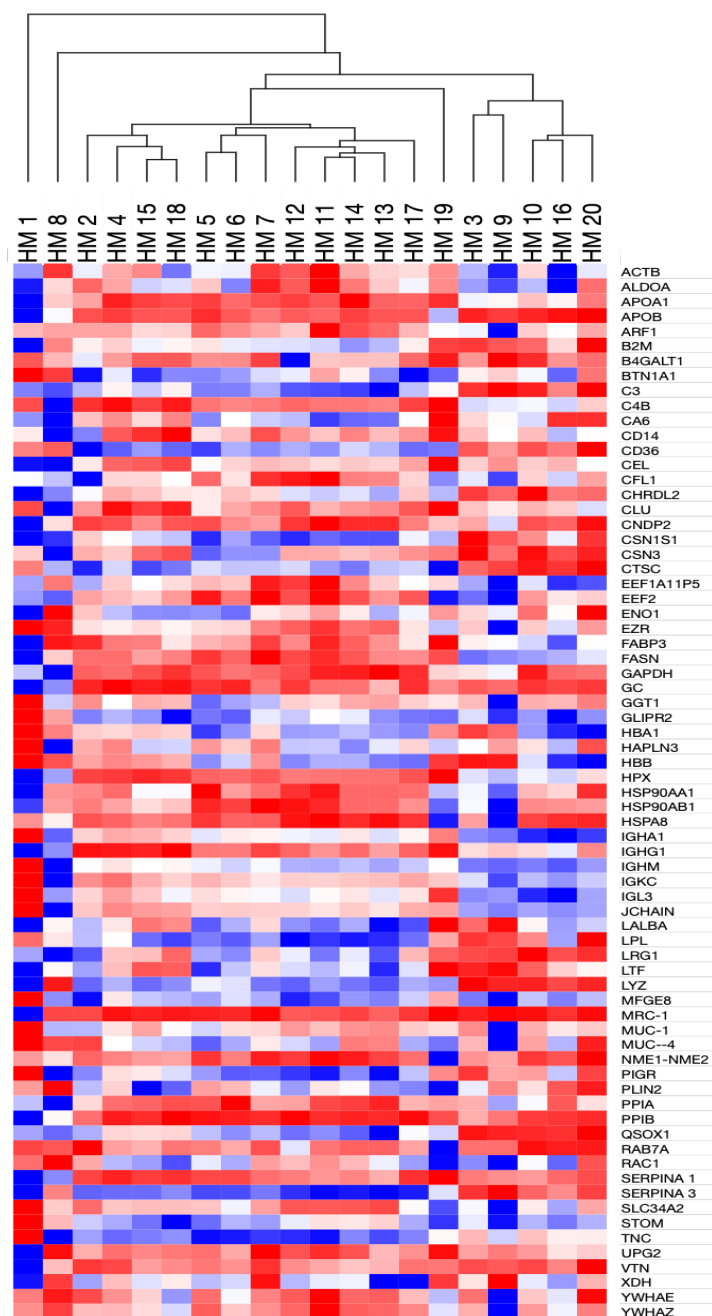


Figure 6.1: EVs proteomic analysis of HM and IMF using LC-MS/MS

Flowchart of mass spectrometry analysis: MaxQuant and Perseus analysis of HM EVs and IMF EVs proteome

a)



b)

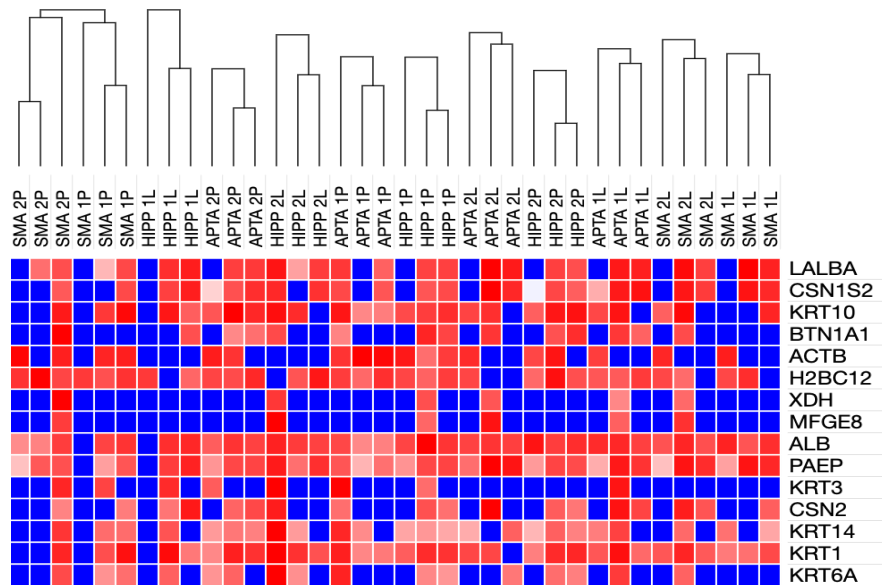
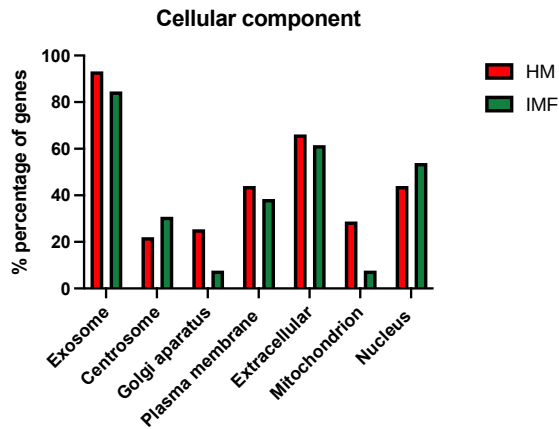


Figure 6.2: Heatmaps of identified proteins in HM EVs and IMF EVs proteome

a) Hierarchical clustering for HM EVs=20 donors. b) Hierarchical clustering for IMF EVs POWDER n=20 and IMF EVs LIQUID n=20. Metric: Euclidean distance; Linkage method; Average. Morpheus database (<https://software.broadinstitute.org/morpheus/>).

Enrichment analysis reported, about cellular component correlated to EVs proteome, IMF had the lowest % of proteins associated to exosomes and extracellular environment when compared to HM. Moreover, most of the proteins were associated with monocytes, B cells, dendritic cells, leucocytes, T cells and lymphocytes which are all involved in the immune response and maturation of immune cells. Interestingly, these proteins were less enriched in IMF EVs compared to HM EVs (Fig. 6.3).

a)



b)

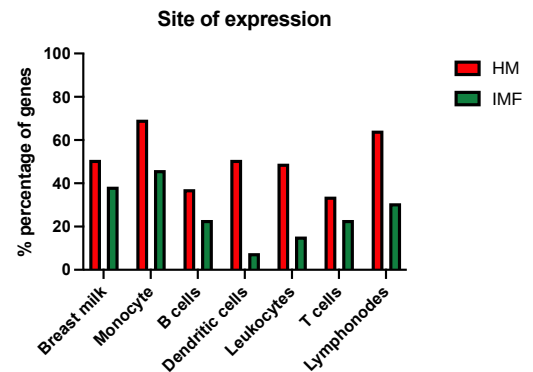


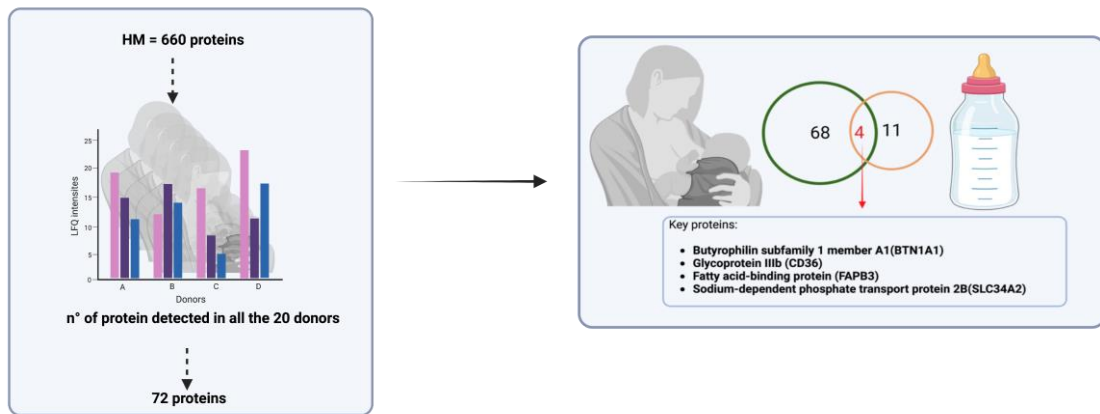
Figure 6.3: Gene enrichment analysis

For each set of genes identified in HM EVs and IMF EVs enrichment analysis was performed using FunRich software where cellular component, biological processes and site of expression were respectively analysed. a) Comparison of % genes in HM EVs vs IMF EVs involved in the cellular component. b) Comparison of % genes identified in HM EVs vs IMF EVs analysed for the site of expression.

6.5.2 LFQ intensities and the expression of common proteins identified in HM EVs and IMF EVs proteome

We investigated among the 660 proteins from HM EVs, which ones were common across all the 20 donors. Here, 72 were presented in all HM EVs samples and so these were further used to classify the key proteins in HM EVs and IMF EVs proteome. Only 4 proteins were shared by the two proteomes (Fig. 6.4a). Specifically, the 4 proteins in common were butyrophilin subfamily 1 member A1 (BTN1A1), glycoprotein IIIb (CD36), sodium-dependent phosphate transport protein 2B (SLC34A2) and fatty acid binding protein (FABP3). For each of these, the relative LFQ intensities, were statistically analysed using GraphPad (Fig. 6.4b). However, for comparison purposes, we presented the IMF EVs proteome in two separated group, powder, and liquid. Overall, BTN1A1; FABP3; CD36 and SLC34A2 were all at significantly ($p < 0.0001$) lower amounts in IMF EVs samples compared to HM EVs samples.

a)



b)

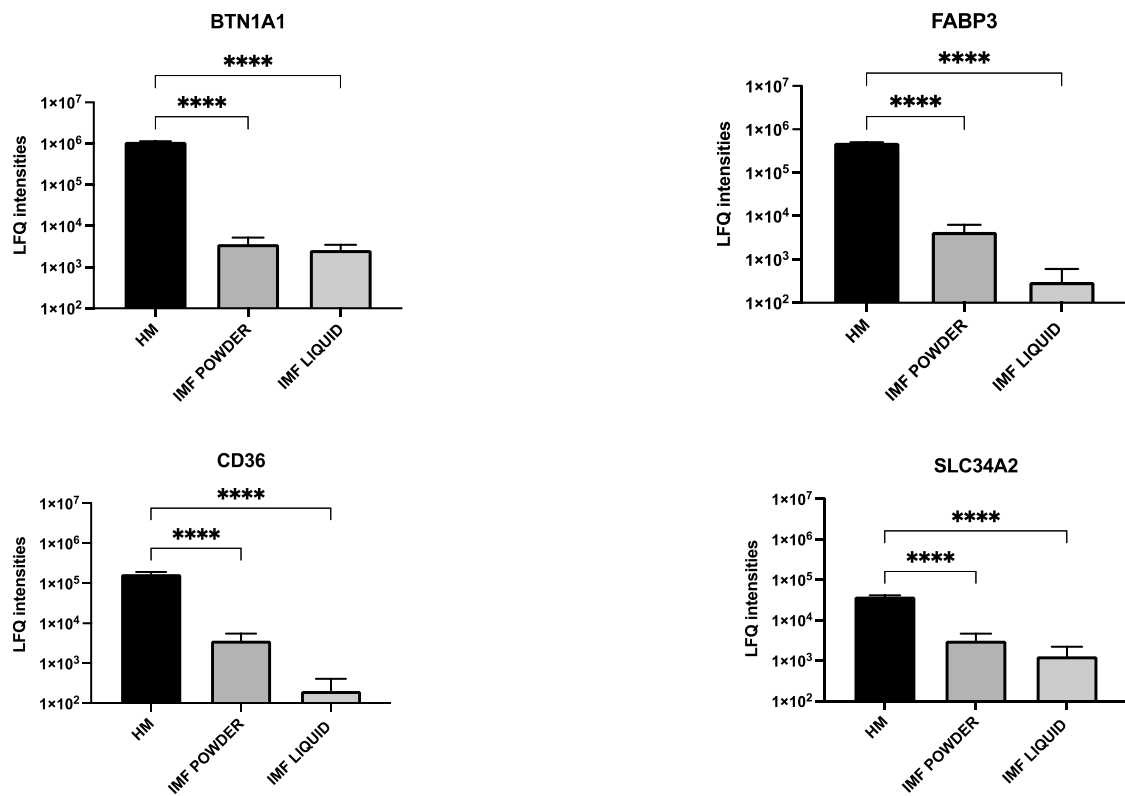


Figure 6.4: Statistical analysis and validation of key proteins in HM EVs and IMF EVs proteome

a) Venn diagram, generated using Venny 2.1, represented 4 key proteins in common between HM EVs and IMF EVs proteome. b) LFQ intensities analysis in HM EVs and IMF EVs of the 4 key proteins: BTN1A1, FABP3, CD36 and SLC34A2 using GraphPad Prism 9. Each dataset was analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; **** indicates $p < 0.0001$.

Additionally, we reported the list of the 68 proteins found in HM EVs in Table 6.4 and the 11 proteins identified in IMF EVs in Table 6.5.

GENE	PROTEIN NAME	GENE	PROTEIN NAME
A2M	Alpha-2-macroglobulin	HSP90AB1	Heat shock protein HSP 90-beta
ACTB	Actin, cytoplasmic 1	HSPA8	Heat shock cognate 71 kDa protein
ALDOA	Fructose-bisphosphate aldolase A	IGHA1	Immunoglobulin heavy constant alpha 1
APOA1	Apolipoprotein A-I	IGHG1	Immunoglobulin heavy constant gamma 1 (Fragment)
APOB	Apolipoprotein B-100	IGHM	Immunoglobulin heavy constant mu
APOD	Apolipoprotein D	IGKC	Immunoglobulin kappa constant
ARF1	ADP-ribosylation factor 1	IGL3	Immunoglobulin lambda constant 3
B2M	Beta-2-microglobulin (Fragment)	JCHAIN	Immunoglobulin J chain
B4GALT1	Beta-1,4-galactosyltransferase 1	LALBA	Alpha-lactalbumin
C3	Complement C3	LPL	Lipoprotein lipase
C4B	Complement C4-B	LRG1	Leucine-rich alpha-2-glycoprotein
CA6	Carbonic anhydrase 6	LTF	Lactotransferrin
CD14	Monocyte differentiation antigen CD14	LYZ	Tyrosine-protein kinase Lyn
CEL	Bile salt-activated lipase	MFGE8	Lactadherin
CFL1	Cofilin-1	MRC-1	Macrophage mannose receptor 1
CHRD2	Chordin-like protein 2	MUC-1	MUC1-CT
CLU	Clusterin	MUC-4	Mucin-4
CNDP2	Cytosolic non-specific dipeptidase	NME1-NME	Nucleoside diphosphate kinase
CSN1S1	Alpha-S1-casein	PIGR	Polymeric immunoglobulin receptor
CSN3	Kappa-casein	PLIN2	Perilipin-2
CTSC	Dipeptidyl peptidase 1	PPIA	Peptidyl-prolyl cis-trans isomerase A
EEF1A1P5	Putative elongation factor 1-alpha-like 3	PPIB	Peptidyl-prolyl cis-trans isomerase B
EEF2	Elongation factor 2	QSOX1	Sulfhydryl oxidase 1
ENO1	Alpha-enolase	RAB18	Ras-related protein Rab-18
EZR	Ezrin	RAB7A	Ras-related protein Rab-7a
FASN	Fatty acid synthase	RAC1	Ras-related C3 botulinum toxin substrate 1
FGA	Fibrinogen alpha chain	SERPINA1	Alpha-1-antitrypsin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	SERPINA3	Alpha-1-antichymotrypsin
GC	Vitamin D-binding protein	STOM	Erythrocyte band 7 integral membrane protein
GGT1	Glutathione hydrolase 1 proenzyme	TNC	Tenascin
GLIPR2	Golgi-associated plant pathogenesis-related protein 1	UPG2	UTP-glucose-1-phosphate uridylyltransferase
HBA1	Hemoglobin subunit alpha	VTN	Vitronectin
HAPLN3	Hyaluronan and proteoglycan link protein 3	XDH	Xanthine dehydrogenase/oxidase
HBB	Hemoglobin subunit beta	YWHAE	14-3-3 protein epsilon
HPX	Hemopexin	YWHAZ	14-3-3 protein zeta/delta
HSP90AA1	Heat shock protein HSP 90-alpha		

Table 6.4: List of unique proteins in HM EVs samples

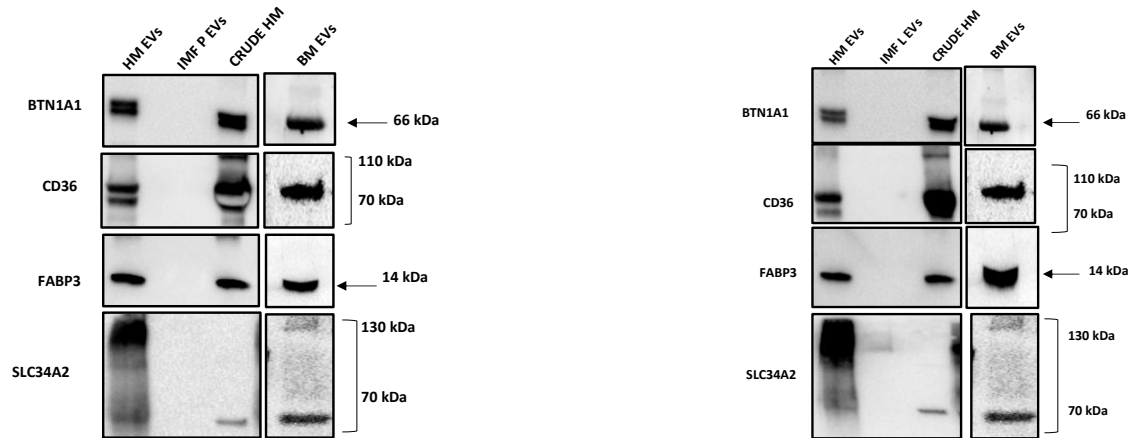
GENE	PROTEIN NAME
CSN1S2	Alpha-S1-casein
KRT10	Keratin, type I cytoskeletal 10
LOC115945178	Histone H4
H2BC12	Histone H2B type 1-K
TUBA1D	Tubulin alpha chain
ALB	Albumin
PAEP	Lipocln_cytosolic_FA-bd_dom domain-containing protein
CSN2	Alpha-S2-casein
KRT14	Keratin, type I cytoskeletal 14
KRT1	Cytokeratin-1
KRT6A	IF rod domain-containing protein

Table 6.5: List of unique proteins in IMF EVs samples

6.5.3 Validation of key proteins classified in HM EVs and IMF EVs proteome using immunoblotting

To confirm the presence of key proteins in EVs samples, the four proteins BTN1A1, CD36, FABP3, SLC34A2 and were investigated by immunoblotting analysis, illustrated in Fig. 6.5a. Specifically, lower levels of BTN1A1, CD36, FABP3 and SLC34A2 were present in IMF EVs powder and liquid compared to HM EVs, of which all the proteins were readily detected (Fig. 6.5a) Moreover, to further confirm that IMF EVs samples had very low amount of these proteins, two positive controls were used, bovine milk EVs (the primary source for the manufacture of IMF) and raw human milk. Both were positive for all the above proteins. Furthermore, in Fig. 6.5b, the densitometric analysis showed significantly lower intensities of the bands for IMF EVs samples for all the proteins analysed compared to HM EVs.

a)



b)

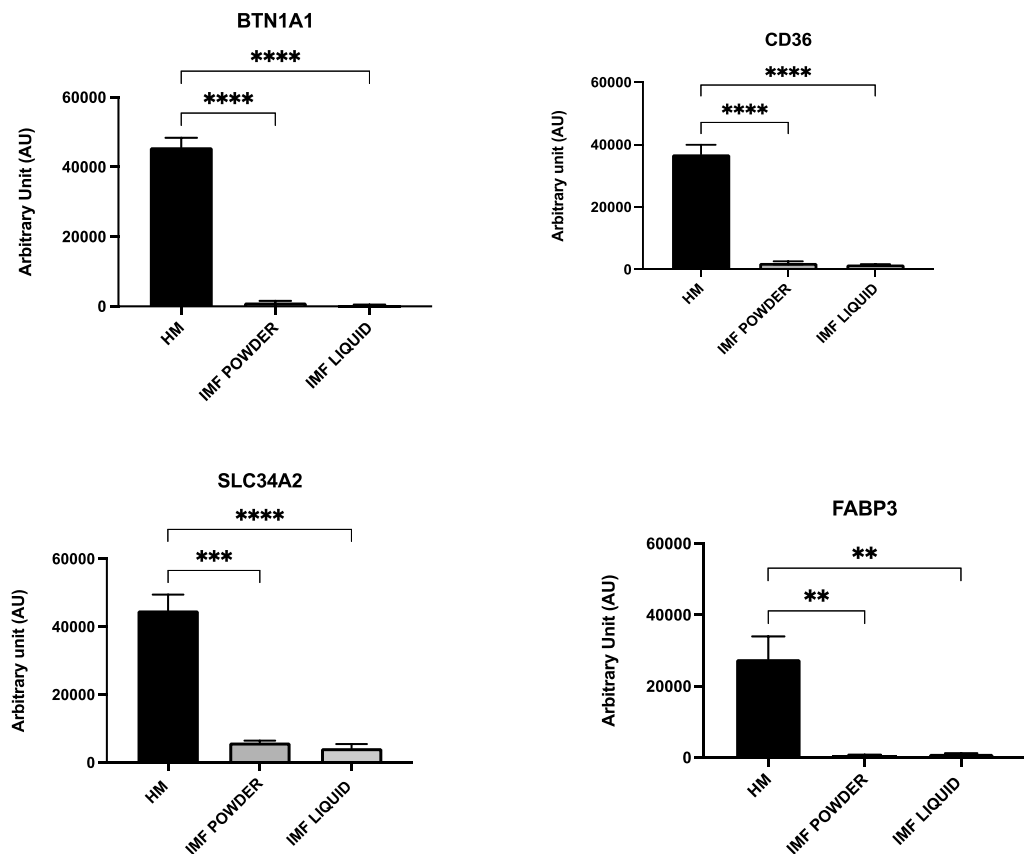


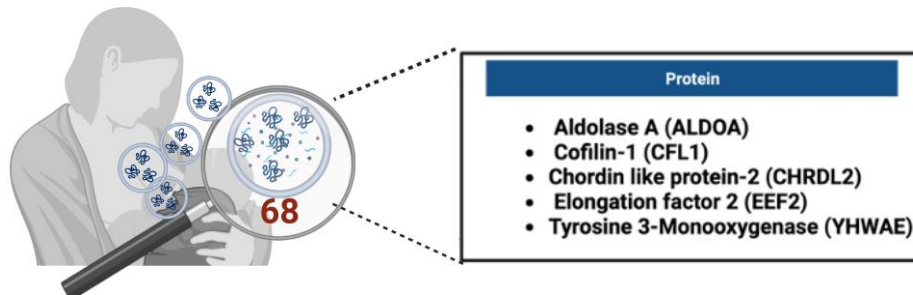
Figure 6.5: Validation of key proteins in HM EVs and IMF EVs proteome

a) Immunoblotting analysis of selected proteins for all the EVs samples, crude HM, and BM EVs; b) Densitometric analysis of each protein analysed. Bars represent the mean of the densities of the signals obtained after immunoblotting (n = 3 ± SEM). Statistical analysis was done using one-way ANOVA test; **p < 0.01; *** p < 0.001; **** p < 0.0001.

6.5.4 Unique protein found in HM EVs and IMF EVs proteome

In total 68 proteins were defined as exclusive proteins in HM EVs proteome analysed by mass spectrometry. Among these it was relevant to select few key proteins which were identified in HM and chosen based on their LFQ intensities (Fig. 6.6a). We selected aldolase (ALDOA), chordin like protein 2 (CHRD2), elongation factor 2 (EEF2), tyrosine-3-monooxygenase (YWHAE). Because of their abundance in our HM EVs samples, these five proteins were further investigated by immunoblotting (Fig. 6.6b). Although all the 20 HM EVs samples carried these proteins, we selected 7 HM samples for further validation and analysis of variability in protein expression in multiple donors. ALDO, CFL1, CHRD2, EEF2 and YWHAE were all readily detected in HM EVs samples and positive controls. Overall, the intensities of the band for all the proteins were homogenous in all samples analysed, confirming that they were approximately equally detected across the representative donors.

a)



b)

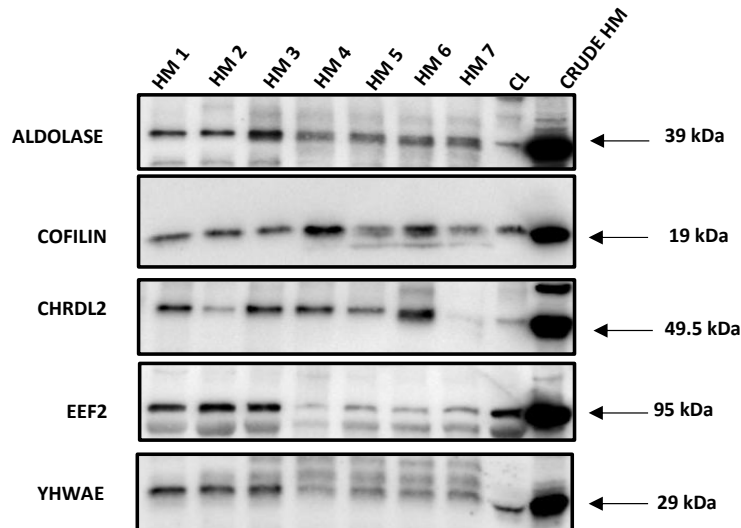


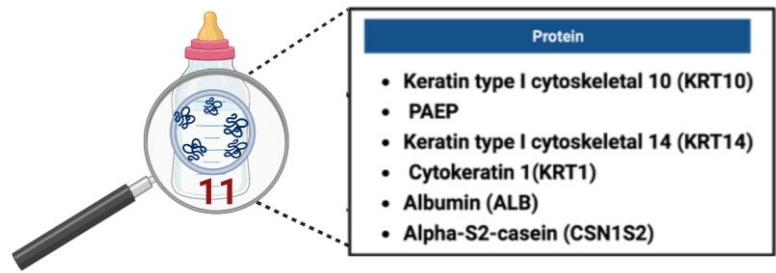
Figure 6.6: Analysis of unique proteins from HM EVs

a) List of 5 unique proteins in HM EVs proteome. b) Validation of selected proteins in HM EVs samples (n=7 donors) by immunoblotting using 10 µg of protein for all the EVs, CL (cell lysate) and crude HM.

Some unique proteins were only detected in IMF EVs samples: keratin type 10 (KRT10), lipocln_cytosolic_FA-bd_dom domain-containing protein (PAEP), keratin type 14 (KRT14), alpha-S2-casein (CSN1S2), cytokeratin-1 (KRT1), and albumin (ALB), as reported in Fig. 6.7a. We analysed and compared the LFQ intensities of the 6 IMF EVs unique proteins in IMF EVs powder and liquid isolates. No statistical differences were observed in any of these proteins, except for PAEP and CSN1S2 where in IMF EVs liquid was significantly ($p < 0.01$) higher amounts, compared

to IMF EVs powder samples. However, the intensities analysed for the rest of the proteins were similar and no differences were observed in both type of formulae (Fig. 6.7b).

a)



b)

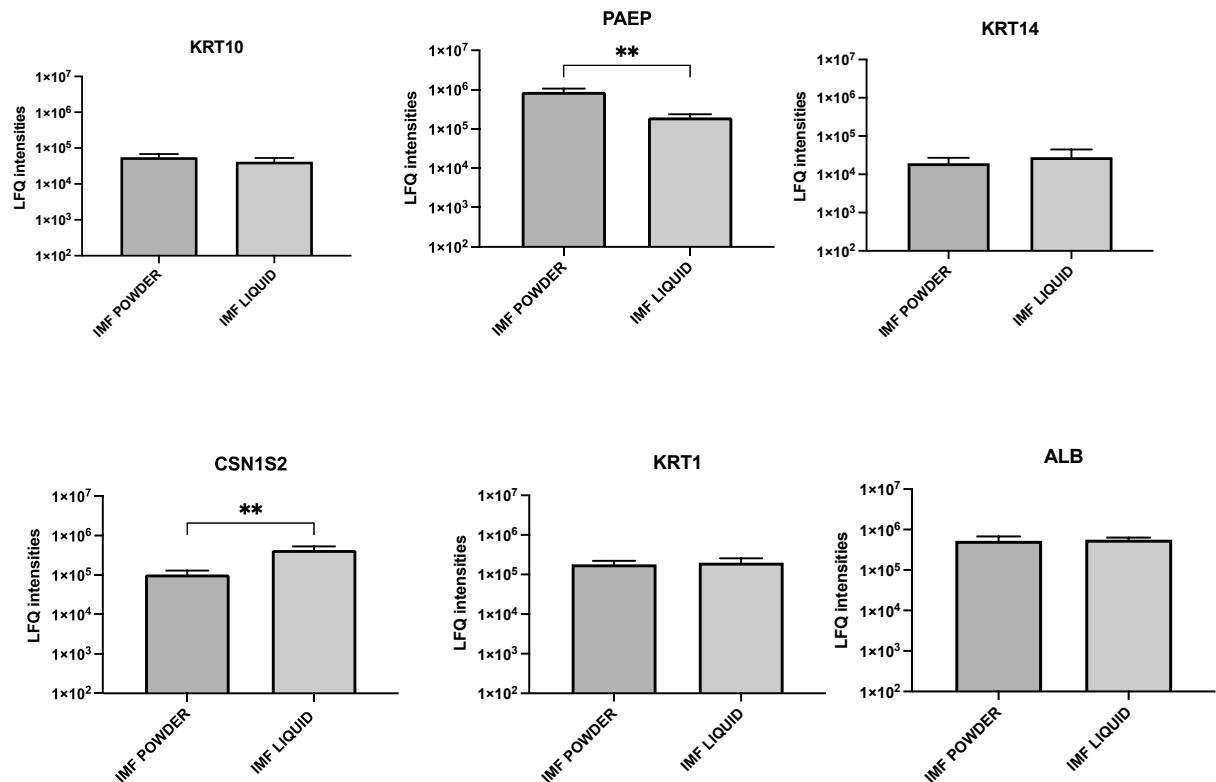


Figure 6.7: Analysis of unique proteins from IMF EVs proteome

a) List of 6 unique proteins in IMF EVs samples. b) LFQ intensities analysis of listed proteins in IMF powder and liquid EVs using GraphPad Prism 9. Data were analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; ** indicates p < 0.01.

6.6 Discussion

In Chapter 5 we observed that IMF EVs were poorly representing EVs quantity/quality as showed already by NTA, TEM and flow cytometry. Here, we investigated the proteomic content from IMF EVs and HM EVs by using mass spectrometry analysis.

6.6.1 Global profiling of HM EVs and IMF EVs proteome

Although, IMF EVs and HM EVs shared some key protein, IMF EVs had a lower number of proteins compared to HM EVs. However, while performing proteomics analysis we observed that only 4 key proteins were in common between IMF EVs proteome and HM EVs proteome. Their LFQ intensities were all significantly lower in IMF EVs compared to HM EVs. BTN1A1 is a member of the Ig superfamily suggested as marker for HM EVs and reported in studies done by others (van Herwijnen et al., 2016; Benmoussa et al., 2019; Kirchner et al., 2020; Xu et al., 2022). It is highly expressed in the lactating mammary gland and secreted into milk within lipid droplets. Butyrophilins are known to have potential immunomodulatory properties (Admyre et al., 2007; Chutipongtanate et al., 2022a), because they modulate T cell activation and development. In fact, specially together with transcription factors, such as TGF- β superfamily, may be responsible for the induction and differentiation of main immune cells (*i.e.* CD4+, CD25+, FoxP3+) (Arnett & Viney, 2014). Most importantly these proteins might be crucial for infants in their first years of life while their immune system is still developing. CD36, potentially detected in milk EVs, it is expressed by many immune and non-immune cells during their maturation, differentiation and activation. SLC34A2 is a sodium-phosphate cotransporter and it is responsible for the sodium-dependent compartment during intestinal phosphate absorption. In fact, it is essential to control the electrolytes balance and absorption during growth and

development, especially in breast fed babies. Interestingly, this protein is involved in the process of maintaining bone integrity (Marks, 2019). FABP3, an intracellular fatty acid-binding protein, its function is to synthesise fatty acids and milk fat. It is involved in the uptake, intracellular metabolism and transport of long-chain fatty acids, and might be responsible in the modulation of cell growth and proliferation (Ye et al., 2022). SLC34A2, along with CD36 and BTN1A1 are listed as the most abundant proteins found in milk EVs proteome (Xu et al., 2022). These results suggest that although traces of these proteins were quantified using LC-MS/MS, their concentration was scarce in IMF EVs.

6.6.2 HM EVs and IMF EVs proteome have unique proteins

Unique EVs protein were detected in IMF and HM isolates. 5 proteins were only associated to HM EVs samples and equally expressed, which could be potentially used as markers for milk EVs. Particularly, aldolase is a glycoprotein which catalyses the conversion of fructose 1-6 phosphate, and it is mainly involved in the actin complex. CFL1 is a well-studied member of the cofilin family, and it is involved mainly in cell motility, apoptosis, and lipid metabolism, but it has been reported to be essential for the maturation of the EVs (Howard et al., 2022). Chordin-like protein 2 (CHRD2) a member of the chordin family of protein, found to play a key role in the TGF- β superfamily. Elongation factor 2 (EEF2) which the main function is for the synthesis of proteins. Tyrosine 3-Monooxygenase (YWHAE) essential for the signal transduction in many pathways such as cell division and regulation of insulin sensitivity. On the other hand, as we also aimed to explore which unique proteins IMF EVs have and 6 proteins were identified. Among these, several isoforms of keratins (KRT1, KRT10, KRT14) while the last two were milk proteins such as Albumin (ALB) and Alpha-S2-casein (CSN1S2). Even though HM EVs and IMF EVs proteome share fewer proteins, their identities are different. In fact, IMF EVs unique proteins were mostly

associated to structural function. Instead HM EVs have many unique proteins involved in growth, development and immune response.

6.7 Conclusion

IMF EVs and HM EVs may share fewer proteins, their concentration and detection was significantly less for IMF isolates in comparison with HM isolates. Therefore, this study may help in narrowing the gaps between IMF and HM composition and gather more knowledges for the consumers/dairy industry and mothers towards improving feeding formulation for infants.

7 Chapter 7: Metabolomic characterisation of EVs from HM and IMF: identifying similarities and differences using high resolution ^1H -NMR

7.1 Abstract

Many efforts are made to narrow the gaps between IMF and human milk HM composition, because IMF is the best alternative of HM. We extended our interest on milk EVs towards metabolomics analysis using high resolution ^1H -NMR, which to date remain still elusive. Therefore, in this study we reported a comprehensive profile of IMF EVs and HM EVs metabolites. HMOs, fatty acids, and nucleotides, normally found in milk, were all less enriched in IMF EVs compared to HM EVs. Furthermore, enrichment analysis showed their key role in specific pathways (*i.e.*, gut maturity, pathogens defence). Our results gather additional information on the dynamic composition of milk EVs.

7.2 Introduction

The characterisation, by IMF, bench-marked against the gold standard HM composition, is essential. Milk EVs are majorly involved in shaping the adaptive immune system in newborns, as well as transmitting specific nutrients to the babies, via breastfeeding (Admyre et al., 2007; Hu et al., 2021; Zonneveld et al., 2021). Although, most of the EVs studies have focused their attention on nucleic acids, proteins, and lipids transported by milk EVs, metabolites (molecules of less than 1.5 kDa) are under-studied. Metabolomics is a golden standard technique to identify chemical composition and quantify metabolites in milk, including sugars, small organic acids, vitamins, nucleotides, and aromatic compounds (Suh, 2022). Metabolomic profiling of human biofluids using EVs sample serve as powerful and non-invasive tool for further characterisation studies (Altadill et al., 2016). Indeed by mimicking pathological condition in vitro, it has been reported that HM EVs reduce intestinal cell inflammation injury, while decreasing apoptosis and promoting cell proliferation (Pisano et al., 2020; S. Yu et al., 2017; Xie et al., 2020). Most importantly, knowledges on the metabolites encapsulated in milk or IMF EVs are lacking. We understand this is the first study to comprehensively describe EVs metabolite content of HM and IMF. Specifically, the aim was to explore the metabolites enclosed in HM EVs and IMF EVs using high resolution nuclear magnetic resonance (^1H -NMR).

7.3 Material and methods

7.3.1 Metabolites extraction from HM EVs and IMF EVs

EVs pellets were resuspended in a mix of deuterium oxide (D_2O) (Sigma; Cat #: 151882-100G), a reporter of magnetic field homogeneity and internal standard, and phosphate buffer stock (115mM sodium phosphate buffer, 23.3 mM NaN_3 and 17.6 mM TSP, pH = 7.4) to a total of 600 μ L which was added to the pellet and vortexed. To achieve full extraction of metabolites encapsulated in the EVs, the resuspended pellet was sonicated using Ultrasonic Cell Disruptor (Microson) at 8 Hz for about 4 min (pulse on time 10 sec, pulse off time 10 sec) to maximise the yield of the EVs metabolite content. Next, ultrafiltration of extracted metabolites was performed using Vivaspin 500 Centrifugal Concentrator with a cut-off of 10 kDa (Sartorius; Cat #: VS101) at 10,000 $\times g$ for approximately 30 min at 4°C. Moreover, to identify the metabolite content of the starting material prior for EVs separation, milk whey samples were processed alongside the EVs isolates. For this, 600 μ L of milk whey was processed for ultrafiltration as described above. Then 65 μ L of D_2O and 85 μ L phosphate buffer stock was added to each ultrafiltrate sample. EVs and milk whey properties were then loaded into an NMR tube (Sigma; Cat #: Z569194) and run with an 800 MHz spectrometer.

7.3.2 Spectra acquisition using 1D NMR Spectroscopy

^1H -NMR spectra were acquired for all samples at 25°C using Agilent Technologies Ultra High Field (UHF) 18.8 T 800 MHz Nuclear Magnetic Resonance (NMR) spectrometer. Once the sample was inserted into the spectrometer, 5 minutes was allowed for equilibration. Pre-processing and acquisition of each spectrum was done using the VnmrJ 4.0, while tuning and matching were performed manually, and the magnet field was shimmed. The 1D Nuclear Overhauser Effect

Spectroscopy (NOESY) pulse sequence was applied for each NMR metabolomics run, given that the NOESY is favoured in metabolomics research because it is effective in solvent suppression, and helps maintain consistency while processing the samples (Mckay, 2011). Each milk samples, either EVs or whey, consisted of 1152 scans, 16 steady state scans for a total acquisition time of two hours and eighteen minutes and spectrum width assigned to 12ppm. After acquisition, the spectra were manually phased, the baseline was corrected, and the chemical shift scale was set by assigning a value to the signal of TSP of $\delta = 0.00$ ppm.

7.3.3 Data pre-processing and NMR procflow analysis of obtained spectra

The spectra were uploaded onto the NMRProcFlow software version 1.4.10 designed to enable baseline correction and binning of a several number of spectra at once. Soft baseline correction method was selected and applied to all samples. Each spectrum was divided (into segments also called 'bins or buckets' which were created) using the uniform binning method. Bins width was set to be 0.01 ppm to cover the spectra in detail whereas the signal-to-noise ratio (SNR) was set to 0. A total of 1043 identical bins were obtained for each spectrum, which covered each spectrum area between 10.0 and -0.5 ppm. Spectral bins obtained were processed for metabolite identification using Chenomx 9.0, next statistical analysis of newly discovered metabolites in milk EVs and whey samples was performed using Metaboanalyst version 5.0 (Pang et al., 2022). Data integrity check, filtration, and normalisation were done before performing statistical analysis (Fig. 7.1).

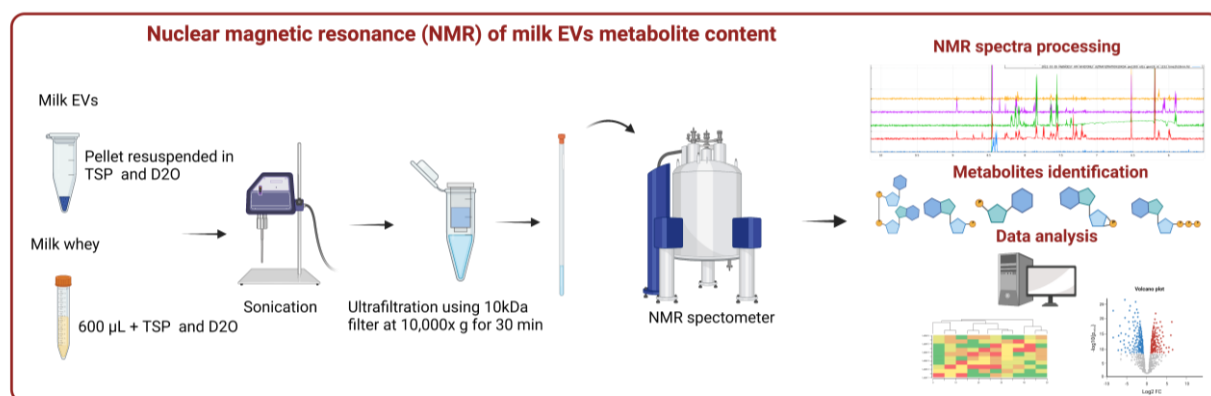


Figure 7.1: Schematic representation of the methodology used in this study

7.4 Data analysis

Descriptive statistics of all reported data was performed using Graph Pad Prism 9 software. Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments ($n=3$). NMRprocflow was used for pre-processing of the spectra, the identification and concentration of single metabolites was obtained using Chenomx 9.0, followed by statistical analysis with Metaboanalyst 5.0 and Graph Pad Prism 9 software. Unpaired two-tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $P < 0.05$ were considered statistically significant.

7.5 Results

7.5.1 Processing of metabolite content analysis in HM EVs and IMF EVs

Pre-processing and uniform binning of raw spectra of HM EVs and IMF EVs samples were processed using NMRproc flow, followed by statistical analysis performed by Metaboanalyst 5.0. Partial Least Squared Discriminant Analysis (PLS-DA) reflected a good separation between the

HM EVs and IMF isolates metabolomes, regardless of powder or liquid origin. In fact, the strengths of the model prediction are designated with the values of Q^2 and R^2 . Chenomx 9.0, containing a library of more than 300 metabolites, was applied to identify within the spectral bins the corresponded metabolites. For this, milk samples (EVs and whey) were processed, and each peak was identified as specific metabolite and their concentration was extrapolated. Subsequently, data were analysed with Metaboanalyst for statistical and enrichment and pathway analysis. Row-wise normalisation, normalisation to constant sum and to auto scaling of the data (mean-centered and divided by standard deviation of each variable) were each performed.

7.5.2 Identification and enrichment analysis of key metabolites in HM EVs and IMF EVs

In total we identified 73 compounds based on features detected using Chenomx. In Fig. 7.2, we illustrate a representative ^1H -NMR spectra of HM EVs (Fig. 7.2a), IMF EVs (Fig. 7.2b) and milk whey (Fig. 7.2c) with metabolites identified by Chenomx 9.0.

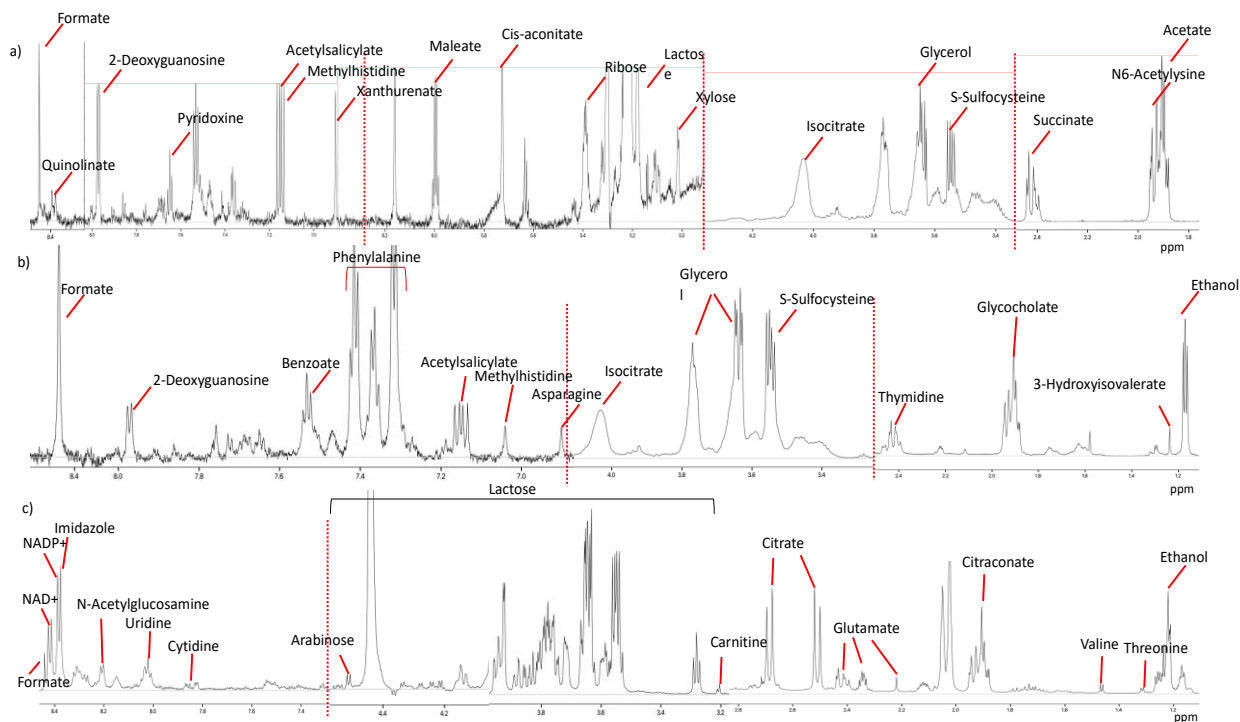
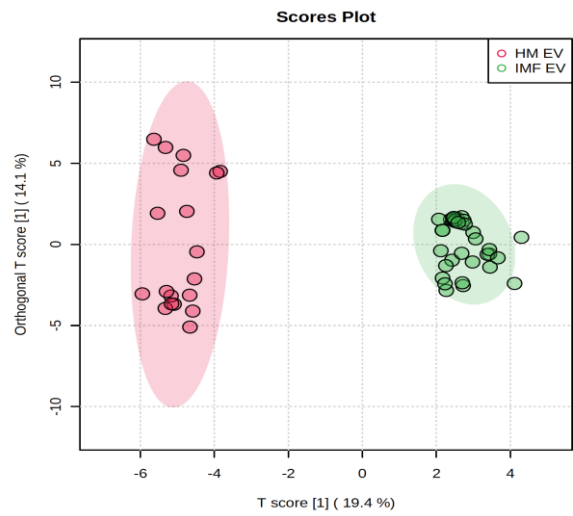


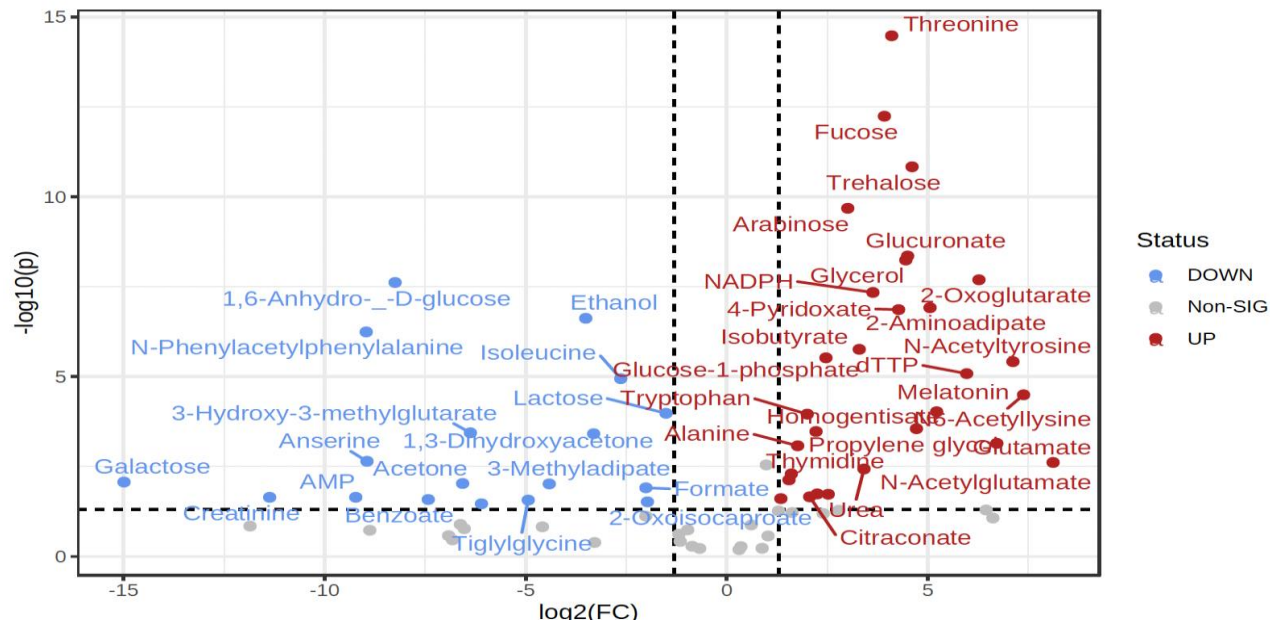
Figure 7.2: Representative ^1H -NMR spectra of EVs metabolite content in HM, IMF and milk whey
a) HM EVs metabolome spectra; b) IMF EVs metabolome spectra; c) milk whey metabolome spectra.

Here we performed Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA), which showed strong separation within set of data, enforcing the singularity of these metabolites in HM EVs and IMF EVs (Fig. 7.3a). Next, two-sample t-tests & Wilcoxon rank-sum tests were performed to assign significant (p -value < 0.05) metabolites based on their concentrations. Using this approach, we identified 48 key features; the top 25 that are upregulated or downregulated in IMF EVs versus HM EVs are shown in the volcano plot (Fig. 7.3b) and enrichment pathway analysis is illustrated in Fig. 7.3c.

a)



b)



c)

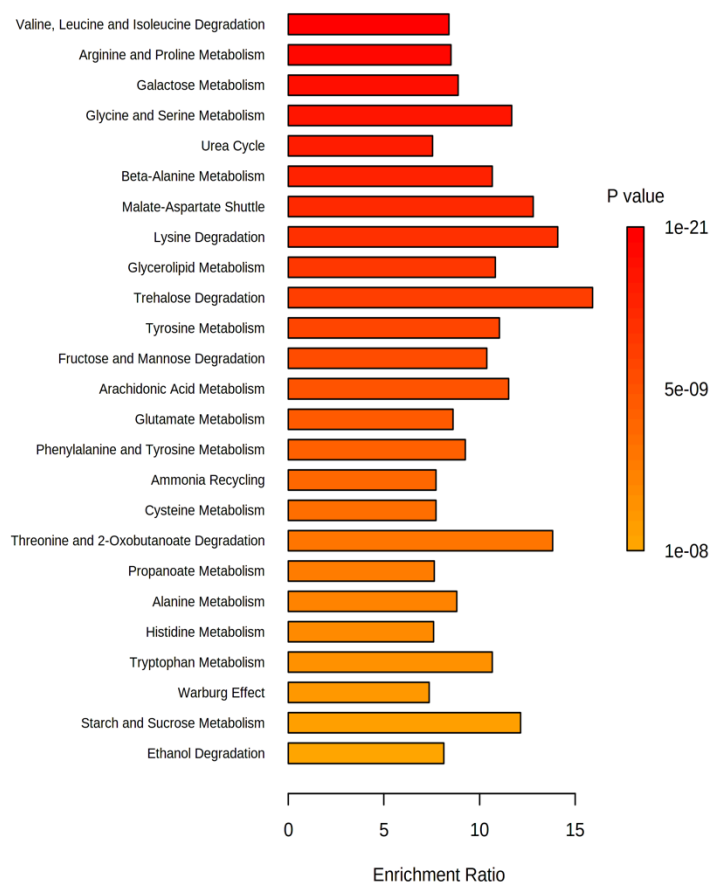
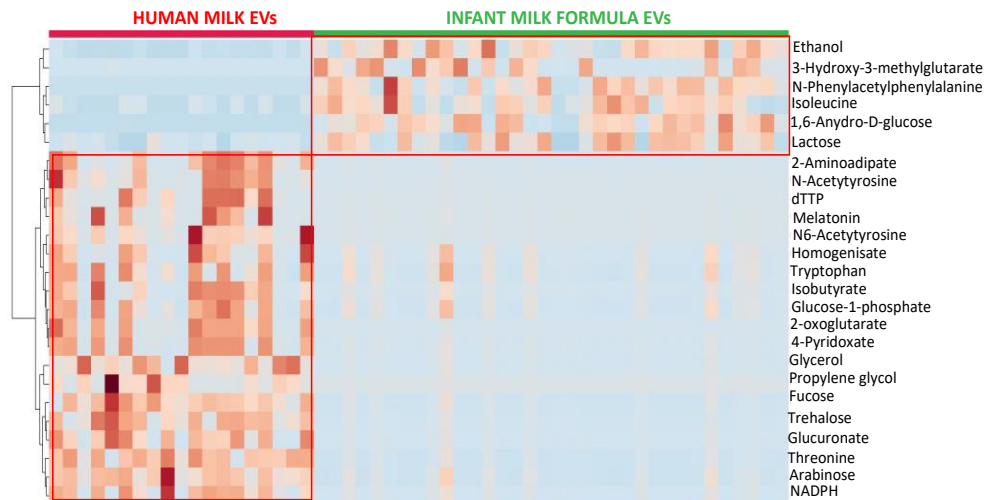


Figure 7.3: Statistical and enrichment analysis of EVs metabolite content in human milk and infant milk formula

a) Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot of all metabolites features in HM EVs and IMF EVs. b) Important features selected by volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.05. c) Metabolite Set Enrichment Analysis (MSEA) of features identified in EVs metabolome.

Accordingly, a signature set of metabolites were substantially lower in IMF EVs compared to HM EVs, illustrated in Fig. 7.4a and Fig 7.4b.

a)



b)

	Compounds	FC	log2(FC)	raw.pval	-log10(p)
1	Threonine	17.1	4.1	3.3e-15	14.4
2	Fucose	15.1	3.9	5.7e-13	12.2
3	Trehalose	24.4	4.6	1.4e-11	10.8
4	Arabinose	8.0	3.0	2.0e-10	9.6
5	Glucuronate	22.6	4.4	4.4e-09	8.3
6	Glycerol	21.9	4.4	5.7e-09	8.2
7	2-Oxoglutarate	77.3	6.2	2.0e-08	7.6
8	1,6-Anhydro-D-glucose	0.0	-8.2	2.4e-08	7.6
9	NADPH	12.4	3.6	4.5e-08	7.3
10	2-Aminoadipate	33.2	5.0	1.2e-07	6.9
11	4-Pyridoxate	19.3	4.2	1.3e-07	6.8
12	Ethanol	0.0	-3.5	2.3e-07	6.6
13	N-Phenylacetylphenylalanine	0.0	-8.9	5.7e-07	6.2
14	Isobutyrate	9.8	3.2	1.7e-06	5.7
15	Glucose-1-phosphate	5.5	2.4	2.9e-06	5.5
16	N-Acetyltyrosine	138.8	7.1	3.8e-06	5.4
17	dTTP	62.5	5.9	8.2e-06	5.0
18	Isoleucine	0.1	-2.6	1.1e-05	4.9
19	N6-Acetyllysine	166.8	7.3	3.1e-05	4.4
20	Melatonin	37.2	5.2	9.4e-05	4.0
21	Lactose	0.3	-1.5	0.01	3.9
22	Tryptophan	4.0	2.0	0.01	3.9
23	Propylene glycol	26.3	4.7	0.01	3.5
24	Homogenisate	4.6	2.2	0.01	3.4
25	3-Hydroxy-3-methylglutarate	0.0	-6.3	0.01	3.4
26	1,3-Dihydroxyacetone	0.1	-3.3	0.01	3.4
27	Glutamate	105.2	6.7	0.01	3.1
28	Alanine	3.3	1.7	0.01	3.0
29	Anserine	0.0	-8.9	0.01	2.6
30	N-Acetylglutamate	277.5	8.1	0.01	2.6
31	Urea	10.6	3.4	0.01	2.4
32	Thymidine	3.0	1.6	0.01	2.2
33	Glucose	2.9	1.5	0.01	2.1
34	Galactose	3.0e-05	-14.9	0.01	2.0
35	Acetone	0.0	-6.5	0.01	2.0
36	3-Methyladipate	0.0	-4.4	0.01	2.0
37	Formate	0.2	-2.0	0.01	1.9
38	N-Nitrosodimethylamine	4.7	2.2	0.01	1.7
39	Succinate	5.7	2.5	0.01	1.7
40	Citraconate	4.1	2.0	0.01	1.6
41	AMP	0.0	-9.2	0.01	1.6
42	Creatinine	0.0	-11.3	0.01	1.6
43	Glycocholate	2.5	1.3	0.01	1.6
44	Benzoate	0.0	-7.4	0.01	1.5
45	Tiglylglycine	0.0	-4.9	0.01	1.5
46	2-Oxoisocaproate	0.2	-1.9	0.01	1.5
47	3-Hydroxyisovalerate	0.0	-6.1	0.01	1.4

Figure 7.4: Heatmap of key EVs metabolite from HM and IMF

a) Significant metabolites in HM EVs and IMF EVs categorised by fold change (FC). b) Heatmap of the top 25 metabolites identified in HM EVs and IMF EVs by t-test. The clustering algorithms used were Euclidean

distance and ward's linkage which generate the represented heatmap. Colours are shading from dark red (higher expression) to dark blue (lower expression).

One sets of metabolites in HM are, human milk oligosaccharides (HMOs). HMOs are made of basic monosaccharides such as glucose, lactose, galactose and fucose. We observed fucose and glucose-1-phosphate to be 4 and 2.5-fold, respectively, lower in IMF EVs compared HM EVs.

Two types of sugars were identified, *i.e.*, trehalose and arabinose. Trehalose was detected at 4.6-fold lower amounts in IMF EVs compared to HM EVs. Likewise, the concentration of arabinose was 3-fold lower in IMF EVs compared to HM EVs. Of note, arabinose is a five-carbon sugar and nutrient source for many bacteria but its function in breast milk is still not understood.

Another set of key metabolites that we detected in the EVs metabolome from IMF and HM were the free amino acids. For example, NADPH was 3.6-fold lower in IMF EVs compared to HM EVs.

Other metabolites identified were threonine, tryptophan, alanine, glutamate, urea and some derivates such as: N-acetyltyrosine, N6-acetyllysine, N-nitrosodimethylamine and N-acetylglutamate which were all significantly lower amounts in IMF isolates compared to HM EVs.

Specifically, N-acetylglutamate was 8.1-fold; N6-acetyllysine was 7.4-fold; N-acetyltyrosine was 7.1-fold; glutamate was 6.7-fold; threonine was 4.1-fold; urea was 3.4-fold; and tryptophan was 2-fold lower in IMF EVs samples. Along with these metabolites, nucleotides thymidine and

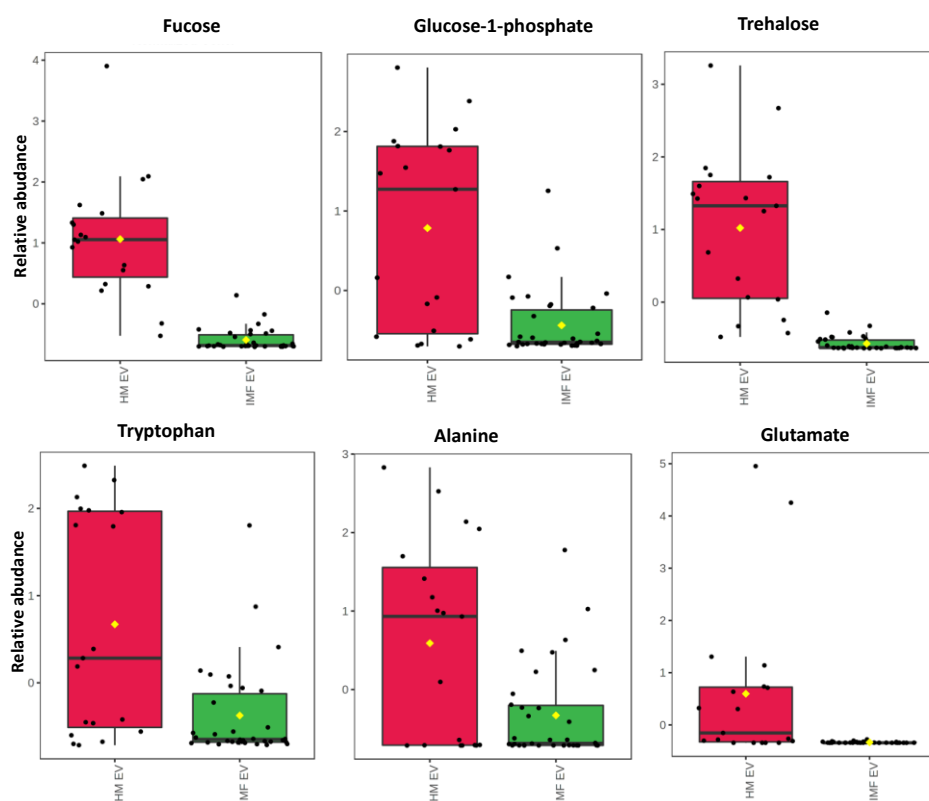
thymidine 5'-triphosphate (dTTP) were also found to be scarce in IMF EVs compared to HM EVs, 1.6-fold and 6-fold lower, respectively. Macromolecules in the form of glycoconjugates

(glycoproteins and glycolipids) were detected, including glucuronate (known as glucuronic acid), which was 4.5-fold lower amounts in IMF EVs compared to HM EVs samples. Essential

metabolites for energy metabolism include 2-oxoglutarate, cis-aconitate, citrate, fumarate, and succinate. While the relative abundances of these metabolites are summarised in Fig. 7.5 and

Table 7.1. Small traces of choline and its free form phosphocholine, were quantified as 3-fold

lower in IMF EVs compared to HM EVs. It is noteworthy to that; we observed some metabolites were more concentrated in IMF EVs compared to HM EVs samples. Specifically, ethanol, N-phenylacetylphenylalanine (9-fold), isoleucine (2-fold), galactose (14-fold), creatinine (11-fold) and 1,6-Anhydro-D-glucose (8-fold), in IMF EVs had higher levels compared to HM EVs.



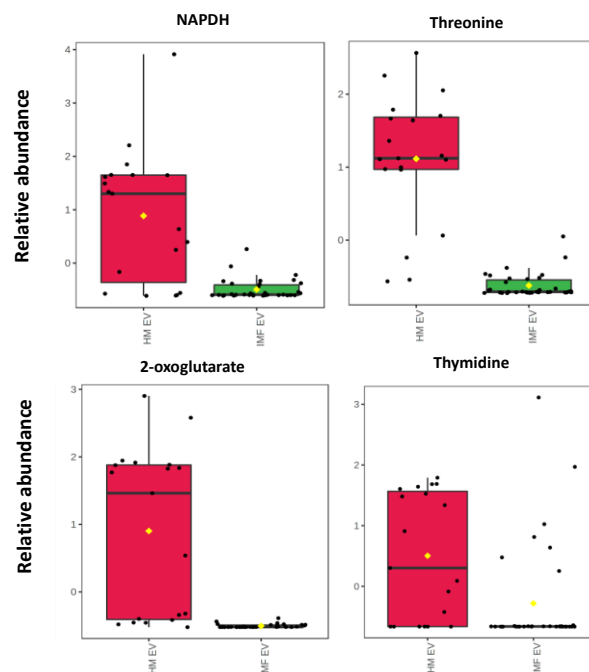


Figure 7.5: Comparison of metabolites enriched in EVs from HM and IMF.

Relative abundance of each metabolite was normalised using auto-scaling and analysed using t-tests. The normalised concentration was reported for all metabolites and the distribution of the samples was represented by a boxplot which was generated using interquartile range, while each sample was reported using black dots. RED= HM EVs; GREEN= IMF EVs.

Key metabolites in IMF EVs and HM EVs	Type	Function	Fold change (Log2x) IMF EVs vs HM EVs
➤ Fucose ➤ Glucose-1-phosphate	Human oligosaccharides (HMOs)	• Promoting bacterial colonization of the infant gastrointestinal tract	➤ 4 lower IMF EVs ➤ 2.5 lower IMF EVs
➤ Trehalose	Sugar	• Improving intestinal innate immunity	➤ 4.6 lower IMF EVs
➤ NADPH	Cofactor	• Glutathione metabolism	➤ 3.6 lower IMF EVs
➤ N-Acetylglutamate ➤ N6-Acetyllysine ➤ N-Acetyltyrosine ➤ Threonine ➤ Glutamate ➤ Tryptophan	Aminoacids and derivatives	• Energy substrates for intestinal epithelial cells and immune cell • Essential during periods of rapid growth	➤ 8.1 lower IMF EVs ➤ 7.4 lower IMF EVs ➤ 7.1 lower IMF EVs ➤ 4.1 lower IMF EVs ➤ 6.7 lower IMF EVs ➤ 2 lower IMF EVs
➤ Thymidine ➤ Thymidine 5'-triphosphate (dTTP)	Nucleotides	• Nonprotein nitrogen fraction of HM • Important cellular and metabolism functions for the infant	➤ 1.6 lower IMF EVs ➤ 6 lower IMF EVs
➤ Choline	Fatty acids	• Crucial for fetal and infant development • Brain development • Essential in the manufacture of acetylcholine	➤ 3 lower IMF EVs
➤ Glucuronate	Glycoconjugates	• Potential role in preventing infection • Natural and sustainable form of disease protection	➤ 4.5 lower IMF EVs
➤ Lactulose	Sugar	• Unique growth-promoting properties for Lactobacilli in the intestinal tract of the infant	➤ 15 lower IMF EVs

Table 7.1 List of metabolites in HM EVs and IMF EVs

Significantly different in IMF EVs compared to HM EVs categorised by type, function, and fold change (FC)

To note, regarding the presence of ethanol, Food Drug Administration (considers 50% ethanol as an appropriate stimulant for infant milk formula (powder and liquid), therefore milk products intended for consumption can be treated with 10% ethanol as a food stimulant.

7.5.3 Pathway enrichment analysis of EVs metabolite content in HM and IMF

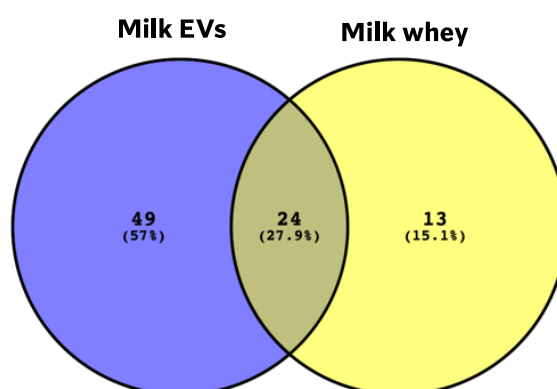
Additionally, we performed quantitative pathway analysis of signature metabolites. We identified multiple key metabolites at lower amounts in IMF EVs compared to HM EVs, illustrated in Fig. 7.6, such as glutamate, 2-oxoglutarate, alanine and succinate which are major components of D-glutamine and D-glutamate metabolism. Furthermore glutamate, 2-oxoglutarate and urea are also involved in the arginine biosynthesis. Glucuronate, also

significantly lower in IMF EVs compared to HM EVs, instead was associated to the ascorbate and alderade metabolism. 2-aminoadipate, also significantly lower in IMF EVs compared to HM EVs, is associated with the lysine degradation metabolism, while glucose 1-phosphate, pyruvate, acetate is substantially involved in one of the most important energy pathways which is glycolysis/gluconeogenesis metabolism.

7.5.4 Milk EVs and milk whey may share some metabolites and have unique metabolites profile

To confirm enrichment of metabolites in milk EVs, this untargeted metabolomics analysis also for included pooled set of milk whey remaining post EVs separation. We found that milk whey and milk EVs had 24 metabolites in common, whereas 49 metabolites were unique in the EVs fraction and 13 were associated with whey samples exclusively, as shown in Fig. 7.6a. PLS-DA was applied to maximise variability within groups, in Fig. 7.6b.

a)



b)

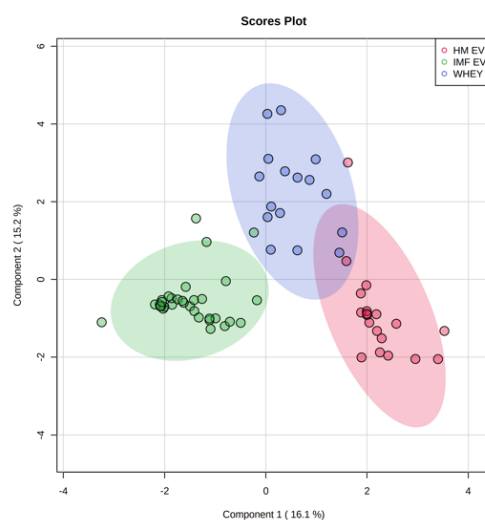
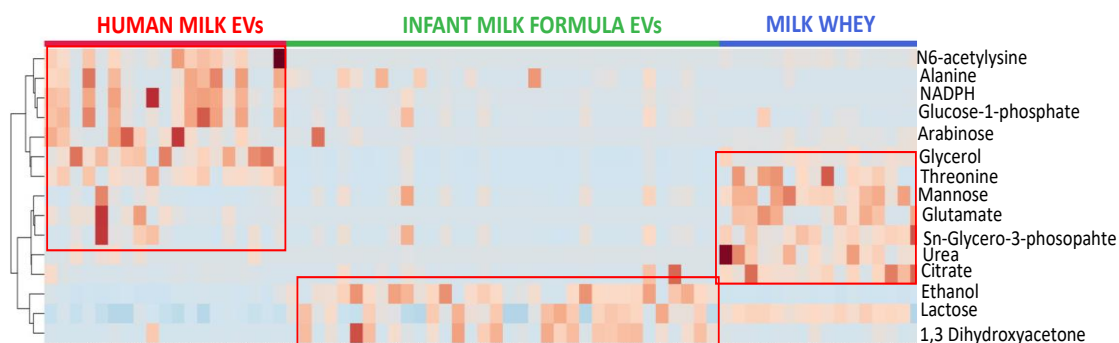


Figure 7.6: Characterisation comparison of metabolites in IMF EVs, HM EVs and milk whey

a) Venn diagram of total metabolites identified in milk EVs (HM and IMF) and milk whey, generated using Venny 2.1. b) Partial Least Squares-Discriminant Analysis (PLS-DA) score plot of all metabolite features in milk EVs and milk whey.

Several metabolites were at lower concentration in milk whey in comparison to milk EVs. Specifically, NADH, NADPH, acetate, alanine, glucose-1-phosphate, glycocholate and N-Acetylglucosamine were all less concentrate in milk whey compared to milk EVs (Fig. 7.7a). Enrichment pathway analysis of identified metabolites are illustrated in Fig. 7.7b. Conversely, citrate, glutamate, mannose, sn-Glycero-3-phosphocholine and urea were enriched in milk whey compared to milk EVs.

a)



b)

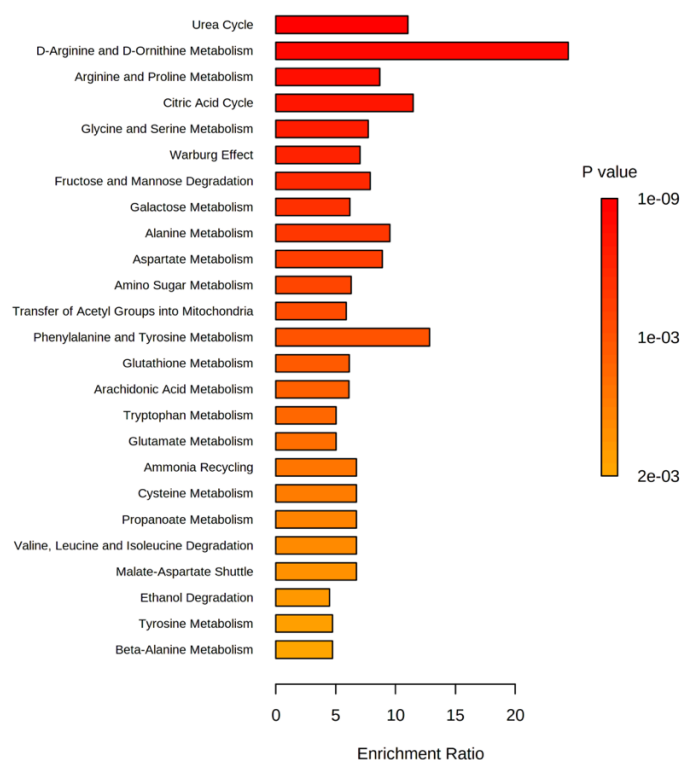


Figure 7.7: Enrichment analysis of metabolite content in IMF EVs, HM EVs and milk whey

a) Heatmap of significant metabolites within HM EVs, IMF EVs and milk whey identified by ANOVA. b) Quantitative Metabolite Set Enrichment Analysis (MSEA) of key features identified within the common metabolites between milk EVs and milk whey.

7.6 Discussion

We evaluated the metabolomic content of EVs separated from HM and IMF. In this matter, as metabolomic analysis of EVs is newly emerging, separation of high purity EVs is required for the true analysis of the entire EVs metabolome (Williams et al., 2019). Therefore, to overcome this challenge, we optimise a new technique to remove any potential contaminants prior to perform high-field ^1H -NMR spectroscopy. The main scope of this study was to apply untargeted metabolomics which is widely used for milk research (Sundekilde et al., 2013; Rocchetti & O'Callaghan, 2021); but, in this regards, towards the comprehensive analysis of important metabolites identified in EVs from IMF and HM. HMOs are the third most abundant components of breast milk (Ramani et al., 2018). Their main purpose is to thrive the colonisation of commensal bacteria (*i.e.*, *Bifidobacteria* and *Lactobacilli*) for infants' gut development. Therefore, many companies have supplemented IMF with a mixtures of galactoligosaccharides and fructoligosaccharides to transfer the same probiotic composition of mother's milk (Wu et al., 2019; Wiciński et al., 2020). Interestingly, mice fed with HMOs have an increased expression of Muc2, decreased bacterial attachment and faster turnover of crypt cells, all potential mechanisms to protect infants, in the first months of life, from several disease such as necrotising enterocolitis (Wang et al., 2019; Wu et al., 2019). In accordance, we identified at lower concentration of HMOs in IMF isolates compared to HM isolates. Sugars are also associated to crucial functions for intestine growth in infants. Lactulose, trehalose and arabinose were all less concentrated in IMF EVs compared to HM EVs samples. Specifically, trehalose and lactulose are both involved in the development of the proximal and mid-small intestine with unique growth-promoting properties (Adachi & Patton, 1961; Collins et al., 2018). Another category of metabolites highlighted in our study were the free aminoacids. They are known to exhibit immunomodulating capacities, but they account only the 10% in IMF in comparison with

HM (He et al., 2020; van Sadelhoff et al., 2020). In IMF EVs we detected lower concentration of those compared to HM EVs. Therefore, our results emphasise the differences in IMF in respective of HM towards metabolites carried by milk EVs. Previous study analysed choline in serum from infants which were either breast-fed or formula-fed. They stated the lower concentration of choline in IMF compared to HM, either in the serum observed in formula-fed infants compared breast-fed infants (Ilcol et al., 2005). Here choline and its amounts were lower in IMF EVs compared to HM EVs. We found glycoconjugates addressed as “immunoglobulins” for their protection from several disease (Peterson et al., 2013) and co-factors (NADPH). It was reported in a cohort study that mice fed with milk enriched in NADPH gained behavioural advantages, including improved spatial memory and enhanced motor learning and performance (Ear et al., 2019). In concordance with our results, one previous study also identified precursors of NADPH in milk EVs from other species, however they used different techniques and none of HM or IMF were used (Mecocci et al., 2020). Metabolites such as nucleotides have been added to nutritional formulas for infants since mid-1980s, to supplement IMF. They are attributed to provide important cellular and metabolism functions for the infant (Hodgkinson et al., 2022). However, we detected lower concentration of nucleotides in IMF EVs in respective of HM EVs. EVs metabolomics studies previously published (Luo et al., 2018; Palviainen et al., 2019; Eylem et al., 2020; Mecocci et al., 2020) are mainly focused towards EVs metabolite for diagnostic purposes and the validation of potential biomarkers for specific disease. Conversely, our study is the first one to compare the EVs metabolome from two milk sources used worldwide.

7.7 Conclusion

EVs metabolomic analysis has been increasingly promising for diagnostic values of various diseases, but knowledges about the composition and the function of the EVs metabolites, especially from HM and IMF, is missing. Here, we demonstrated that IMF has consistent lower concentration of metabolites compared to the gold standard HM, which are correlated to important pathways. Therefore, our data support the future application of EVs in the food industry for their potential advantages.

8 Chapter 8: Detection of miRNAs in HM EVs and IMF EVs

8.1 Abstract

EVs are transporting numerous information in form of proteins, lipids, metabolites, and nucleic acid like miRNAs. Particularly, miRNAs derived from milk EVs are associated to immunomodulatory functions which regulate genes associated to growth and development of the immune system. As IMF, in infants, is widely used as a substitute of HM, we asked if IMF EVs express similar miRNAs which are naturally presented in HM EVs. Therefore, here we proposed a comparison of 5 out of the top ten most abundant miRNAs from milk. We analysed the amplification of miR-148a-3p, miR-182-5p, miR-30b-3p, miR-151a-3p and miR-200a-3p in IMF EVs in respective of HM EVs. Our results demonstrated that in IMF EVs, the miRNAs expression is significantly reduced in comparison with that in HM EVs.

8.2 Introduction

MicroRNAs (miRNAs) are short (~22 nt) endogenous noncoding RNA molecules, normally found in various fluids such as blood, saliva, urine, and milk. However among those, human milk (HM) is one of the richest sources of miRNAs (Shah et al., 2021). Generally, miRNAs regulate the expression of targeted mRNAs either by modulating their expression or potentially by inducing their degradation (Li et al., 2022). Furthermore, miRNAs in milk EVs are designated as 'dietary' since they are ingested by infants during feeding. Therefore miRNAs, especially in milk EVs, have gathered a lot of attention in last decades, because they are protected from enzymatic degradation, acidic environment, typical conditions of the GI tract, usually mimicked by low pH and RNases treatment; and consequently reach the intestine where they elicits their designated functions (Liao et al., 2017; Manca et al., 2018). For instances, miR-148a-3p and miR-30b-3p play a key role in infant growth and fat acquisition during the first month of life, particularly miR-148a-3p is directly involved in establishing the neonate's immune system and have other beneficial effects on infant gut health (Shah et al., 2021; Vélez-Ixta et al., 2022). There is a clear evidence that miRNAs, such as miR-148a-3p, miR-30b-3p, miR-200a-3p, miR-182-5p and miR-151a-3p are carried by HM EVs and captured by intestinal cells in infants (Zhou et al., 2011; Guo et al., 2022; López de las Hazas et al., 2022; Shah et al., 2021). However, because infants are also fed by formula, it is relevant to evaluate if the same nutrients delivered by HM are provided by IMF too. Although a study demonstrated that miRNAs expression in IMF was drastically reduced compared to HM, it was also confirmed in a pilot study, where the amount of miRNAs was lower in the gastric content of formula-fed infants than that of breastfed infants (Kakimoto et al., 2020; Vélez-Ixta et al., 2022). Nevertheless, it is still debating if miRNAs present in IMF EVs are compromised or lacking compared to HM EVs. Therefore, in this study, we quantified extracted total RNA in IMF EVs and HM EVs and subsequently compared the expression of 5

immunoregulatory miRNAs to investigate similarities and differences in EVs cargo from the two sources.

8.3 Material and Methods

8.3.1 Total EVs RNA extraction

Total RNA was extracted from milk EVs pellet using miRNeasy Serum/Plasma Advanced kit (Qiagen; Cat: # 217204) and following manufacturer instructions with slightly modifications, as showed in Fig 8.1. Milk EVs pellet from HM and IMF was resuspended in 180 μ L of Buffer RPL and vortexed for > 5 s. Then, samples were left at RT (15–25°C) for 3 min, additionally, 3 μ L of exogenous miRNA (33 fmol) cel-miR-39 (cel-miR-39 Spike-In Kit, Cat : # 59000, Norgen, Canada) were added to each sample before the lysis buffer, following manufacturer instruction. Then 60 μ L of Buffer RPP was added and samples were mixed mix vigorously by vortexing for > 20 s and incubated at RT for 3 min. After this stage, first centrifugation at 12,000 x *g* for 3 min was performed at RT to pellet the precipitate, therefore the pellet was discarded. Instead, colorless supernatant was first transferred to a new centrifuge tube and mixed with 1 volume of isopropanol (Sigma; Cat :# I9516-500ML), whereas total sample was then added to the RNeasy UCP MinElute column followed by centrifugation at 8,000 x *g* for 15s. Next 700 μ L Buffer RWT was added to the same column and centrifuge at 8,000 x *g* for 15s. Same procedures was applied for the the last two steps of which 500 μ L Buffer RPE and 500 μ L of 80% ethanol were added, respectively after each centrifugation step and the flow-through was discarded. Particularly, in the last step where the 80% ethanol was used samples were centrifuged for 2 min and the RNeasy UCP MinElute spin column was carefully removed from the collection tube to avoid carryover of ethanol. The columns were placed in a new 2mL collection tube and centrifuged for

5 min to dry the membrane. Finally, 20 μ L of RNase-free water was added directly to the centre of the spin column and incubate for 1 min. Sample were centrifuged for 1 min at max speed to elute the RNA. In order to maximise the RNA extraction, we performed this step twice with longer incubation time (10 min) as recommended by the manufacturer, then same procedure as before was applied. The dead volume of the RNeasy UCP MinElute spin column was 2 μ L, so elution with 20 μ L RNase-free water resulted in an 18 μ L eluate. Finally, RNA was quantified using Qubit 2.0.

8.3.2 cDNA synthesis of extracted EVs RNA

cDNA synthesis was made from < 5 ng/ μ L of extracted RNA, using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems™; Cat: # A28007), as recommended by manufacturer instructions we used 2 μ L of sample eluent. Samples and cDNA synthesis reagents were thawed in ice and subsequently centrifuged to spin the contents. Each step of the cDNA synthesis was performed using thermocycler Veriti 96 (Well thermocycler; Applied Biosystems™).

8.3.3 Poly(A) tailing reaction

The Poly(A) reaction was performed, and volume of each reagent used for this step are reported in Table 8.1. 3 μ L of the reaction mix was added to 2 μ L of sample, previously prepared in 0.2mL PCR tube (Fisher Scientific; Cat: # 951010006) mixed thoroughly and centrifuged. Poly(A) reaction consisted of three steps: 1) Polyadenylation at 37°C for 45 mins; 2) Stop reaction at 65°C for 15 mins; 3) Hold at 4°C.

Component	Volume per sample (μL)
10X Poly(A) buffer	0.5
ATP	0.5
Poly(A) Enzyme	0.3
RNAase-free water	1.7
Total Poly(A) Reaction Mix Volume	3.0

Table 8.1: Poly(A) tailing reaction reagents

8.3.4 Adaptor ligation reaction

Samples processed for Poly(A) were prepared for the adaptor ligation reaction and volume of each reagent used for this step are reported in Table 8.2. 10 μL of the ligation reaction mix was added to each sample (total volume 15 μL), following by mixing and centrifuged. Adaptor ligation reaction consisted of two steps: 1) Ligation at 16°C for 60 mins; 2) Hold at 4°C. Once the reaction was done, we proceeded immediately with the next step.

Component	Volume per sample (μL)
5X DNA Ligase Buffer	3
50% PEG 8000	4.5
25X Ligation Adaptor	0.6
RNA Ligase	1.5
RNAse free water	0.4
Total Ligation Reaction Mix	10

Table 8.2: Adaptor ligation reaction reagents

8.3.5 Reverse transcription (RT) reaction

Samples processed for ligation reaction were prepared for reverse transcription reaction and volume of each reagent used for this step are reported in Table 8.3. 15 μL of the RT mix was added to each sample (total volume 30 μL), following by mixing and centrifuged. RT reaction consisted of three steps: 1) Reverse transcription 42°C for 15 mins; 2) Stop reaction at 85°C for 5 mins and 3) Hold at 4°C. To note, only the RT the product can be stored at -20°C for up to 2 months. Once the reaction was completed, we proceeded immediately with the final step.

Component	Volume per sample(μL)
5X RT Buffer	6
dNTP Mix (25mM each)	1.2
20X Universal RT Primer	1.5
10X RT Enzyme Mix	3
RNAse free water	3.3
Total RT Reaction Mix volume	15

Table 8.3: RT reaction reagents

8.3.6 miR-Amp reaction

For the last step only 5 μL of the RT product was used for each sample. Therefore, reagents for miR-Amp reactions were prepared and related volume are reported in Table 4. 45 μL of the miR-Amp reagent mix was added to each sample (total volume 50 μL), following by mixing and centrifuged. miR-Amp reaction consisted of four steps: 1) Enzyme activation 95°C for 5 mins; 2) Denature at 95°C for 3 sec and Anneal/Extend at 60°C for 30 sec (both repeated for 14 cycles); 3) Stop reaction at 99°C for 10 mins and 4) Hold at 4°C. Undiluted miR-Amp was stored at -20°C for up to 2 months or directly used for qPCR.

Component	Volume per sample(μL)
2X miR-Amp Master Mix	25
20X miR-Amp Primer Mix	2.5
RNAse free water	17.5
Total RT Reaction Mix volume	45

Table 8.4: miR-Amp reaction preparation

8.3.7 miRNAs quantification by qPCR

Sample for qPCR were made using 5 µL of 1:10 dilution of cDNA (miR-Amp product) and TaqMan™ Fast Advanced Master Mix (Applied Biosystem™, Cat. # 4444557) as described by the manufacturer. We used its universal reverse primer, and miR-specific forward primers reported in Table 8.5. TaqMan® Advanced MicroRNA Assay (Applied Biosystem™, Cat. # A25576) were employed, specifically for the following miRNAs: hsa-miR-148a-3p, hsa-miR-182-5p, hsa-miR-30b-5p, hsa-miR-151a-3p, hsa-miR-200a-3p. To note, the nucleotide sequences of *Homo sapiens* and *Bos taurus* were identical. Thus, each reagent used, and associated volumes are reported in Table 5. Reactions were done using MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode 0.1 mL (Applied Biosystems™, Cat: # 4346906). Therefore, 15 µL of PCR master mix was carefully added to each well followed by 5 µL of diluted cDNA, final volume (20 µL) and the plates were sealed MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Cat: # 4311971). qPCR was run using ViiA™ 7 Real-Time PCR System with 96-Well Block (Applied Biosystems™, Cat: # 4453534) according to the two-step cycling protocol: Enzyme activation at 95°C for 20 sec followed by 40 cycles for Denaturation (95°C for 1s) and Anneal/Extend (60°C for 20 sec). Synthetic *Caenorhabditis elegans* miR-39 was used to normalise the efficiency of the qPCR amplification and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Thus, quantitation was made in triplicate and each experiment was repeated a minimum of three times.

Component	Volume per sample(μL)
TaqMan™ Fast Advanced Master Mix (2X)	10
TaqMan™ Advanced miRNA Assay (20X)	1
RNAse free water	4
Total RT Reaction Mix volume	15
miRNAs	Sequence (homologous to <i>Bos taurus</i>)
hsa-miR-148a-3p	5'-UCAGUGCACUACAGAACUUUGU-3'
hsa-miR-30b-5p	5'-UGUAAACAUCCUACACUCAGCU-3'
hsa-miR-182-5p	5'-UUUGGCAAUGGUAGAACUCACACU-3'
hsa-miR-200a-3p	5'-CAUCUUACCGGACAGUGCUGGA-3'
hsa-miR-151a-3p	5'-UCGAGGAGCUCACAGUCUAGU-3'

Table 8.5: qPCR preparation and miRNAs sequence

The methodology applied for miRNAs characterisation is illustrated in Fig 8.1.

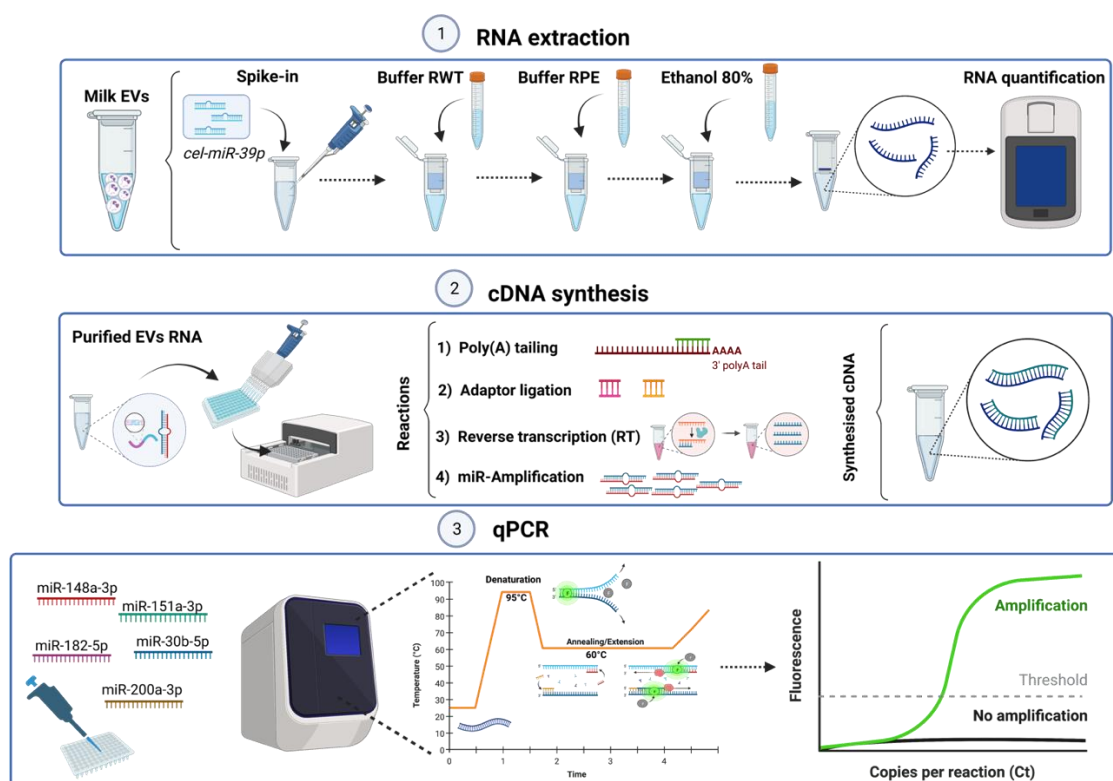


Figure 8.1: Flowchart of RNA extraction, cDNA synthesis and qPCR in milk EVs

8.4 Data analysis

Descriptive statistics of all reported data was performed using Graph Pad Prism 9 software. Data is presented in form of mean $\pm$ standard error of the mean (SEM) for at least three experiments (n=3). Unpaired two-tailed Student's t-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $P < 0.05$ were considered statistically significant.

8.5 Results

8.5.1 Total RNA quantification in HM EVs and IMF EVs

Total RNA was extracted from HM EVs and IMF EVs powder and liquid formula using miRNeasy Serum/Plasma Advanced kit and measured with Qubit 2.0. In Fig 8.2, we reported the comparison of the RNA yield in HM EVs and IMF EVs samples. Specifically, we observed that RNA concentration was significantly ($p < 0.0001$) lower in IMF EVs powder (433.3 ± 17.6 ng/mL) and liquid (663.3 ± 19.5 ng/ μ L) in comparison with extracted RNA in HM EVs which was equal to 1560 ± 73.7 ng/mL. Of note, extracted RNA was also quantified in the control sample that was 1965 ± 35 ng/mL.

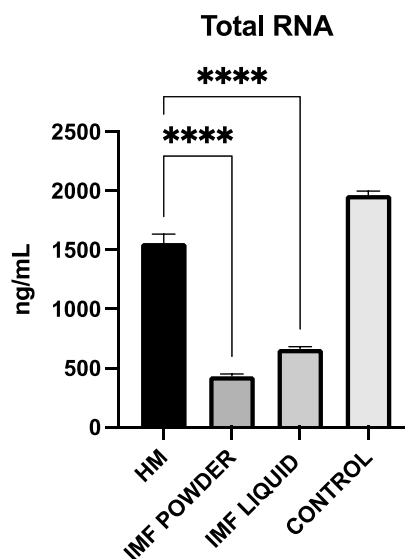


Figure 8.2: Quantification of RNA in milk EVs

Total RNA extraction from HM EVs and IMF EVs samples using miRNeasy Serum/Plasma Advanced kit. RNA concentration was reported in ng/mL. Each graph represent three replicate and statistical analysis was performed using one-way ANOVA. Significance was assigned as **** $p < 0.0001$.

8.5.2 Global profiling of miRNA in HM EVs and IMF EVs

Next, extracted RNA from milk EVs was first reverse transcribed in cDNA using TaqMan™ Advanced miRNA cDNA Synthesis Kit. Subsequently, we performed the qPCR to quantify the amplification of selected miRNAs in this study: miR-148a3p; miR-182-5p; miR-30b-5p; miR-151a and miR-200a-3p. We reported the results for each miRNA as delta Ct value and to do so normalised for the internal control cel-miR-39p, which was added during RNA extraction, as illustrated in Fig. 8.1. To note, for the purpose of this study a positive control was employed only to validate the data but excluded from the statistical analysis. In Fig. 8.3, we compared the number of cycles (Ct value) of the above miRNAs in HM EVs and IMF EVs. We observed that the Ct value of miR-148a-3p was significantly higher in IMF EVs powder ($p < 0.001$) and IMF EVs liquid ($p < 0.0001$) compared to HM EVs, therefore lower number of copies. Similar trend was obtained for the Ct value of miR-182-5p which was significantly ($p < 0.001$) higher IMF EVs powder compared to HM EVs, whereas no amplification was detected in IMF EVs liquid. Instead, for miR-30b-5p no amplification was detected for both IMF EVs samples in respective of HM EVs and the positive control, where the Ct value was below 20. Likewise, the Ct value for miR-151a-5p was significantly higher in all IMF EVs powder ($p < 0.01$) and liquid ($p < 0.001$) in comparison with the Ct value of HM EVs. Interestingly, no significant differences were observed for the Ct value for miR-200a-3p when comparing the three sets of EVs samples. Additionally, we reported the mean of the three experiments for all the samples and selected miRNAs in Table 8.6.

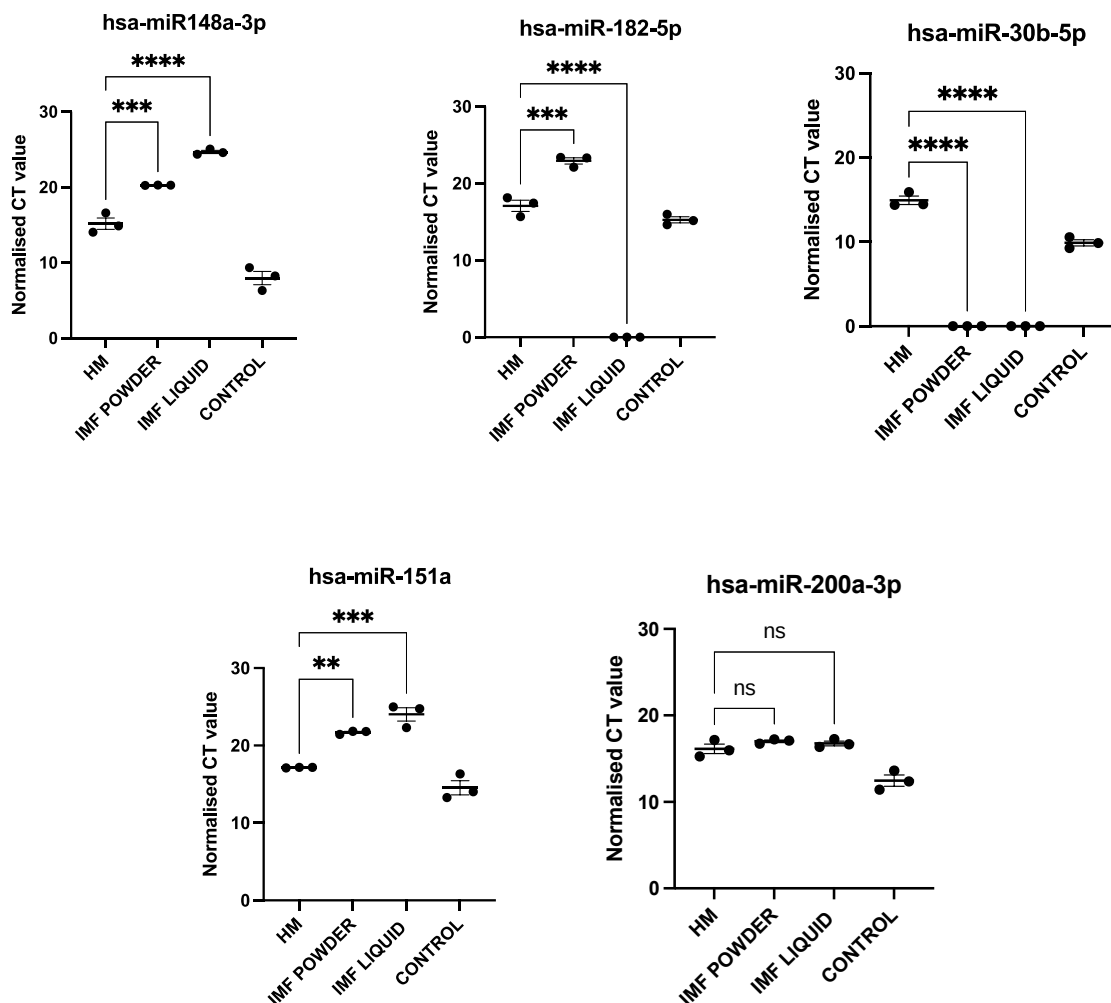


Figure 8.3: Ct value statistical analysis of selected miRNAs in HM EVs and IMF EVs.

a) qPCR data analysis of hsa-miR-148a-3p, hsa-miR-182-5p, hsa-miR-30b-5p, hsa-miR-151a-5p, hsa-miR-200a-3p were reported as normalised Ct value using *C.elegans* as synthetic control. Detection limit was set to 40 cycles.

	HM EVs		IMF P EVs		IMF L EVs		Control	
miRNAs	CT value	SEM	CT value	SEM	CT value	SEM	CT value	SEM
148a-3p	15.1	0.7	20.3	0.01	24.7	0.2	8.0	0.8
30b-5p	15.0	0.5	/	/	/	/	9.9	0.4
182-5p	17.0	0.7	23.0	0.4	/	/	15.3	0.4
151a-5p	17.2	0.02	21.7	0.1	24.0	0.9	14.5	0.9
200a-3p	16.1	0.5	17.0	0.1	16.8	0.3	12.5	0.6

Table 8.6: Ct value of miRNAs from IMF EVs and HM EVs.

Normalised Ct value (mean of 3 experiments) $\pm$ SEM for each miRNA in HM EVs, IMF P (powder) EVs, IMF L (liquid) EVs and the control.

Accordingly, we evaluated the fold change reported in Log2x, based on the $2^{-\Delta\Delta Ct}$ method of the selected miRNAs (Fig. 8.4). Therefore, similar results were observed for all the miRNAs. Specifically, the most relevant was miR-148a-3p, where we detected a 3-fold and 10-fold lower in IMF EVs powder and liquid, respectively, in comparison with HM EVs. However, a significant downregulation of miR-182-5p, miR-30b-5p and miR-200a-3p, was estimated in IMF EVs samples compared to HM EVs samples. Instead, for miR-151a-3p we didn't observe any significant differences, however in IMF EVs powder and liquid samples it was less enriched in respect of HM EVs.

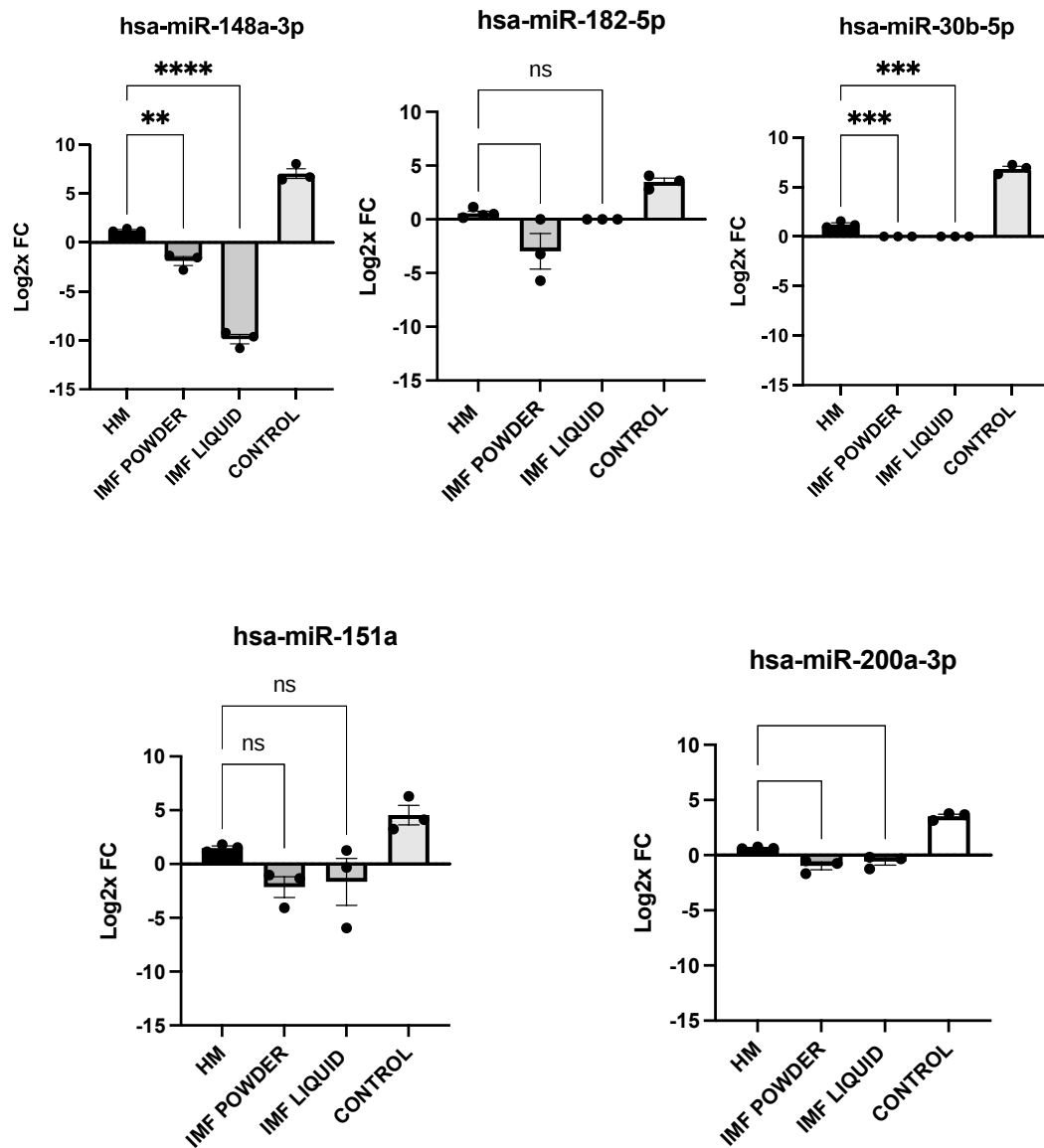


Figure 8.4: Fold change analysis of selected miRNAs in HM EVs and IMF EVs.

Relative expression comparison of miRNAs in HM EVs and IMF EVs selected in this study. Data were reported in Log2x following the $2^{\Delta\Delta Ct}$ method. Each graph is a representation of three replicates. One-way ANOVA was performed to obtain statistical difference where significance *p value was assigned. * $p < 0.05$; * $p < 0.01$; ** $p < 0.001$; *** $p < 0.001$; **** $p < 0.0001$.

8.6 Discussion

In Chapter 6 and 7, we explored the protein and metabolomic contents of EVs in HM and IMF. Both analyses showed different pattern and characteristics which defined differences between the two EVs samples. Although, among all EVs cargo miRNAs got the highest interest, a profile of miRNAs enclosed in IMF EVs compared to HM EVs is still missing. Therefore, in this section we wanted to estimate the amplification of multiple miRNAs in IMF EVs and HM EVs, which it had not been reported yet. We selected five miRNAs as the most expressed in milk EVs and involved in specific pathway often correlated to ameliorate gut maturity and development as well as immune defences in infants. For instances, miR-148a-3p, miR-30b-5p and miR-200a-3p were identified as in the top 10 miRNAs found in milk EVs (Zhou et al., 2011; Herwijnen et al., 2018). Firstly, we observed that miR-148a-3p was significantly less enriched in both IMF EVs samples with higher Ct value compared to HM EVs. Interestingly, a study where they used same methodology for RNA extraction but on whole (not EVs) HM and IMF showed similar results, where the miR-148a-3p expression was less than 1/500th in IMF in comparison with HM whole milk (Chiba et al., 2022). In this regard, one of the main targets of miR-148a-3p is the DNA methyltransferase 1 (DNMT1) and it is known that DNMT1 regulates FOXP3, where DNMT1 deficiency leads to higher expression of FOXP3 (Josefowicz et al., 2009). Thus, miR-148a-3p acting on DNMT1, is negatively modulating the recruitment of T-cells in case of inflammation (Melnik et al., 2016). Additionally, miR-148a-3p is involved in inhibiting colitis-associated tumorigenesis by interfering with STAT3 and NF- κ B, also known as inflammation-associated factors, and their pathways, thus preventing bowel diseases and less chance to encounter in cells inflammation (Zhu et al., 2017). Similar observation were reported by Guo et al (2022) where miR-148a-3p by regulating the expression of Tp53 in intestinal cells, reduced significantly the nuclear translocation of NF- κ B thus implementing the recovery in case of necrotising

enterocolitis. We also reported another well-known miRNA in milk EVs which is miR-182-5p. Likewise the previous one, miR-182-5p had significantly higher Ct value in IMF EVs powder, whereas no amplification was detected for IMF EVs liquid formula when compared to HM EVs samples. As for miR-148a-3p, miR-182-5p has also anti-inflammatory properties while modulating the TLR4/NF- κ B signalling pathway and it was demonstrated to be present in milk EVs from other species (*i.e.* cow, donkey)(Mecocci et al., 2021). Besides miR-182-5p enhances T cell activity by modulating the immune responses (Hatmal et al., 2022). We evaluated the presence of miR-30b-5p, but in this case the trend was slightly different, because we could not detect any amplification of it in both IMF EVs samples in respective to HM EVs and the control. Interestingly, miR-30b-5p induces immunosuppression which is the opposite effect exploit by miR-182-5p (Hatmal et al., 2022). One of the specific targets for miR-30b-5p is SIRTUN-1 (SIRT1). In fact, Xiao et al (2022) demonstrated that in cells treated with EVs, miR-30b-5p modulated the release of two interleukin IL-1 β and IL-6 which further reduced the expression of SIRTUIN-1. Consequently, in a study on bovine milk EVs derived miRNA, it was showed that miR-30b-5p regulates genes expression belonging to the RAS family and to do so can control the stress responses and cell survival (Colitti et al., 2019). For miR-151a-5p, differently from the other miRNAs, no significant differences in fold change and amplification were observed between IMF EVs and HM EVs. Although, it does not rule out if present in IMF or not. Indeed, Kakimoto et al (2020) performed a pilot study of miRNAs on gastric content in infants to address if they were breast-fed or formula-fed. They showed that miR-151a-3p was significantly elevated in breast-fed groups compared to formula-fed ones and because it is a key regulator of innate immunity and inflammation, potentially, infants can benefit from miR-151a-3p which was 2.2 less enriched in formula-fed babies (Kakimoto et al., 2020b). IMF EVs samples had higher Ct value for miR-200a-3p compared to HM EVs samples. Specifically, miR-200a-3p from bovine milk EVs, has a key role in alleviating caecal inflammation by downregulating the expression of the

proinflammatory chemokine, which was showed in mice with IBD syndrome (Wu et al., 2019). Altogether, we demonstrated that IMF EVs were less enriched in all important miRNAs in respective of HM EVs. Furthermore, the relevance of studying dietary miRNAs in EVs relies on the enormous potentiality of EVs to protect miRNAs from degradation and consistent loss during ingestion of milk. In fact Liao et al (2017) and López de las Hazas et al (2022) have extensively demonstrate that EVs are stable under extreme condition, such as gastric digestion and gut environment, but most importantly they showed that miRNAs like miR-182-5p; miR-30b-3p; miR-200a-3p and miR-148a-3p enclosed in milk EVs, are taken up successfully by intestinal cells and can elicit their original functions.

8.7 Conclusion

IMF EVs had lower amounts of miRNAs associated to immunomodulatory activity. The five miRNAs we analysed here were found at the lowest expressions (less number of copies) in IMF EVs compared to HM EVs. Hence, we hypothesised that the damage caused by the IMF manufacturing might be permanent for the cargo transported by EVs as well as for EVs concentration and structure.

9 Discussion

9.1 EVs from commercial milk and raw bovine milk

Milk of any kind and across species has unique composition which makes EVs separation and characterisation very challenging. As casein micelles are approximately 120 nm in diameter, both size and concentration overlapping with EVs (Fox & Brodkorb, 2008; O'Regan et al., 2009). Hence, identification of an optimal protocol using acid treatment and ultracentrifugation techniques to separate EVs from milk was the primary goal. Initially, we used commercially available sources of SM and IMF to approach a new methodology which combined the acid treatment of milk with HCl for casein removal and gradient ultracentrifugation to obtain enriched EVs samples. We demonstrated that isoelectric precipitation successfully precipitated most if not all the contaminants, but most importantly gradient ultracentrifugation increased EVs yield. Although, there are several studies done on EVs separated from commercial SM or bovine milk (Pieters et al., 2015; Zemleni et al., 2017; Benmoussa et al., 2017; Somiya et al., 2018; Samuel et al., 2021) very less is known about EVs in IMF. As described in Chapter 4, IMF production involves harsh industrial processes, we demonstrated that EVs may be lost and/or compromised compared to those in SM. In Chapter 2, our results indicate that irrespective of the treatment, the concentration of EVs present in IMF were substantially compromised compared to SM and the lower amount of EVs or EVs-like particle present in IMF do not carry EVs markers. The results also suggest that both AA and IP pre-treatments remove milk proteins efficiently, whereas the GUC technique is the optimum method for isolation of high yields of intact EVs without any effect on their morphology. Therefore, once our methodology for milk EVs separation was established, we wanted to apply the same technique on raw bovine milk. As described in Chapter 1, Section 1.2.2, the molecular composition of milk is influenced by several factors, such as lactation stage, breed, nutrition, and energy balance. In fact, COL and FM are

higher in fats, immunoglobulins and protein. Therefore, we wondered if EVs concentration and characteristics may change during the lactation period. Between COL, FM and MM samples analysed, COL represented the highest concentration of EVs which was slightly diminishing in FM and maintained steady in mature milk with no significant differences in the following months. The protein content and EVs marker, were enriched in COL compared with the rest of the samples. Moreover, we did not detect any potential contaminants (BTN1A1, MUC- 1 and MFG1) in any of our milk EVs samples. Furthermore, while bovine milk (either commercial or raw) is defined as the most available source of EVs (Zeng et al., 2019; Mukhopadhyaya et al., 2021), it is not the case for IMF. In Chapter 1, Section 1.2.3, we generally discussed IMF manufacturing. However, as IMF is the final product of a complex industrial process, we aimed to investigate at which step the loss of EVs might occur. Therefore, we analysed EVs separated from milk samples after ultra-high treatment (UHT), commonly used by the dairy industry for producing IMF from bovine milk. Interestingly, EVs were intact, quantified and EVs marker were readily detected only before heat treatment, instead in post UHT samples, EVs were in the range from compromised morphologically to absent EVs in the spray dried formula. Furthermore, their quantity and protein quality were at the lowest range compared to the starting material. Similarly Kleinjan et al (2021) reported no intact EVs in the UHT milk.

9.2 Comparison of IMF EVs and HM EVs

While UHT treatment is necessary to guarantee a safe product and a long shelf life, it is disturbing EVs structure and altering their recovery. In this project, we performed EVs comparison between IMF and “the gold standard” HM, essential for infants’ nutrition. IMF is the best alternative of HM, but EVs quantity and quality differs tremendously. We used commercial IMF of known brands which are easily available on the market and generally preferred by

mothers. Our results showed that IMF EVs samples, irrespective of the formulation (powder or liquid), were significantly less enriched in EVs and proteins compared to HM EVs samples, hence the original EVs structure was deeply affected. Moreover, EVs marker like TSG101 and CD63 had lower detection in IMF isolates compared to HM isolates. In addition, we performed multi-omics characterisation of EVs for in depth analysis of proteomic and metabolite content, followed by miRNA profiling in all IMF EVs and HM EVs samples. To note, this is the first study where EVs basic characterisation and high throughput analysis of IMF EVs and HM EVs are combined.

9.3 Multi-omics analysis of EVs content in HM and IMF

Firstly, we identified 660 proteins in HM EVs and only 18 in IMF EVs. Proteins identified by LC-MS/MS in milk EVs, such as MHC and butyrophilins, are known to have potential immunomodulatory properties (Admyre et al., 2007; Chutipongtanate et al., 2022), but were lacking in IMF EVs compared to HM EVs. Only 4 proteins (BTN1A1, FABP3, CD36 and SLC34A2) were in common between the two milk EVs proteome and most relevantly we observed by immunoblotting that no one of them was detected in IMF EVs compared to HM EVs. Accordingly, in the first proteomic study on HM EVs, evidenced also CD36 and other proteins such as polymeric-Ig receptor precursor and immunoglobulins in milk EVs (Admyre et al., 2007). However, they did not include IMF EVs. Furthermore, within the 11 proteins found in IMF EVs, most of them were associated to structural functions, whereas in HM EVs were associated to intestinal growth and development of the immune system (Chen et al., 2022; Ye et al., 2022). While many proteins found in the EVs proteome might be not exclusive of a specific EVs type, milk EVs contain unique milk proteins, such as CD36, BTN1A1 and FABP3 as reported previously (Samuel et al., 2017), and corroborate by our study. Subsequently, we explored the EVs metabolite content in both sources. Currently, EVs metabolomic analysis is emerging as new

type of 'omics', thus a specific methodology should be applied, especially for milk EVs. ¹H-NMR spectroscopy couple with 1D NOESY methodology was performed because it is much more sensitive. One of the most important metabolites found in our study were the HMOs which are absent in infant milk formula but a mix of those is generally added to mimic HM (Wiciński et al., 2020). However, we observed that IMF EVs were lacking in HMOs compared to HM EVs. Other compounds, like trehalose, L-alanine, glutamine, glutamate and some nucleotides were overall less enriched in IMF EVs compared to HM EVs. These metabolites are essential for the development of the gut microbiome during infancy maturation and function of the infant's immune system via lymphocytes maturation (Mantziari & Rautava, 2021). Similarly, FAAs exhibit immunomodulating capacities, indicating they contribution to neonatal immune development exclusively correlated to the protective effects of breastfeeding (van Sadelhoff et al., 2020). Additionally, nucleotides and choline provide important cellular and metabolism functions for the infant, including neurological and brain development (Hodgkinson et al., 2022). We extended our interest in miRNAs carried by milk EVs, which have gathered huge interest but a comparison of specific miRNAs between IMF EVs and HM EVs, was not reported yet. Five miRNAs (miR-148a-3p, miR-30b-5p, miR-182-5p, miR-200a-3p and miR-151a) were selected from the top ten list of miRNAs found in milk EVs. We reported a significant loss of the selected miRNAs in IMF EVs compared to HM EVs. For instances, miR-148a-3p was more than 10-fold lower in IMF EVs, while for miR-30b-5p, there was no amplification in both IMF EVs and similar results were obtained for the others. In literature miR-148a-3p is the most expressed miRNAs in milk EVs and its main role is to regulate the expression of DNA methyltransferase enzyme DNMT1 by modulating the expression and Forkhead box P3 (FOXP3) (Melnik et al., 2016).

10 Conclusion

Our journey to investigate milk EVs started with the optimisation of the EVs separation protocol from commercial milk which achieved enriched EVs samples in bovine milk, human milk and infant milk formula as well. Once we established our methodology we could perform basic characterisation of EVs but most relevantly, multi-omics analysis of EVs cargo. We aimed to gather new features and characteristics of milk EVs, essential for the dairy and agriculture industry as well as for consumers of milk/milk derivatives. Particularly, we reported that COL is an enriched source of EVs compared to FM and MM. Subsequently, by comparing IMF EVs and HM EVs, we explored the composition of IMF and HM and gather information for mothers who are either breastfeeding or not. It is important to be aware of how representative of human milk, IMF is, especially for infants in the first years of life. Moreover, milk EVs contribute for health benefit; a future approach to improve IMF consistently, could be towards the use of milk EVs as food supplement.

10.1 Future directions

Milk EVs are highly promising because milk is cheap, available, scalable and it is possible to get high EVs sample volumes. The essential role of milk in infant and adult nutrition along with the abundance of EVs makes milk EVs a particular candidate of interest with the potential therapeutic application. Furthermore we showed that enriched EVs samples can be obtained directly from the source just after the removal of major contaminants. Thus, future applications of milk EVs are focused on the possibility of engineering EVs as a drug delivery vehicles, particularly for their abundance, non-toxic nature and non-immunogenic behaviour (Mecocci et al., 2022). In fact, the efficacy of milk EVs loaded with drugs was proved to be much higher

against lung tumour xenografts in vivo compared with stand-alone drugs (Agrawal et al., 2017). Additionally, milk EVs were applied for treating osteoarthritis (OA), which showed a significant decrease in inflammation and cartilage consumption when human chondrocytes were co-cultured with milk EVs by suggesting a promising treatment for OA patients (Pieters et al., 2022). Similarly Yu et al (2023) used milk EVs engineered with icariin (ICA) and they observed that the proliferation and differentiation of osteoblasts was driven better by milk EVs-ICA than the free drug which provide a new research direction for the clinical treatment of bone defects. Alternatively, incorporating specific nucleic acid and/or protein cargos in milk EVs is another area of application of EVs in therapeutics (EL Andaloussi et al., 2013). Generally, a new-born mammal receives all its energy, essential nutrients needed for the proper growth and development from milk. Thus the transfer of immunity from mother to the child starts in-utero and breast milk continues the process, thereby helping in the development of the infant intestinal mucosa, microbiota and immune defence, which makes this topic of high scientific and commercial interest (Zempleni et al., 2017; Le Doare et al., 2018). Hence, dairy-related products are also considered an important constituent of a balanced diet for all age groups (Fox 2001, Mukhopadhyaya and Sweeney 2016). Therefore, the addition of EVs as an ingredient to resemble the characteristics of HM could potentially overcome the challenges related to industrial processing . In conclusion, this work was intended to shed a light on newly discovered characteristics on milk EVs for their future application in vitro and in vivo study. In fact, their stability to transverse the gastrointestinal tract and survive to digestion has been proved by different groups (Benmoussa et al., 2016; Tong et al., 2020; Yun et al., 2020; Maghraby et al., 2021; Samuel et al., 2021). Therefore, their capacity to influence recipient cells by delivering efficiently bioactive molecules (*i.e.*, proteins, metabolites) and immunoregulatory miRNAs to the recipient cells make milk EVs a versatile and powerful tool.

11 APPENDIX I- Supplementary material

11.1 Chapter 2: An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve

a) Nanoparticle Tracking Analysis

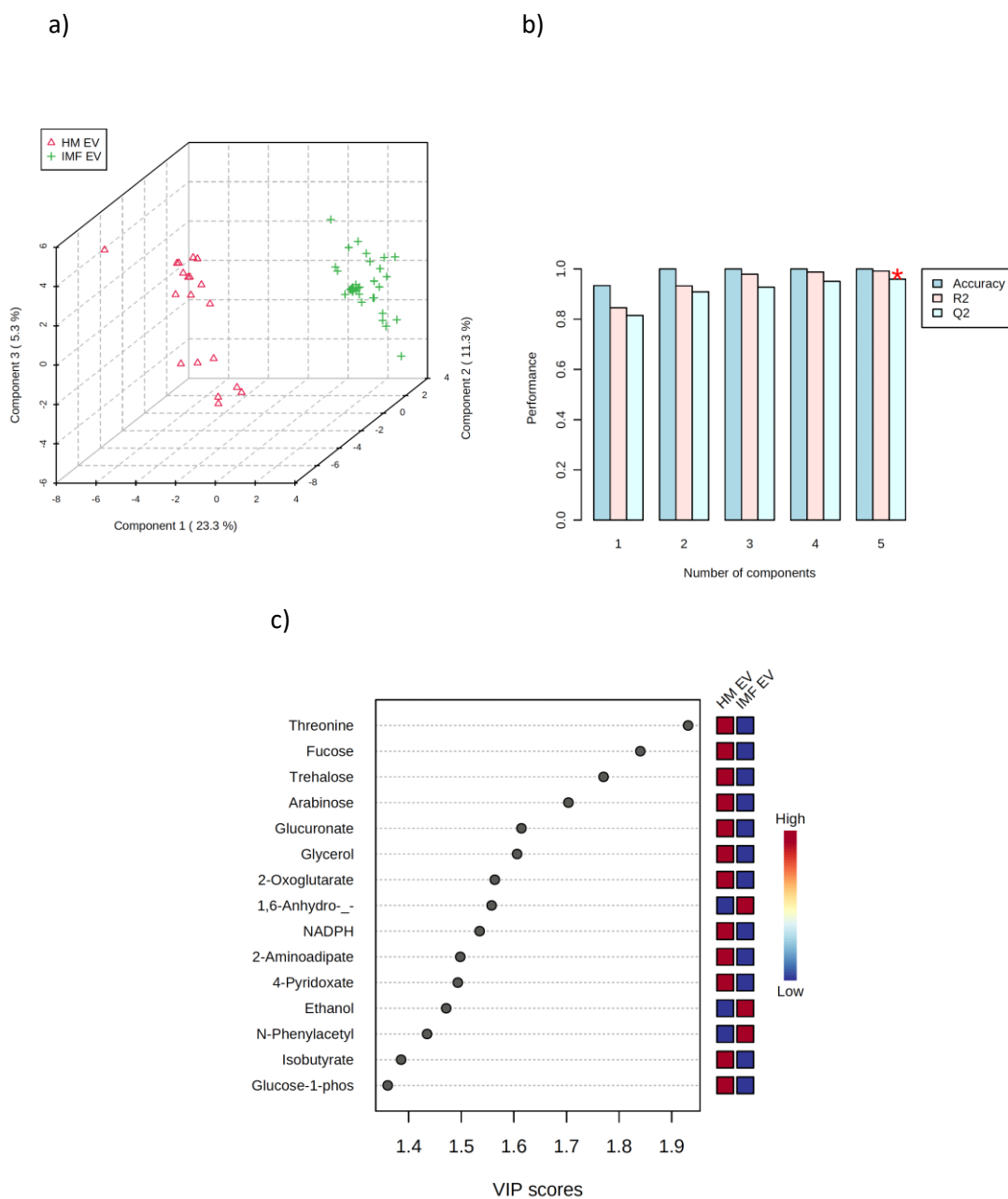
Shapiro-Wilk test					
W	0.9816	0.9762	0.8387	0.9832	0.8905
P value	0.7405	0.7039	0.2106	0.7517	0.3558
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
Shapiro-Wilk test					
W	0.9301	0.8614	0.9975	0.9984	0.9739
P value	0.4890	0.2714	0.9051	0.9231	0.6904
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes

b) Imaging Flow Cytometry analysis

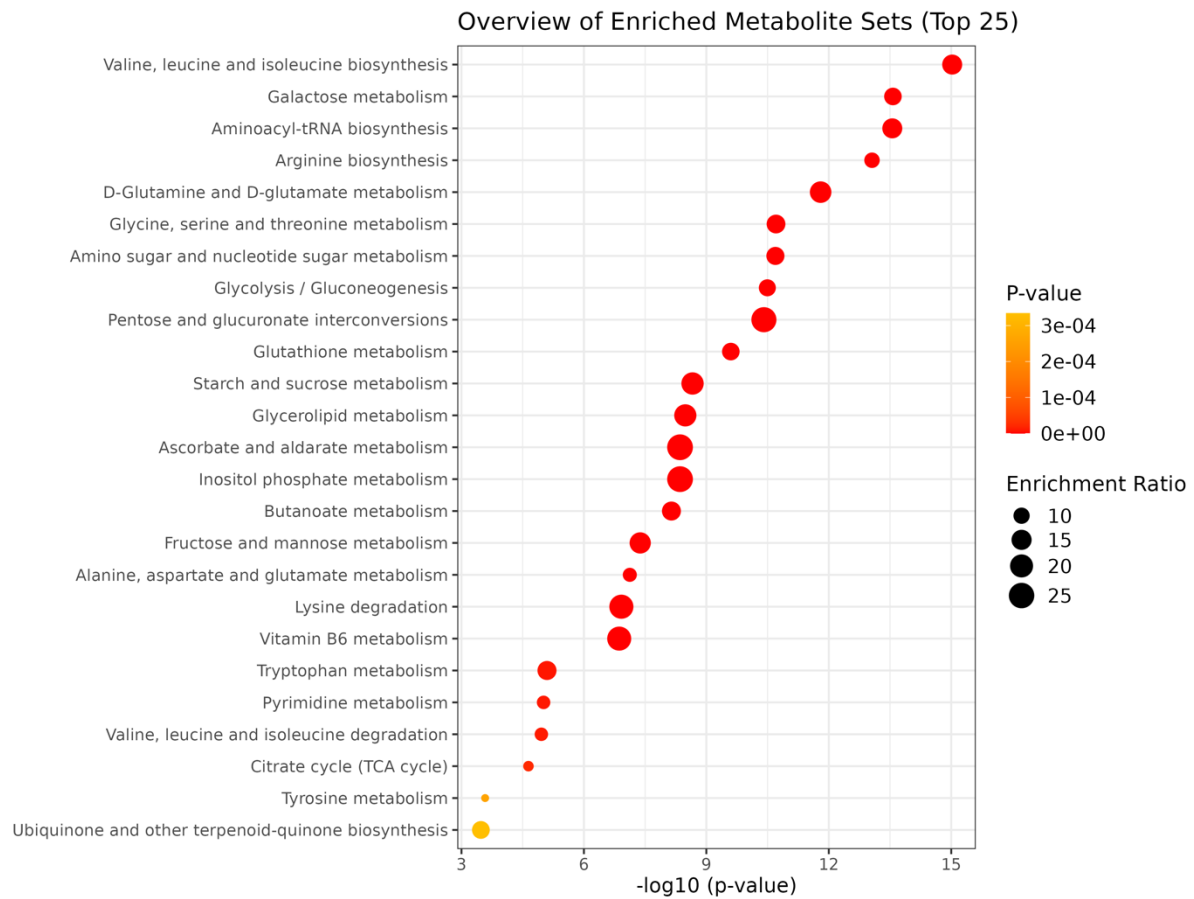
Shapiro-Wilk test					
W	0.9814	0.9277	0.9231	0.7788	0.8855
P value	0.7390	0.4802	0.4634	0.0647	0.3408
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes

Supplementary Figure A.1: Shapiro-Wilk test for normal distribution, showing as examples the outcomes for a) Shapiro-Wilk test of NTA data analysis, and b) Shapiro-Wilk test of flow cytometry data analysis.

11.2 Chapter 7: Metabolomic characterisation of EVs from HM and IMF: identifying similarities and differences using high resolution ^1H -NMR



Supplementary Figure A.2: Multivariate analysis of HM EVs and IMF EVs metabolome. a) 3D scores plot between the selected PCs. The explained variances are shown in brackets. b) PLS-DA classification using different number of components. The red star indicates the best classifier ($Q^2 > 0.4$; $R^2 > 0.9$). c) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

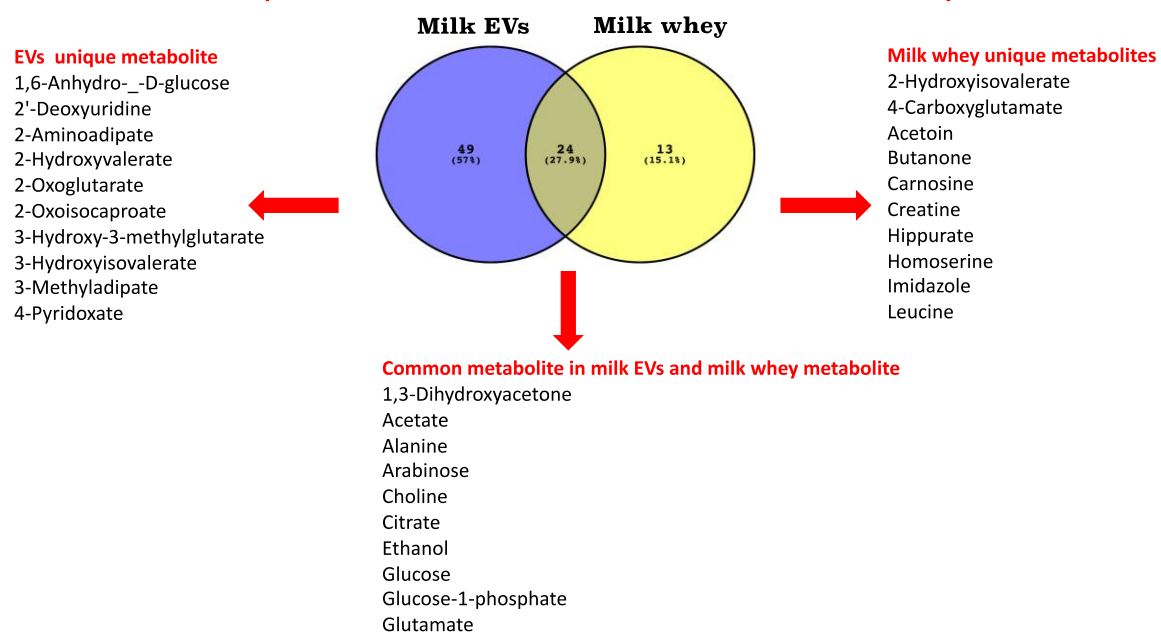


Supplementary Figure A.3. Overview of enriched metabolite sets in HM EVs and IMF EVs. The top 25 metabolites significantly concentrated. Each node represents a metabolite set with its color based on its p-value, and its size based on number of hits matched.

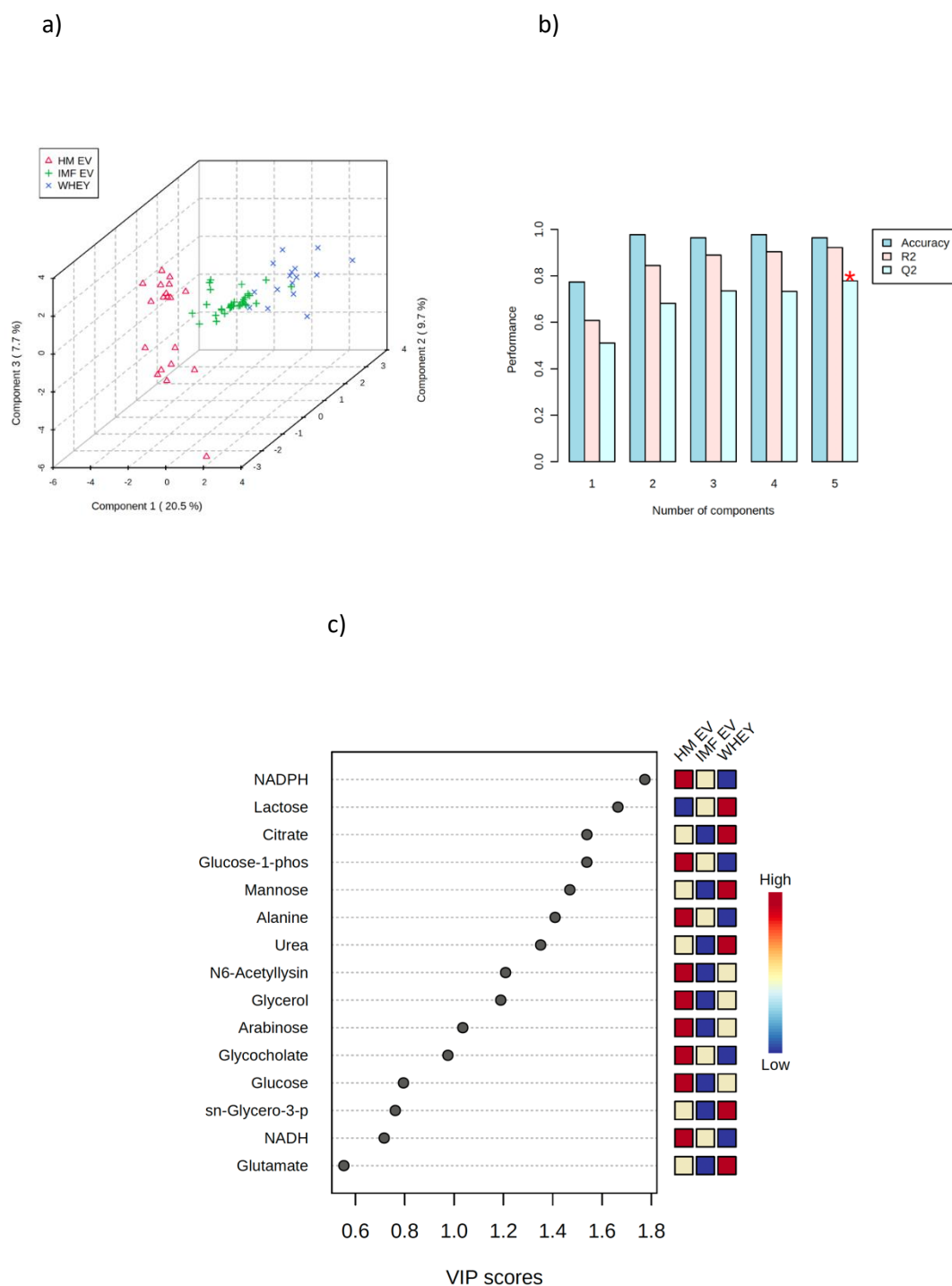
Sugars and HMOs	Amino acids and derivatives	Fatty acids and derivatives	Energy metabolites
Fucose	Isobutyrate	Acetate	2-Oxoglutarate
Glucose	Alanine	Choline	Acetate
Glucose-1-phosphate	Glutamate	O-phosphocholine	Cis-aconitate
Lactose	N-acetylglutamate	sn-Glycero-3-phosphocholine	Creatinine
Lactulose	N-6-acetyllysine	2-hydroxyisovalerate	Fumarate
Galactose	Isoleucine	3-hydroxyisovalerate	Pyruvate
	Threonine		Succinate
	Urea		
	Tiglylglycine		
	Valine		

Supplementary Table A.1: Classification of metabolites in HM EVs and IMF EVs. Enriched metabolites identified in HM EVs and IMF EVs categorised based on their characteristics.

Top 10 identified metabolites between milk EVs and milk whey



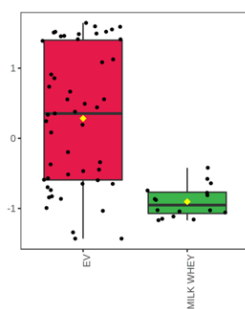
Supplementary Figure A.4: List of top 10 metabolites identified in milk EVs and milk whey. Venn diagram of milk EVs and milk whey unique metabolites. Among these 24 are shared and the top 10 are reported for each circle, representing milk EV unique metabolites (49; violet circle) and milk whey unique metabolites (13; yellow circle).



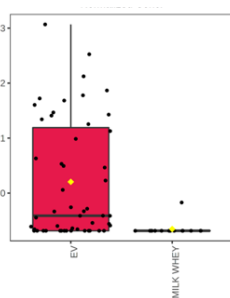
Supplementary Figure A.5: Multivariate analysis of milk EVs and milk whey metabolome. a) 3D scores plot between the selected PCs in milk EVs and milk whey. The explained variances are shown in brackets. b) PLS-DA classification using different number of components. The red star indicates the best classifier ($Q^2 > 0.4$; $R^2 > 0.7$). c) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

a)

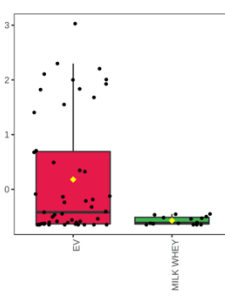
Glycerol



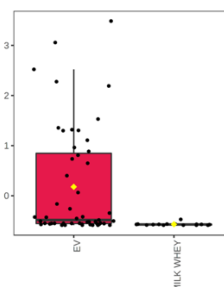
Alanine



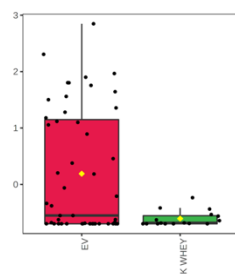
N-Acetyllysine



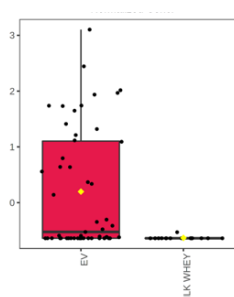
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Glycocholate

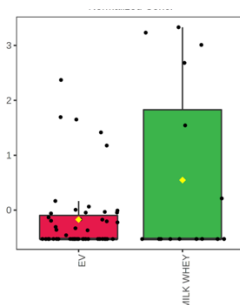


NAPDH

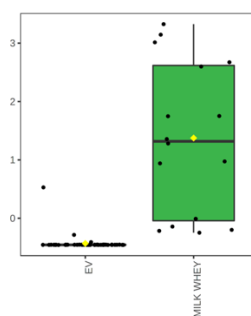


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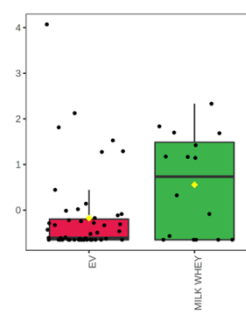
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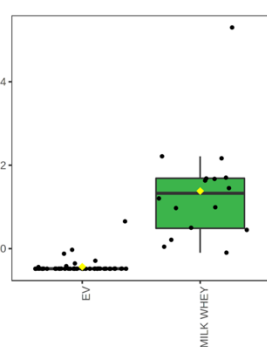
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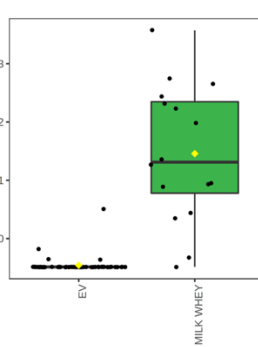
Glutamate



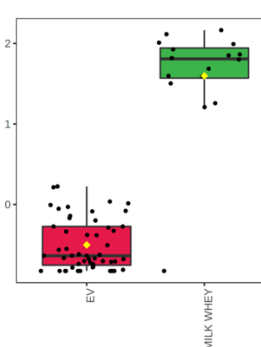
Sn-Glycero-3-phosphocholine



Mannose

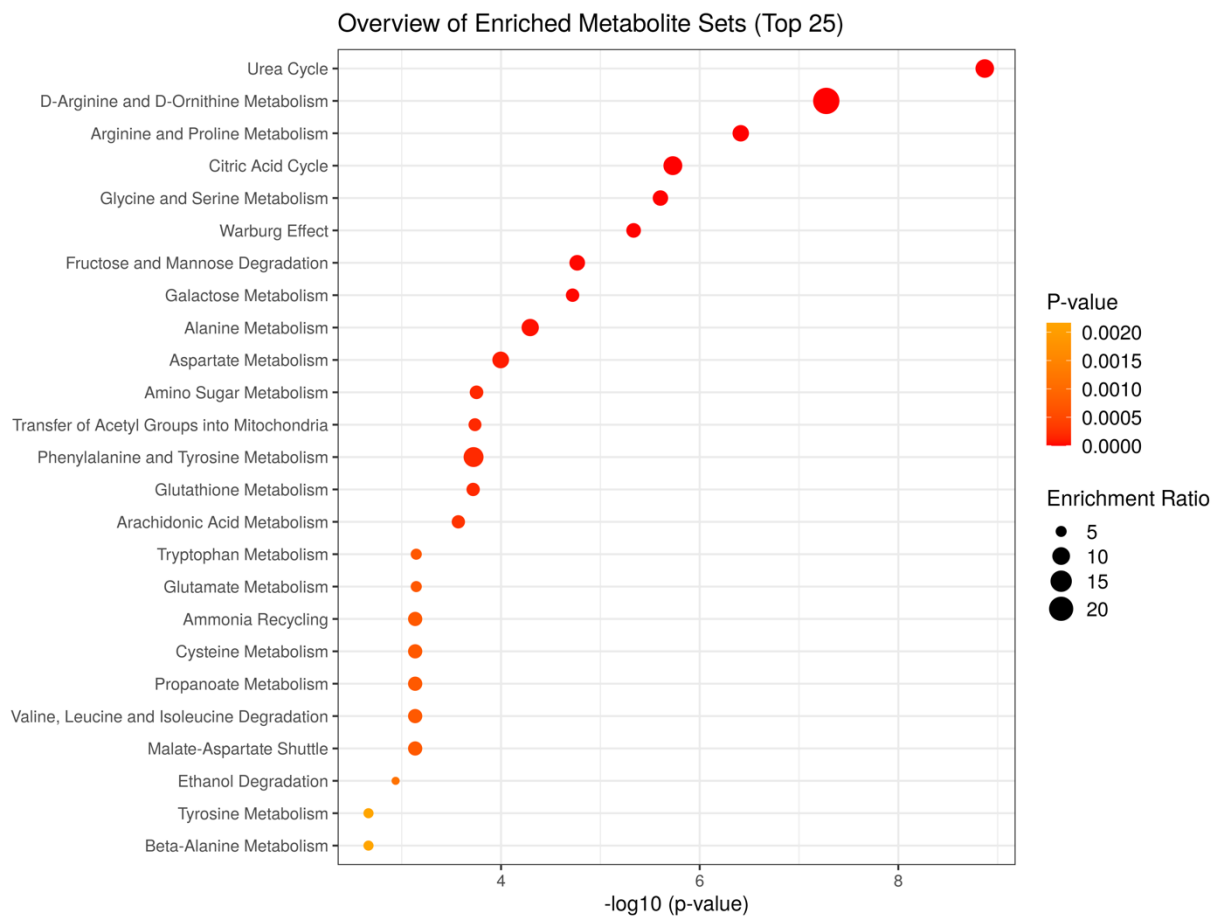


Lactose



Supplementary Figure A.6: Comparison of metabolites enriched in milk EVs and milk whey

a) Relative abundance of metabolites enriched in milk EVs compared to milk whey. b) Relative abundance of metabolites enriched in milk whey compared to the milk EVs fraction. For both analysis, normalisation was performed by auto-scaling and analysed using the t-test. The normalised concentration was reported for all metabolites and the distribution of the samples was represented by a boxplot which was generated using interquantile range, while each sample was reported using black dots. RED= MILK EVs; GREEN= MILK WHEY.



Supplementary Figure A.7: Overview of enriched metabolite sets in milk EVs and milk whey. The top 25 metabolites significantly concentrated. Each node represents a metabolite set with its color based on its p-value, and its size based on number of hits matched.

12 Appendix II- Publications, oral presentations, and other contribution

Full Peer-Reviewed Publications:

Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk. Anindya Mukhopadhyaya, **Jessie Santoro**, Barry Moran, Zivile Useckaite, Lorraine O'Driscoll. Food Chemistry Volume 353, 15 August 2021, 129309 <https://doi.org/10.1016/j.foodchem.2021.129309>

Extracellular vesicle separation from milk and infant milk formula using acid precipitation and ultracentrifugation", STAR PROTOCOL Anindya Mukhopadhyaya, **Jessie Santoro**, and Lorraine O'Driscoll

"An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve", Food Chemistry **Jessie Santoro**, Anindya Mukhopadhyaya, Charlotte Oliver, Andre Brodkorb, Linda Giblin and Lorraine O'Driscoll Food Chemistry Volume 401 1 February 2022, 134029 <https://doi.org/10.1016/j.foodchem.2022.134029>

Conferences:

49th Annual Food Science and Technology Conference, Virtual Meeting: "*Extracellular vesicles in skim milk and infant milk formula: a separation and characterisation comparison*" presented by **Jessie Santoro**

RAMI ,Virtual Meeting: "*Separating and comparing characteristics of the extracellular vesicles from human milk and it's best available commercial alternative, Infant Milk Formula*" presented by **Jessie Santoro**

TRAIN EV Virtual Conference Oral Presentation: "*Separation and Characterisation Comparison of Milk Extracellular Vesicles (MilkEVs) from Bovine Colostrum, First Milk and Mature Milk Throughout The Lactation Period*" presented by **Jessie Santoro**

Published abstract:

"Optimising methods for separation and characterisation of extracellular vesicles from skim milk and infant milk formula" , JEV Authors : Anindya Mukhopadhyaya, **Jessie Santoro**, Lorraine O'Driscoll. ISEV2020 Abstract Book , Journal of Extracellular Vesicles, 2021 (PS08.02)

"Characterisation of Milk Extracellular Vesicles separated from Bovine Colostrum, First Milk and Mature Milk over the Lactation Curve" **Jessie Santoro**, Anindya Mukhopadhyaya, Charlotte Oliver, Andre Brodkorb, Linda Giblin and Lorraine O'Driscoll. ISEV2021 Abstract Book , Journal of Extracellular Vesicles, 2021 (PS11.02).

Poster presentation:

All Ireland School of Pharmacy Conference Poster presentation : *“Investigating Extracellular Vesicles: how representative of milk is infant milk formula”* Authors : **Jessie Santoro**, Anindya Mukhopadhyaya, Lorraine O’Driscoll

Extracellular vesicles: From Biology to Biomedical Applications Poster presentation: *“Exploring Milk Extracellular Vesicles (EVs): Isolating EVs using different pre-treatment and isolation method ”* Authors: **Jessie Santoro**, Anindya Mukhopadhyaya, Lorraine O’Driscoll

ISEV, Virtual Annual Meeting Poster presentation: *“Optimising methods for separation and characterisation of extracellular vesicles from skim milk and infant milk formula”* Authors: Anindya Mukhopadhyaya, **Jessie Santoro**, Lorraine O’Driscoll

ISEV, Virtual Annual Meeting Poster presentation: *“Characterisation of Milk Extracellular Vesicles separated from Bovine Colostrum, First Milk and Mature Milk over the Lactation Curve”* Authors: **Jessie Santoro**, Anindya Mukhopadhyaya, Charlotte Oliver, Andre Brodkorb, Linda Giblin and Lorraine O’Driscoll

Biennial Congress International Society for Research on Human Milk and Lactation (ISRHML), Virtual Meeting Poster presentation : *“Characterisation of the extracellular vesicles from human milk and its best commercial alternative, Infant Milk Formula”* Authors : **Jessie Santoro**, Anindya Mukhopadhyaya, Lorraine O’Driscoll

Other contributions:

RAMI Student Research Award, Virtual Meeting Abstract submitted: *“Extracellular Vesicle Separation and Characterisation from Bovine Colostrum, First Milk and Mature Milk throughout the Lactation Period ”* Authors: Jessie Santoro, Anindya Mukhopadhyaya, Charlotte Oliver, Andre Brodkorb, Linda Giblin and Lorraine O’Driscoll

All Ireland School of Pharmacy Conference Abstract submission : *“Characterisation of milk EV separated from skim milk and infant milk formula”* Authors : Jessie Santoro, Anindya Mukhopadhyaya, Lorraine O’Driscoll

18th Annual IMGC VIRTUAL Symposium on MILK Science and Health Abstract submitted: *“Milk Extracellular Vesicles (Milk EVs) in bovine colostrum, first and mature milk: a characterisation study throughout the lactation curve ”* Authors : Jessie Santoro, Anindya Mukhopadhyaya, Lorraine O’Driscoll

EMBO Practical Course

Extracellular vesicles: from biology to biomedical applications. EMBL Heidelberg, Germany 2nd-7th June 2019

13 Appendix III – Research published articles

“Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk”

Anindya Mukhopadhyaya ^a, Jessie Santoro ^a, Barry Moran ^b, Zivile Useckaite ^a, Lorraine O'Driscoll ^a

^aSchool of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute, Trinity College Dublin and Trinity St. James's Cancer Institute, Dublin 2, Ireland

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<https://doi.org/10.1016/j.foodchem.2021.129309>Get rights and content

Abstract

Many infants are fed infant milk formula (IMF). However, IMF production from skim milk (SM) involves harsh treatment. So, we hypothesised that the quantity and/or quality of extracellular vesicles (EVs) in IMF may be reduced. Thus, firstly, we aimed to optimise separation of EVs from IMF and SM and, secondly, we aimed to compare the EV isolates from these two sources. Prior to EV isolation, abundant casein micelles of similar sizes to EVs were removed by treating milk samples with either acetic acid or hydrochloric acid. Samples progressed to differential ultracentrifugation (DUC) or gradient ultracentrifugation (GUC). EV characterisation included BCA, SDS-PAGE, nanoparticle tracking (NTA), electron microscopy (TEM), immunoblotting, and imaging flow cytometry (IFCM). Reduced EV concentrations were found in IMF. SM-derived EVs were intact, while IMF contained disrupted EV-like structures. EV biomarkers were more abundant with isolates from SM, indicating EV proteins in IMF are compromised. Altogether, a suitable method combining acid pre-treatment with GUC for EV separation from milk products was developed. EVs appear to be substantially compromised in IMF compared to SM.

Keywords

Extracellular vesicles (EVs); Milk; Infant milk formula (IMF); Pre-treatment; Developed methodologies

Introduction

Bovine milk is considered as a major source of nutrition for humans and consumed, across the globe, by all age groups. The proposed biological function of extracellular vesicles (EVs) in milk, including their role in cell growth and immune system modulation, is gathering increasing interest (Benmoussa & Provost, 2019). Infant milk formula (IMF), typically made from bovine skim milk (SM), is frequently used to fulfil the nutritional requirement of infants and toddlers. However, although the industrial processing stages of IMF manufacture from SM involves extensive thermal treatments steps -including sterilisation, evaporation and spray-drying that can involve temperatures of up to 280 °C (Burke et al., 2018, Gathercole et al., 2017)- the influences of these harsh conditions on EVs within IMF has not been well investigated. This may be, at least in part, due to the fact that milk and IMF are very complex fluids and a suitable method that is in keeping with MISEV2018 guidelines for separating and characterising EVs from both has not yet been reported. However, as the EU Commission Directive (2006/141/EC) specifies that IMF composition must be of sufficient biological quality, IMF and dairy ingredient industries need to know how representative IMF is of SM. They need to know whether EVs are present and maintained as in SM or, alternatively, if efforts would be needed to retain/supplement EVs. Simply put, industry -and consumers- need to know what's in their IMF product. Substantial efforts have been invested in analysing EVs from bovine milk (Arntz et al., 2015, Hata et al., 2010, Munagala et al., 2016, Reinhardt et al., 2013, Samuël et al., 2017, Somiya et al., 2018, Vaswani et al., 2017, Wolf et al., 2015, Yamada et al., 2012) and human milk (Admyre et al., 2007, Lasserre et al., 2011; van Herwijnen et al., 2018, Zonneveld et al., 2014). The removal of some "debris", including casein, before proceeding to EV separation from milk has been attempted with differential centrifugation, filtration, or treatment with EDTA or acids (Jensen et al., 2012). However, although extremely high concentrations *i.e.* 10^{14} – 10^{16} /mL of casein exist in milk (Fox, 2001, O'Rega, 2009) which -because of their size (approx. 150 nm), fall well within the size range of EVs- a standardised pre-analytical protocol for casein micelles removal prior to EV analysis is lacking. Furthermore, attempts to remove casein micelles from IMF prior to subsequent EV analysis have yet to be reported. Based on a survey in 2016, differential ultracentrifugation (DUC) is the most commonly used EV separation technique, with alternative techniques such as density gradient ultracentrifugation (GUC), size exclusion chromatography, and precipitation techniques used to a varying degree (Gardiner et al., 2016). Following a report by Admyre et al. (2007) on the separation of EVs from breast milk using DUC, a number of subsequent studies used DUC-based approaches (Izumiet al.,

2012, Kusuma et al., 2016, Manca et al., 2018, Reinhardt et al., 2012; Sun et al., 2013). However, as reviewed by Witwer et al. (2013), EV samples separated by DUC methods are often quite impure as DUC does not take into consideration the density of EVs. GUC, especially the ‘bottom-up’ approach, was proposed to much more efficiently separate EVs from proteins and other such contents that have similar densities to EVs. Some subsequent milk EV studies used GUC-based approaches as their method of EV separation (Benmoussa et al., 2016; Sun et al., 2015; van Herwijnen et al., 2016, Yamauchi et al., 2018). Of course, every research method has its pros and cons and so, ideally, such methods should be run in parallel on the same samples and critically compared. This is something that we addressed in this study where our main hypothesis was that industrial processing of SM to IMF leads to loss of EVs’ quantity and/or quality. We also proposed that using ultracentrifugation alone may not be adequate to remove most casein micelles from either milk or IMF and so a pre-treatment would be needed to separate the most enriched EVs possible from these sources. So here, after investigating options of pre-treatment (acetic acid or isoelectric precipitation) to remove casein micelles - that would otherwise confuse EV analysis- from SM and IMF, we compared SM and IMF EVs that were separated by GUC or by DUC. The evaluated pre-treatments, EV separation techniques, and characterisation methods used to help test our hypothesis are summarised in Fig. 1.

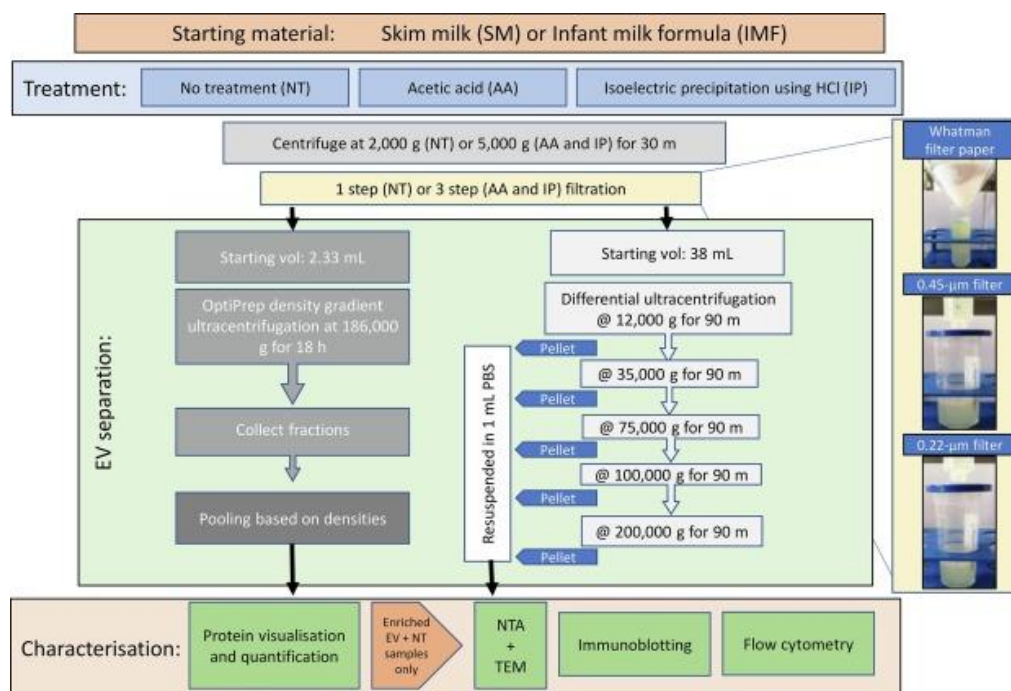


Fig. 1. Overview of methodology used. Methodology used in EV separation from skim milk (SM) and infant milk formula (IMF), including the starting materials used, treatments performed, ultracentrifugation techniques used and characterisation of the samples.

Materials and methods

All relevant information was submitted to EV-TRACK knowledgebase (Van Deun et al., 2017). The EV-TRACK ID: **EV190096** was assigned and the score achieved was **100%**.

Milk samples

Bovine skim milk ($n = 3$ independent batches) and IMF, prepared as recommended by the manufacturer, ($n = 3$ independent batches) were compared. Samples were commercial samples purchased from local vendors, as these are the sources that would be available to consumers to provide to infants/children. The preparation of IMF from its powder form was exactly as recommended by its manufacturer, in order to replicate the product that would be prepared to feed to infants. Specifically, 4.4 g of IMF powder was mixed with 30 mL of water that had been freshly boiled and then allowed to cool naturally. Crucially, a fresh batch of IMF was prepared for each biological repeat experiment. When prepared following the instruction on the container, the IMF was described by its manufacturer as a 'nutritionally complete' breast milk substitute with 0.7 mg per 100 mL feed iron content. Each independent batch of SM and IMF was divided into 3 equal aliquots. One was untreated (NT), and the others were pre-treated with either acetic acid (AA) or with HCl for isoelectric precipitation (IP) in an effort to remove abundant casein micelles and from the resultant whey, 38 mL was used for differential ultracentrifugation and 2.33 mL for gradient ultracentrifugation. All subsequent analyses detailed in the study were performed a minimum of 3 times.

Filtration and treatment for casein micelles removal

For the untreated samples, 50 mL of SM or IMF were centrifuged at 2,000 g for 20 min at 4 °C. The supernatant was carefully collected and the pellet containing any remaining fat globules or debris was discarded. This supernatant was filtered using Whatman™ Grade 1 filter paper (diameter 150 mm) (GE Healthcare Life Sciences, Ireland). The filtrate obtained from untreated SM was designated as SM_NT and the filtrate obtained from untreated IMF was designated as IMF_NT. The AA-treatment of SM and IMF (50 mL in both cases) was performed using a modification of a method previously reported for defatted bovine milk (Somiya et al., 2018). The modifications were that 6 N acetic acid was used (equivalent to the HCl) and additional filtration and ultracentrifugation steps were introduced in an effort to remove all casein protein

aggregates and casein micelles, which reportedly was not achieved in the earlier study (Somiya et al., 2018). In brief, 6 N acetic acid (Sigma-Aldrich, Ireland) was used to adjust the SM or IMF to pH 4.6 and further incubated for 10 min at RT and centrifuged at 5,000 g for 30 min at 4 °C. Furthermore, instead of performing only a 0.22- μ m filtration and ultracentrifugation of the resulting whey at 210,000 g, a 3-step filtration was performed prior to subsequent ultracentrifugation steps. Specifically, the supernatant was passed through a Whatman™ Grade 1 filter paper (diameter 150 mm) (GE Healthcare Life Sciences, Ireland), followed by 0.45- μ m membrane filtration and then 0.22- μ m membrane filtration (Thermo Fisher Scientific Ltd, Waltham, MA, US). The filtrate from SM was designated as SM_AA and that from IMF was designated as IMF_AA. Isoelectric precipitation (IP) was performed using a modification of a method previously reported by Yamauchi et al. (2018) for raw bovine milk. In brief, here the pH of SM or IMF were adjusted to pH 4.6 using 6 N HCl (Sigma-Aldrich, Ireland) to remove caseins. The pH adjusted SM or IMF was placed on ice for 10 min followed by centrifugation at 5,000 g for 30 min at 4 °C. Resulting whey was subject to the 3-step filtration procedure. The filtrate from SM was designated as SM_IP and that from IMF was designated as IMF_IP. While the protocol used by Yamauchi et al. (2018) then proceeded to a sucrose gradient we, instead, used OptiPrep™ gradient and differential ultracentrifugation techniques, detailed in the following sections.

Of note, neither Somiya et al. (2018) nor Yamauchi et al. (2018) analysed IMF.

Separation of EVs by ultracentrifugation (UC) using either gradient UC or differential UC

Gradient ultracentrifugation (GUC) was performed using iodixanol solution OptiPrep™ (60% (w/v)) (Sigma Aldrich, Ireland; Cat. #D1556) following the bottom-up technique (Kowal et al., 2016). Briefly, a 40% (w/v) bottom layer was made by diluting 60% OptiPrep solution with each of the 6 sample types (4.66 mL OptiPrep + 2.33 mL sample = 7 mL). Further layering was done by a discontinuous gradient of 30% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol, made by diluting a stock solution of OptiPrep (60% (w/v)) with sterile PBS (Sigma Aldrich, Ireland). The gradient was formed by adding 3 mL of the iodixanol solutions to a 17 mL polypropylene tube (Beckman Coulter, Brea, CA, US). Ultracentrifugation was performed using a SW Type 32.1 Ti rotor in Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 186,000 g for 18 h at 4 °C. Post 18 h centrifugation, the supernatant was carefully pipetted out in 1 mL fractions. Their refractive indexes were recorded, and densities were calculated for all fractions. Based on previous studies (Colombo et al., 2014, Kowalec et al., 2016),

in this study fractions with densities of 1.05–1.19 g/mL were pooled for further analyses. Of note, as we aimed to isolate all EVs present in SM and IMF and considering that Kowal et al. (2016) observed positive signals for EV-specific proteins in fractions with densities of 1.08 g/mL, we chose 1.05–1.19 g/mL fractions. Post-pooling, the sample was washed in sterile PBS at 110,000 g for 90 min at 4 °C before resuspending the pellet in 1 mL of sterile PBS. Differential ultracentrifugation (DUC) separation of EVs from the untreated or treated SM and IMF samples was performed based on protocol published by Benmoussa et al. (2017) with addition, in this study, of a final step of ultracentrifugation at 200,000 g. A similar separation of EVs from urine (Gonzales, 2010, Musante et al., 2013) and milk (Somiya et al., 2018) at 200,000 g have been reported previously. In brief, samples were filled into 38 mL open-top polypropylene tubes and subjected to DUC using a SW Type 32 Ti swinging bucket rotor (Beckman Coulter, Brea, CA, US) in an Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA). Consecutive spins were at 12,000 g (12 K), 35,000 g (35 K), 75,000 g (75 K), 100,000 g (100 K) and 200,000 g (200 K) for 90 min each at 4 °C. After each centrifugation step, the resulting pellets (12 K, 35 K, 75 K, 100 K or 200 K) were collected, resuspended in 1 mL filtered PBS and stored in –80 °C for further analysis, while the supernatant was poured in a fresh tube and centrifuged at the next speed.

SDS-PAGE for protein visualisation

Effects on milk proteins of pre-treatment with AA or IP compared to NT, prior to EV separation by ultracentrifugation, was visualised on a 10% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, US). For this, 15 µg of protein samples were loaded on a 10% polyacrylamide gel, run at 120 V, stained with coomassie blue (Sigma Aldrich, Ireland) and visualised using a Chemidoc XRS system (Bio-Rad Laboratories, Hercules, CA, US).

Protein quantification

A bicinchoninic acid (BCA) reagent kit (Thermo Fisher Scientific, Waltham, MA, US; Cat. #10249133), with a bovine serum albumin (BSA) standard (0–800 µg/mL) (Sigma Aldrich, Ireland) curve, was used to determine total protein in samples.

Nanoparticle tracking analysis

Particle concentration and sizes were estimated using NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK). EV samples were automatically injected into the NTA system under constant

flow conditions (flow rate = 50) and videos of the particles in motion were recorded and analysed using NTA 3.1.54 software.

RNA isolation and quantification

Total RNA isolation from the EV isolates was performed using a previously published protocol (Pieters et al., 2015). Briefly, 1.0 mL TRI reagent (Invitrogen, Carlsbad, CA, US) was added with 200 μ L chloroform (Sigma Aldrich, Ireland) to the pelleted EV isolates and mixed vigorously for 15 s and incubated for 2 min at RT. The suspension was centrifuged at 12,000 g for 15 min at 4 °C and the clear supernatant was transferred to a fresh tube and mixed with 500 μ L isopropanol (Sigma Aldrich, Ireland) before storing at –80 °C overnight. Following overnight incubation, the mixture was centrifuged at 12,000 g for 30 min at 4 °C followed by two washes with 75% ethanol. The ethanol was removed by centrifugation at 12,000 g for 5 min at 4 °C and the pellet was air-dried at room temperature and resuspended in RNase-free water. RNA quantity was measured using the Qubit HS RNA Assay kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham, CA, US) on a Qubit fluorometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, CA, US). The Qubit HS RNA assay is described as highly selective for RNA and will not quantitate DNA, protein, or free nucleotides.

Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis following previously published protocol (Visnovitz et al., 2019). Specifically, 5 μ L of sample was suspended in 5 μ L of 0.22 μ m-filtered PBS and placed onto carbon coated grids (Ted-Pella B 300 M, Mason Technology Ltd., Ireland; Cat. #: 01813-F) for 10 min at RT before fixing with 4% glutaraldehyde (Sigma Aldrich, Ireland) and contrasting with 2% phosphotungstic acid (Sigma Aldrich). The grids were examined at 100 kV using a JEOL JEM-2100 TEM (JOEL, Peabody, USA), as we previously described (O'Brien et al., 2013).

Immunoblotting

Immunoblotting was performed as we previously described (O'Brien et al., 2013), except that 35 μ g of lysed EV protein was used here. For this, EVs were lysed using SDS lysis buffer (250 mM Tris-HCL, pH 7.4; 2.5% SDS), lysates (35 μ g) were resolved on 10% SDS gels (Bio-Rad) and the protein was transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, US; Cat. #1620177). Blots were blocked with 5% (w/v) BSA in PBS containing 0.1% Tween-20 and incubated overnight at 4 °C with primary antibodies to anti-human TSG101 (1:1000; Abcam,

Cambridge, UK; Cat. # ab30871), anti-human CD63 (1:500; Abcam, Cambridge, UK; Cat. #: ab68418) or anti-human Actinin 4 (1/1000, Abcam, Cambridge, UK; Cat. #: ab108198). Secondary antibodies used were anti-mouse (1/1000 in 5% BSA/PBS-T, Cell Signalling, Danvers, MA, US; Cat. #: 7076) or anti-rabbit (1/1000 in 5% BSA/PBS-T, Cell Signalling, Danvers, MA, US; Cat. #: 7-74). Cell lysate from the Hs578Ts(i)₈ human cell line was included in all gels as a positive control. SuperSignal West Femto Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Waltham, CA, US; Cat. # 11859290) was used for detection, imaging was performed using an automated Chemidoc exposure system (Bio-Rad Laboratories, Hercules, CA, US) and densitometric analysis was performed using ImageJ tool.

Imaging flow cytometry

The imaging flow cytometry (IFCM) analysis of EVs separated from SM and IMF was performed following a protocol previously published (Ricklefs et al., 2019). Briefly, EVs surface antigens were exposed to antibodies diluted in 0.22 µm-filtered PBS with 2% EV-depleted-FBS supplemented with protease inhibitor and phosphatase inhibitor (IFCM buffer). The specific antibodies used were anti-human CD63 conjugated with FITC (CD63-FITC; 1:150, Cat. #: 353006, Biolegend, San Diego, CA, US); anti-human CD9-PE (1:1500, Cat. #: 312106, Biolegend, San Diego, CA, US); anti-human CD81-PE-Cy7 (1:150, Cat. #: 349,512 Biolegend, San Diego, CA, US); anti-human HLADR-BV421 (1:600, Cat. #: 307636, Biolegend, San Diego, CA, US) and anti-human ADAM10-APC (1:150, Cat. #: 352706, Biolegend, San Diego, CA, US). The selection of the antibodies was made based on the presence of surface markers on the EVs. Anti-CD9, anti-CD63 and anti-CD81 antibodies were selected as EV specific tetraspanins present on their surface, along with HLADR, an MHC II immunoregulatory molecule present on EV surface. Additionally, ADAM10 was also selected as in a previous study, Kowal et al. (2016) reported the presence of ADAM10 in EVs. The EVs were incubated with the antibodies for 45 min at RT in the dark, and washed using a 300 kDa filter (Nanosep, Pall Biotech, Ireland; Cat. #: 516-8531), resuspended in 50 µL IFCM buffer and acquired within 2 h on the ImageStream X Mk II imaging flow cytometer (Amnis/ Luminex, Seattle, USA) at 60x magnification and low flow rate, as previously described (Ricklefs et al., 2019). EV-free IFCM buffer, unstained EVs, single-stained controls and fluorescence minus one (FMO) controls were run in parallel. Fluorescence was within detection linear range in the following channels: FITC was measured in channel 2 (B/YG_480–560 nm), PE in channel 3 (B/YG_560–595 nm filter), PE-Cy7 in channel 6 (B/YG_745–780 nm filter), BV421 in channel 7 (R/V_435–480 nm filter), APC in channel 11 (R/V_642–745 nm,

filter), Brightfield in channel 1 and 9 (B/YG_435-480 and R/V_560-595 nm filter respectively) and Side Scatter Channel (SSC) in channel 12 (R/V_745-780 nm filter). Data analysis was performed using IDEAS software v6.2 (Amnis/ Luminex, Seattle, USA). EVs were gated as SSC-low vs fluorescence, then as non-detectable brightfield (fluorescence vs Raw Max Pixel Brightfield channel), gated EVs were confirmed in the IDEAS Image Gallery.

Statistical analysis

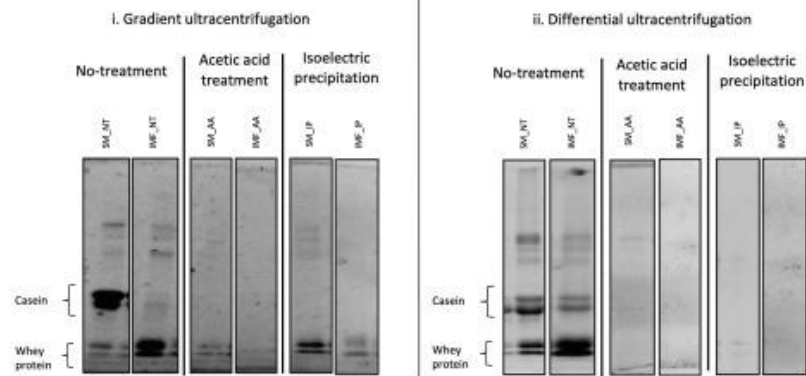
All data presented are in form of the means of ≥ 3 experiments $\pm$ SEM. Using Graph Pad Prism 8 software, unpaired two-tailed Student's *t*-test was performed when comparing two groups and one-way or two-way ANOVA was performed when comparing more than two groups. A value of $P < 0.05$ was considered statistically significant.

Results

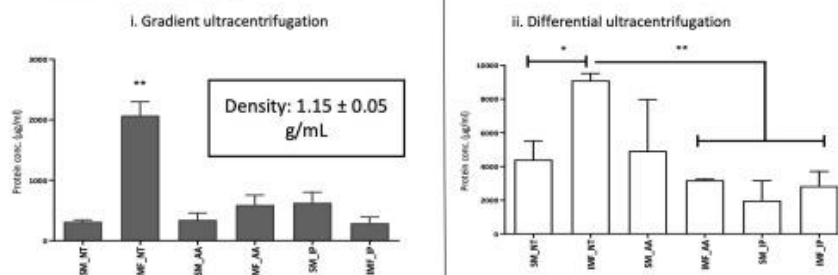
Analysis of milk protein removal by acid treatment and ultracentrifugation

Total protein separation by polyacrylamide gel showed that the major groups of milk proteins, including caseins and whey proteins, were present post-GUC at high levels in the SM_NT samples and to a lesser extent in IMF_NT samples (Fig. 2a i), while pre-treatment with AA or IP quite efficiently removed caseins. Post-DUC, the SM_NT and IMF_NT had strong bands representing caseins and whey proteins (Fig. 2a ii); while, again, AA and IP pre-treatment efficiently removed these proteins. While compared to NT, AA and IP have efficiently removed casein proteins, faint bands were still observed indicating seepage of contaminant proteins in the enriched EV fractions.

a. SDS-PAGE for protein visualisation



b. Protein concentration analysis



c. Transmission electron microscopy analysis

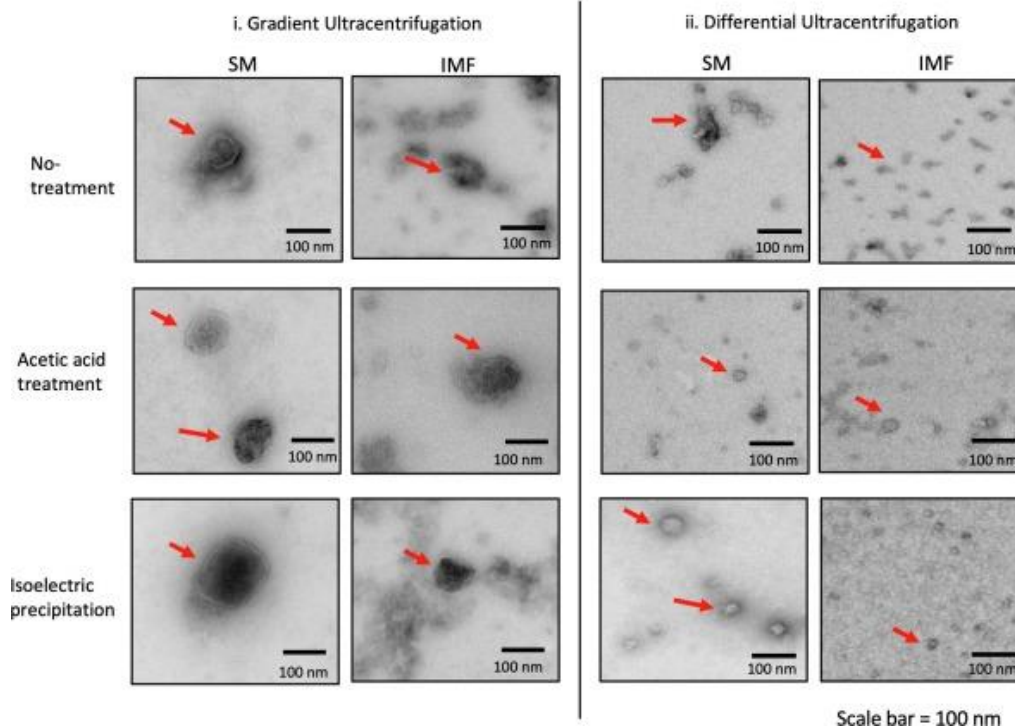


Fig. 2. Protein and particle characterisation of skim milk and IMF EVs a) Milk proteins were visualised on 10% acrylamide gels. Efforts to remove milk proteins were performed by acetic acid (AA) and isoelectric precipitation (IP) treatment and the resulting whey was used for either gradient ultracentrifugation (GUC) or differential ultracentrifugation (DUC). Untreated milk (NT) was used as a control to compare the

removal of milk proteins by AA or IP pre-treatment. b) Isolates from skim milk (SM) and infant milk formula (IMF) post i) gradient ultracentrifugation (GUC) and ii) differential ultracentrifugation (DUC) were analysed for protein concentrations using bicinchoninic acid (BCA). Protein concentration (in $\mu\text{g/mL}$) of the three repeats were analysed using one-way ANOVA test and presented as mean bars $\pm$ SEM, * indicates a significance value of $P < 0.05$, ** indicates a significance value of $P < 0.01$. c) Representative transmission electron microscope (TEM) images from i) gradient ultracentrifugation (GUC), and ii) differential ultracentrifugation (DUC) samples. Red arrows point towards vesicular structures in the sample. Scale bar = 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Due to the persistent presence of some casein even after AA or IP, the DUC generated 12 K, 35 K, 75 K and 100 K resuspended pellets or the crude EV samples were not used for further extensive analysis, whereas the 200 K samples were further characterised (representative SDS-PAGE blots on all samples are provided in Supplementary information, Fig. S1 a&b). It is also important to note that in the untreated SM (SM_NT) and IMF (IMF_NT) samples, post-GUC or after 200,000 g DUC step, casein and whey proteins were still present to varying extents and so were present during further characterisation steps as these samples serve as control samples for further characterisation steps.

Protein concentration analysis of isolates from untreated or AA- or IP- treated SM and IMF

The total protein concentrations of samples post-GUC and -DUC are presented in Fig. 2b. Considering the pooled fractions (1.05–1.19 g/mL), the total protein content of IMF_NT appeared to be significantly ($P < 0.01$) higher compared to all other treatment samples analysed (Fig. 2b i). The total protein contents on post-DUC samples are presented in Fig. 2b ii. The IMF_NT samples were associated with significantly higher total protein content compared to SM_NT samples ($P < 0.05$) and IMF_AA, SM_IP and IMF_IP samples ($P < 0.01$).

Milk EV concentration, size characterisation and RNA quantity

The sizes and concentrations of EVs separated from pre-treated or untreated SM and IMF, analysed by NTA are presented in Table 1. Considering size distribution, post-GUC particles from the SM samples were 156.13 ± 5.87 nm and from IMF samples were 159.90 ± 12.96 nm, while those post-DUC of SM_200K were 159.10 ± 5.27 nm and IMF_200K were 175.73 ± 8.44 nm. No significant differences were observed between these EV sizes. The representative size distribution graphs with mean and modal sizes (as observed with the NTA software) are provided in Supplementary information Table S1 a&b. Regarding the concentration of particles in each sample, significant differences were observed between SM and IMF samples. As presented

in Table 1a (i), post-GUC, the concentration of EVs in SM_NT samples was higher (7.10-fold; $P = 0.05$) compared with IMF_NT samples. Similarly, the EV concentration of SM_IP samples was significantly higher (6.71-fold; $P = 0.01$) when compared with IMF_IP samples. In turn, the concentration in SM_AA samples was substantially higher (4.93-fold) than in IMF_AA, although not significantly different ($P = 0.26$) as a result of quite large differences in biological repeat SM_AA values (*i.e.* a large error bar).

Table 1a. Yields of EVs separated from skim milk (SM) and infant milk formula (IMF) by gradient ultracentrifugation (GUC) or differential ultracentrifugation (DUC), as estimated NTA.

i) Gradient Ultracentrifugation					
Empty Cell	Skim milk		Infant milk formula		P value
Size (in nm)	Mean	Mode	Mean	Mode	
Non-treated (NT)	147.00 ± 8.39	113.60 ± 10.34	166.15 ± 2.75	140.45 ± 1.25	ns
Acetic acid treated (AA)	159.83 ± 5.55	135.46 ± 5.28	180.16 ± 16.77	133.53 ± 16.22	ns
Isoelectric precipitation (IP)	162.90 ± 13.51	141.03 ± 11.93	152.40 ± 45.10	109.50 ± 18.80	ns
Concentration (Particles/mL starting material)	Skim milk		Infant milk formula		
Non-treated (NT)	4.35 × 10 ¹⁰ ± 1.40 × 10 ¹⁰		6.13 × 10 ⁰⁹ ± 8.17 × 10 ⁰⁸		0.05
Acetic acid treated (AA)	1.39 × 10 ¹⁰ ± 8.25 × 10 ⁰⁹		2.82 × 10 ⁰⁹ ± 2.33 × 10 ⁰⁹		ns
Isoelectric precipitation (IP)	2.16 × 10 ¹⁰ ± 2.47 × 10 ⁰⁹		3.22 × 10 ⁰⁹ ± 1.45 × 10 ⁰⁹		0.01
ii) Differential Ultracentrifugation					
	Skim milk		Infant milk formula		P value

i) Gradient Ultracentrifugation					
Empty Cell	Skim milk		Infant milk formula		P value
Size (in nm)	Mean	Mode	Mean	Mode	
Non-treated (NT)	173.60 ± 29.20	123.40 ± 60.00	180.40 ± 7.50	152.50 ± 50.10	ns
Acetic acid treated (AA)	150.10 ± 16.40	146.90 ± 47.33	149.28 ± 18.37	137.30 ± 68.22	ns
Isoelectric precipitation (IP)	149.23 ± 23.50	130.81 ± 46.11	184.19 ± 15.63	117.10 ± 67.70	ns
Concentration (Particles/mL of starting material)	Skim milk		Infant milk formula		
Non-treated (NT)	$1.97 \times 10^{10} \pm 8.90 \times 10^{09}$		$4.65 \times 10^{07} \pm 2.50 \times 10^{07}$		0.04
Acetic acid treated (AA)	$2.78 \times 10^{09} \pm 1.00 \times 10^{09}$		$1.69 \times 10^{08} \pm 3.30 \times 10^{07}$		0.03
Isoelectric precipitation (IP)	$3.65 \times 10^{09} \pm 5.43 \times 10^{08}$		$4.66 \times 10^{08} \pm 2.02 \times 10^{08}$		0.01

ns = not significant.

Similarly, post-DUC -as presented in Table 1a (ii)- the EV/particle concentration in SM_NT_200K samples was significantly higher (423.66-fold; $P = 0.04$) than in IMF_NT_200K. Likewise, the EV/particle concentration in SM_AA_200K samples was 16.45-fold higher ($P = 0.03$) compared to that in IMF_AA_200K and the SM_IP_200K samples had 7.83-fold more particles ($P = 0.01$) when compared to the IMF_IP_200K samples.

Directly comparing yields of EVs/particles from SM and IMF, respectively, following GUC compared to DUC, as illustrated in Table 1b, the yields were always many folds higher with GUC compared to DUC. Within SM, the yields with GUC ranged from 2.2 to 5.91 folds higher than when compared to aliquots of the same samples separated by DUC. Similarly, with the IMF samples. Here the yields from IMF when using GUC ranged from 6.92 to 131.7 folds higher than when compared to aliquots of the same samples separated by DUC. In some, but not all cases, statistical significance was found. As can be observed from the SEM values, there were differences between biological repeat samples. This variability when grouped together would have contributed to statistical significance not being reached in all cases.

Table 1b. Comparison of concentration of particles/ mL (analysed by NTA) from SM and IMF by GUC *versus* DUC.

i) Skim milk					
Concentration of particles/mL of starting material	GUC	DUC	Fold Higher Yield with GUC vs. DUC	P value	
Non-treated (NT)	$4.35 \times 10^{10} \pm 1.40 \times 10^{10}$	$1.97 \times 10^{10} \pm 8.90 \times 10^9$	2.2	0.06	
Acetic acid treated (AA)	$1.39 \times 10^{10} \pm 8.25 \times 10^9$	$2.78 \times 10^9 \pm 1.00 \times 10^9$	5.02	ns	
Isoelectric precipitation (IP)	$2.16 \times 10^{10} \pm 2.47 \times 10^9$	$3.65 \times 10^9 \pm 5.43 \times 10^8$	5.91	0.01	
ii) IMF					
Concentration of particles/mL of starting material	GUC	DUC	Fold Higher Yield with GUC vs. DUC	P value	
Non-treated (NT)	$6.13 \times 10^9 \pm 8.17 \times 10^8$	$4.65 \times 10^7 \pm 2.50 \times 10^7$	131.7	0.01	
Acetic acid treated (AA)	$2.82 \times 10^9 \pm 2.33 \times 10^9$	$1.69 \times 10^8 \pm 3.30 \times 10^7$	16.7	ns	
Isoelectric precipitation (IP)	$3.22 \times 10^9 \pm 1.45 \times 10^9$	$4.66 \times 10^8 \pm 2.02 \times 10^8$	6.92	ns	

ns = not significant

As EVs typically carry RNA, but casein micelles do not, RNA was isolated from the SM and IMF isolated following GUC and DUC separation. The RNA concentration/mL of starting material obtained and RNA/particle post GUC and DUC are presented in Table 1c(i). Following GUC, the total RNA and quantity of RNA/particle was always greater with SM compared to IMF. However, statistically significant differences were not always observed, due to the relatively large difference between biological repeats. Doing the same comparison but post-DUC, results were less consistent. When directly comparing ng RNA/particle following DUC *versus* GUC Table 1c(ii)., with SM the amounts were typically great following GUC compared to DUC. However, the differences between repeat biological experiment contributed to no statistical significance been reached. In the case of IMF, the amounts of RNA per particle was significantly greater ($P = 0.01$) following GUC compared to DUC.

Table 1c. **(i).** Comparison of RNA (ng) total yield/mL and per particle separated from SM) *versus* IMF by (a) GUC and (b) DUC.

a) GUC - RNA yield							
Samples Analysed	Skim milk		Infant milk formula		P value		
	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i.	ii.	
Non-treated (NT)	93.00 ± 21.33	$2.14 \times 10^{-09} \pm 4.90 \times 10^{-10}$	7.67 ± 1.87	$1.25 \times 10^{-09} \pm 2.15 \times 10^{-10}$	0.01	ns	
Acetic acid treated (AA)	68.67 ± 3.00	$4.94 \times 10^{-09} \pm 2.16 \times 10^{-10}$	46.90 ± 6.10	$1.66 \times 10^{-08} \pm 1.77 \times 10^{-09}$	ns	ns	
Isoelectric precipitation (IP)	90.33 ± 14.00	$4.18 \times 10^{-09} \pm 6.48 \times 10^{-10}$	5.10 ± 1.90	$1.58 \times 10^{-09} \pm 5.90 \times 10^{-10}$	0.01	ns	
b) DUC - RNA yield							
Samples Analysed	Skim milk		Infant milk formula		P value		
	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i. Total yield (ng/mL)	ii. RNA conc. (ng)/particle	i.	ii.	
Non-treated (NT)	43.53 ± 1.67	$2.21 \times 10^{-09} \pm 8.46 \times 10^{-11}$	7.30 ± 2.97	$1.57 \times 10^{-07} \pm 6.38 \times 10^{-08}$	ns	ns	
Acetic acid treated (AA)	6.60 ± 2.33	$2.37 \times 10^{-09} \pm 8.39 \times 10^{-10}$	11.37 ± 1.17	$6.73 \times 10^{-08} \pm 6.90 \times 10^{-09}$	ns	ns	
Isoelectric precipitation (IP)	51.00 ± 0.67	$1.40 \times 10^{-08} \pm 1.83 \times 10^{-10}$	22.40 ± 19.20	$4.81 \times 10^{-08} \pm 4.12 \times 10^{-08}$	ns	ns	

= comparing Total RNA yields (ng/mL); ii = comparing RNA concentration (ng/particle); ns = not significant.

Complementary information was obtained by TEM and presented in Fig. 2c. Following GUC separation, (Table 1d.) the SM EVs (SM_NT, SM_AA and SM_IP) appeared intact with limited background debris. IMF-derived EVs appeared more diffuse with less smooth surfaces, and with

much more debris in the background (Fig. 2c). Post-DUC, in SM EVs samples (SM_NT, SM_AA and SM_IP), multiple smaller vesicles were observed, although sometimes in clumps (arrows, Fig. 2c). The IMF samples (IMF_NT, IMF_AA and IMF_IP) had very few EV-like structures, but much more background debris.

Table 1d. **(ii)** Comparison of RNA (ng) /particle separated from SM and IMF by GUC *versus* DUC.

i) Skim milk			
RNA conc. (ng)/particle	GUC	DUC	P value
Non-treated (NT)	$2.14 \times 10^{-09} \pm 4.90 \times 10^{-10}$	$2.21 \times 10^{-09} \pm 8.46 \times 10^{-11}$	ns
Acetic acid treated (AA)	$4.94 \times 10^{-09} \pm 2.16 \times 10^{-10}$	$2.37 \times 10^{-09} \pm 8.39 \times 10^{-10}$	ns
Isoelectric precipitation (IP)	$4.18 \times 10^{-09} \pm 6.48 \times 10^{-10}$	$1.40 \times 10^{-08} \pm 1.83 \times 10^{-10}$	ns
ii) IMF			
RNA conc. (ng)/particle	GUC	DUC	P value
Non-treated (NT)	$1.25 \times 10^{-09} \pm 2.15 \times 10^{-10}$	$1.57 \times 10^{-07} \pm 6.38 \times 10^{-08}$	0.01
Acetic acid treated (AA)	$1.66 \times 10^{-08} \pm 1.77 \times 10^{-09}$	$6.73 \times 10^{-08} \pm 6.90 \times 10^{-09}$	ns
Isoelectric precipitation (IP)	$1.58 \times 10^{-09} \pm 5.90 \times 10^{-10}$	$4.81 \times 10^{-08} \pm 4.12 \times 10^{-08}$	ns

ns = not significant.

Immunoblotting analysis of EV specific positive and negative markers

In line with MISEV2018 guidelines (Thery et al., 2018), immunoblotting was performed on lysed samples for two EV positive markers, TSG101 and CD63, and a negative marker Actinin 4. The GUC generated SM_NT, SM_AA and SM_IP samples were typically positive for both TSG101 and CD63, but not Actinin 4 (Fig. 3a, with densitometric analysis of $n = 3$ presented in Fig. 3b). Although equal quantities of protein were loaded, TSG101 and CD63 were always at higher levels

in SM-derived compared to IMF-derived samples and Actinin 4 was undetected in all EV samples (Fig. 3a).

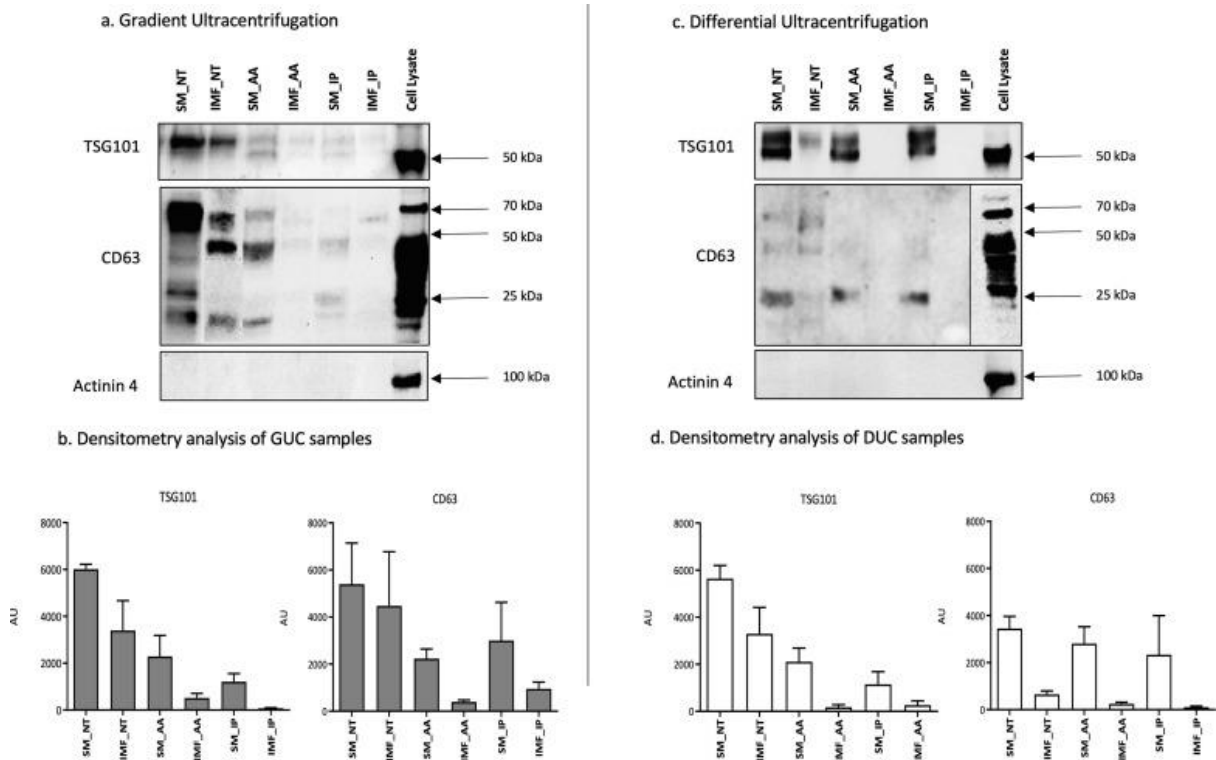


Fig. 3. Immunoblotting of EV specific markers. Immunoblotting of skim milk (SM) and infant milk formula (IMF) EVs separated using gradient ultracentrifugation (GUC) and differential ultracentrifugation (DUC) and their densitometric analysis. Equal amount of protein (35 µg) were loaded for all the samples and analysed for TSG101, CD63 and Actinin 4. Representative blots for GUC samples are presented in (a) and DUC samples are presented in (c), densitometric analysis of GUC samples are presented in (b) and those of DUC samples in (d). Each bar represents the mean of the densities of the signals obtained post immunoblotting (mean of $n = 3$ biological repeats $\pm$ SEM). All the EVs/particles collected post-DUC of SM and IMF samples were also lysed and analysed for TSG101, CD63 and Actinin 4 (Fig. 3c, with densitometric analysis of $n = 3$ presented in Fig. 3d). Again, no signal for Actinin 4 was observed for either SM or IMF samples generated by DUC. Conversely, SM_NT, SM_AA and SM_IP samples were positive for both TSG101 and CD63. IMF_NT samples compared to SM_NT had faint TSG101 and CD63 signals. Unlike SM_AA and SM_IP samples, neither IMF_AA or IMF_IP samples showed bands for either TSG101 or CD63.

Detection of EVs using imaging flow cytometry analysis

Subsequently, analysis of intact EVs based on presence of EV surface markers CD9, CD63, CD81, ADAM10 and HLADR was performed using imaging flow cytometry (IFCM). In line with a recent report on the use of IFCM for EV analysis (Ricklefs et al., 2019), we have provided the gating strategy used in Supplementary Fig. S2 and representative EV images from IDEAS Image Gallery in Supplementary Fig. S3. In all cases, post-GUC and post-DUC, higher quantities of EV-like particles were separated from SM compared to IMF. Specifically, post-GUC (Fig. 4a), significant differences in the particles/mL of SM_AA vs. IMF_AA ($P < 0.01$) and SM_IP vs. IMF_IP ($P < 0.05$)

samples were observed; SM_NT had higher quantities than IMF_NT, but statistical significance was not reached ($P = 0.08$). In post-DUC 200 K samples, significant differences were observed in particles/mL of SM_NT vs. IMF_NT ($P < 0.01$) and SM_AA vs IMF_AA ($P < 0.05$); statistical significance was not obtained between SM_IP vs. IMF_IP samples (Fig. 4b). In keeping with this trend, and as illustrated in Fig. 4c, d, e, f and g that show the approximate number of + EVs/mL of sample that are positive for each of five individual EV-specific markers (CD63, CD9, CD81, ADAM10 and HLADR), post-GUC and post-DUC -where there was a difference in surface marker detected- the levels were always higher with the SM EV compared to the IMF EVs.

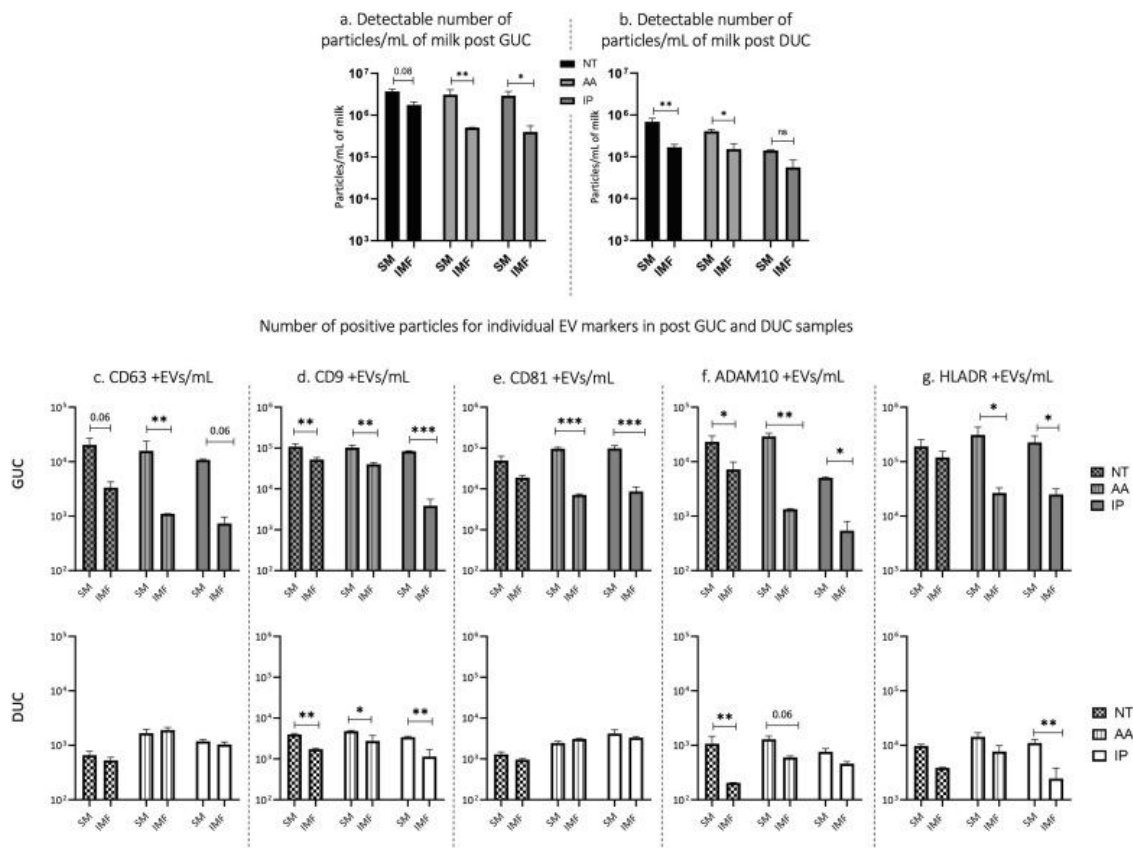


Fig. 4. Imaging flow cytometry analysis of EV specific markers. Number of positive events (particles) in skim milk (SM) and infant milk formula (IMF) samples analysed by imaging flow cytometry (IFCM). The events were gated for low side scatter channel (SSC) signals and no signals on brightfield, based on EV size and events positive for these gating was acquired as objects (+EVs) in low SSC. The total number of detectable particles in gradient ultracentrifugation (GUC) samples are presented in a) and those from differential ultracentrifugation (DUC) samples are presented in c), data presented as mean and individual values $\pm$ SEM. The number of positive CD9, CD63, CD81, ADAM10 and HLADR particles in GUC samples are presented in b) and DUC samples are presented in d). The IDEAS software was used to analyse the data, data presented as means and individual values $\pm$ SEM. A two-way ANOVA test was performed to compare differences in EVs in SM and IMF samples from each treatment groups; *indicates $P < 0.05$. **indicates $P < 0.01$ and ***indicates $P < 0.001$.

Discussion

The aim of this study was to compare EVs present in SM and IMF. As IMF production involves harsh industrial processes, we hypothesised its EVs may be lost and/or compromised compared to those in SM. Comparisons were also made based on both milk pre-treatment and EV isolation methods to identify the optimal procedure to separate enriched EVs from SM and IMF. Our results indicate that irrespective of the treatment, the concentration and the quality of EVs present in IMF are substantially compromised compared to SM. This suggests that in IMF, proteins carried by EVs that are present may be damaged or lost. Our results also suggest that both AA and IP pre-treatments remove casein micelles quite efficiently, and that the GUC technique is the optimum method for isolation of high yields of intact EVs without any effect on the EV morphology.

To perform detailed characterisation of milk- or IMF- derived EVs, as pure/enriched EVs as possible need to be separated. However, using complex biofluids in conjunction with ultracentrifugation typically leads to contamination of EVs with proteins (Langevinet al., 2019, Liet al., 2019). Ultracentrifugation of milk is reported to lead to sedimentation of dense casein micelles (Fox, 2003). It has been reported that following milk fractionation by ultracentrifugation at 100,000 g, casein remnants exist in milk serum because ultracentrifugation alone does not break-up or remove casein micelles and the small casein micelles are difficult to sediment (Jensen, Poulsen, Møller, Stensballe, & Larsen, 2012). In agreement with this, by SDS-PAGE we observed strong casein -and to a lesser extent, whey- protein bands in samples that were collected after multiple ultracentrifugation steps of DUC in both SM and IMF samples, with similar observations made for SM and IMF samples that underwent GUC. Therefore, substantial consideration needs to be given towards removal of caseins micelle whose size and concentration overlapping with EVs, if claiming to study EVs.

In industry, depending on the subsequent use of the product, casein is sometimes removed by isoelectric precipitation (Fox & Brodkorb, 2008) using acids that generate 'acid-caseins' (Atamer et al., 2017). Acidification of milk reduces its pH to the isoelectric point of casein (pH 4.6) resulting in irreversible destruction of the casein micelle structure and further coagulation and precipitation of the casein complex that can be sedimented at low speed centrifugation (Chinprahastet al., 2015, Fox and Brodkorb, 2008). In fact, a recent study investigated acidification effects on defatted raw bovine milk and observed some morphological changes in EVs due to acid treatment. However, the use of acid to remove milk proteins and separate large scale EVs remains their preferred method (Rahman et al., 2019). In our study, a

combination of acid treatment and ultracentrifugation was evaluated with both SM and IMF which, to the best of our understanding, has not previously been done. It is noteworthy that a recent study did compare IMF EVs to milk EVs and reported EVs to be undetectable in IMF (Leiferman, Shu, Upadhyaya, Cui, & Zemleni, 2019). However, that study did not remove casein micelles, did not analyse samples by TEM, and only used DUC for EV collections. So, while it is an important study, it is very different in many respects to the work we presented here.

Further orthogonal steps were performed to analyse the EVs separated from SM and IMF and to explore the effects of pre-treatment on EV morphology or protein composition. Characterisation was performed by NTA, a technique widely used to estimate number of particles as well as the size of particles in a medium (Szatanek et al., 2017; Thery et al., 2018). Interestingly and as we hypothesised, post-GUC and -DUC, the number of particles/mL of IMF samples (all treatment groups) were significantly lower than in the corresponding SM samples, while no differences in the particle sizes were observed. Additionally, to rule out this being because of milk pre-treatment, we included the non-treated SM and IMF in our characterisation steps. Similar differences between non-treated SM and IMF samples were observed, where SM samples had higher concentration of EVs compared to IMF. However, the lack of significance in this difference may be due to a combined analysis of EVs and casein micelles confusing the results. Of note, regardless of whether SM or IMF were pre-treated or non-treated, when directly comparing GUC and DUC, GUC always yielded many folds more EVs/particles with GUC compared to DUC. Experimental variability between biological repeats, however, meant that statistical significance was not always reached.

While NTA is a widely used technique in EV research, it has certain limitations including its incapability to distinguish between particle sizes in a heterogeneous mixture being one (Witwer et al., 2013). Therefore, to further validate our observations, the EVs isolated from SM and IMF were imaged using TEM. The TEM analysis of GUC samples suggested that no negative effect of the pre-treatment or ultracentrifugation on the EVs separated from SM *i.e.* all the EVs derived from SM samples had a very smooth outer surface, round shaped and evenly distributed on the grid. Compared to SM samples, however, GUC generated IMF samples were sparsely distributed across the grids and showed rough surfaces and shapes indicating to signs of disruption or disintegration. Interestingly in all the SM DUC samples, very small particles (approximately 30 nm) were detected that did not have resemblance to the EVs that were observed in GUC samples. In IMF samples, some EV structures were presented but compromised, with high

amount of debris present in all IMF samples. The presence of high amount of debris in IMF might be related to the industrial processing during IMF production. Emami et al. (2018) reviewed the physicochemical degradation of protein formulations that undergo harsh spray drying, lyophilisation and heating steps; this possibly leads to high number of insoluble protein particles or aggregates that may pellet at the highest speed. Additionally, these harsh industrial conditions potentially also lead to disruption of EVs present in milk and so intact EV-like structures were typically not observed in IMF samples.

As EVs typically carry RNA, but casein micelles do not, the separation of enriched EV samples were evaluated quantifying RNA yield (and RNA amount per particle) from the isolates from GUC and DUC. Interestingly, SM_NT and SM_IP samples, post GUC, were associated with the highest total RNA yield compared to their corresponding IMF samples. Additionally, GUC isolates typically carried more RNA/particle. While statistical significance in these comparisons was not always reached, at least partly contributed to by the quite substantial differences between biological repeats of the experiment, overall, the data does support GUC rather than DUC producing the more EV-enriched isolates.

Further characterisation of the SM and IMF samples were done by immunoblotting for EV-positive markers. Two positive markers for EVs (tetraspanin CD63 and TSG101) and a negative milk EV marker (Actinin 4) were evaluated based on MISEV2018 guidelines. In the GUC generated lysed samples, all SM samples, irrespective of their treatment, were positive for CD63 and TSG101 and negative for Actinin 4. Interestingly, IMF samples were associated with notably weaker signals indicating extremely low concentration of EVs present in the sample. Since the same amount of protein (35 µg) was loaded on the gel for each sample, lower density of bands indicates a very low EV to protein ratio indicating absence of EVs in the sample. A similar observation was also made in DUC samples where all IMF samples were again associated with faint bands for both CD63 and TSG101 compared to SM samples. These results, again, support our hypothesis that due to the use of harsh processing steps on milk in an industrial setting, EVs in the starting material are negatively affected. Of note, the doublet observed in some samples for TSG101 may be due to internal initiation at MET₁₀ (VerPlank et al., 2001); whereas, in case of CD63, due to multiple glycosylation sites, bands within the range of 25–70 kDa were observed which is consistent with published literature (Metzelaaret al., 1991, Van Deunet al., 2014).

Subsequently, the EVs separated from SM and IMF samples were analysed by flow cytometer (Ricklefs et al., 2019). Only events that had very low SSC signal and no brightfield signals were

considered as a positive EV like particle and presented as particles/mL of milk. Following the observations from our previous characterisation steps, we observed that all SM samples - generated both by GUC and DUC- have a higher EV like particle counts compared to all IMF samples. Further, enumeration of surface markers on EVs that include tetraspanins CD9, CD81 and CD63, ADAM10 and HLADR were also carried out. In GUC generated samples, several significant differences were observed; all indicating that SM samples have higher EV specific surface markers compared to IMF samples, indicating again to the lower number of EVs present in IMF samples. In contrast, the SM samples generated by DUC recorded a higher CD9, ADAM10 and HLADR + EVs/mL compared to IMF samples. This observation may indicate that isolates resulting from DUC, in comparison to GUC, - are not as enriched in EVs.

While this study is focussed primarily on comparing the differences between SM and IMF samples and we clearly demonstrated that IMF has lower amounts of EVs/EV-like particles, comparison between GUC and DUC were also made. The findings from this study are summarised in Table 2 and it is evident that GUC is a laborious process, with approximately 18 h required just for ultracentrifugation added to the time for sample preparation. However, the favourable aspect of this technique is the small volume of sample required compared to DUC. In fact, based on our NTA and IFCM results, GUC is associated with the highest yield of EVs compared to DUC; there is a log difference between the number of EVs detected in GUC compared to DUC. Additionally, the EV samples generated post-GUC, can be used in a multiple range of characterisation technique and generates highly reproducible data; apart from immunoblotting. In contrast, DUC yields high amount of visible pellet/suspension at the end of each centrifugation step, however, this suspension contains a high number of contaminant proteins that interferes with the further EV characterisation. The results from this study strongly indicate that a combination of AA/IP and GUC could be the best strategy to separate the most enriched EVs from milk. In fact, GUC technique is a well-accepted method for isolation of EVs, especially the bottom-up approach, as previously reviewed (Witwer et al., 2013).

Thus, returning to our hypothesis that EVs in IMF may be compromised, we can clearly state that IMF contains some but significantly fewer EVs/EV-like particles; many of which have damaged morphology and do not carry intact EV protein markers. Additionally, we can also conclude that our methodology that includes IP pre-treatment and then GUC takes longer times but requires comparatively less starting material and separated EVs can be successfully analysed by techniques such as NTA, TEM and IFCM. Thus, on balance, although we observed merit as well

as challenges with both GUC and DUC, in our opinion GUC does seem to be somewhat more beneficial here even though it is certainly not perfect (*e.g.* as shown by some variability between biological repeat experiments, especially on EV-RNA analysis); but both techniques are possible suitable options. We encourage other researchers to use the information included here to inform their own choices as to which they consider to be the better method. Future studies analysing samples taking through the industrial processing of IMF may help to identify ways of preventing or minimising the EV loss from IMF. The outcome from this study suggests that a functional study to compare the effects of the SM and IMF EV isolates is now warranted.

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“An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve”

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Abstract

Extracellular vesicles (EVs) in milk have claimed benefits ranging from conveying immunological privilege to infants to being suitable as natural delivery vehicles for therapeutic drugs. However, a longitudinal study of bovine EVs quantities and characteristics in colostrum (COL), first milk (FM) and throughout the lactation curve of mature milk (MM) had never been performed and so was our aim. COL, FM and 9 months of MM samples were collected. Caseins -overlapping size

with EVs- were removed. EVs were collected by density gradient ultracentrifugation and characterised by SDS-PAGE, Bradford assay, nanoparticle tracking analysis, immunoblotting, imaging flow cytometry analysis, and transmission electron microscopy. COL and FM had substantially more EVs than MM, with COL enriched in small EVs. No significant differences were observed between months 1–9 of MM. Altogether, although COL and FM are particularly rich sources of EVs, mature milk throughout the lactation curve is also an abundant source of intact EVs.

Keywords

Extracellular vesicles (EVs); Colostrum; First milk; Mature milk; Lactation curve

Introduction

The milk secreted by mammary glands in the time immediately after birth is known as colostrum (COL). It is considered to be a very rich source of nutritional, growth and immunological factors, vitamins and minerals and bioactive compounds (O'Sullivan et al., 2019, VandeHaar et al., 2006). Within cows, COL is gradually replaced by first milk (FM) from the 2nd to the 8th day post-calving (McGrath et al., 2016). Although FM continues to be a rich source of nourishment for infant growth, it has a substantial reduction in immunological compositions compared to COL (Sanwlani et al., 2020). Mature milk (MM) is then produced from approximately-one-month post-parturition. Its composition differs from COL and FM which are higher in fat and lactose (O'Sullivan et al., 2019). The lactation period ends around 9 months post-calving (Butler, 2014). The composition of total milk solids, milk proteins, and antibodies is reported to change substantially throughout this period *i.e.*, COL includes 23.9% total solids, 14% proteins, and 6% antibodies, compared to 16%, 6.8% and 3.3% in FM and 12%, 3.1% and 0.09% in mature milk, respectively (Foley et al., 1978, Gu, et al., 2012). Milk, the primary source of vital nutrients and bioactive components contributing to the growth and development of the newborn, is a source of extracellular vesicles (EVs) with biological process modulating capabilities (Admyre et al., 2007, Hata et al., 2010). EVs are membrane-surrounded vesicles that are released from cells into biofluids, such as milk. EVs, including those from milk, are considered to play a fundamental role in intercellular communication. This is highly relevant as EVs are abundant in milk and they carry quite extensive cargo including proteins, lipids, and nucleic acids that contribute to them being considered as bioactive components of milk (Manca et al., 2018). The fact that milk EVs have been demonstrated to be stable under conditions of low pH *i.e.* maintaining their integrity in

challenging conditions representing the gastrointestinal tract, further supports their bioactive relevance (Sanwlani et al., 2020). Indeed, for example, characterisation of milk EVs' cargo (mRNA and miRNA) has highlighted their anti-inflammatory and immunomodulatory potential for attenuating symptoms of inflammatory bowel diseases (Mecocci et al., 2021). Milk EVs are reported to transmit maternal information to the infant, helping to shape the adaptive immune system in newborns (Zempleni et al., 2017). Similarly to human babies, the health of calves is believed to benefit substantially from the transfer of fats, proteins, immune cells, and apparently EVs, that nourish and offer protection against infection and inflammation (Wu et al., 2016, Arslan, et al., 2021). Indeed, bovine milk is readily available, cheap, safe, and serves as a starting material for various dairy products including nutritional beverages such as infant milk formula, (Zeng et al., 2019). However, although studies -by ourselves and others- of EVs from milk of several mammalian species, such as human, bovine, and porcine have been reported (Admyre et al., 2007, Gu, et al., 2012, Samuel et al., 2021, Somiya et al., 2018, Zonneveld et al., 2014), knowledge of comparative EVs quantities and characteristics throughout the lactating stages are lacking. For the dairy industry, for consumers of milk/milk products and, indeed, for those investigating milk EVs as natural delivery vehicles for therapeutics, it is relevant to know if and how EVs change over the lactation curve. Hence, we hypothesised that as COL is considered a particular rich source of nutrients and antibodies that it contains most EVs. To investigate this, we collected bovine COL, FM, and MM; we separated EVs following our recently developed protocol (Mukhopadhyaya et al., 2021a); and then performed comparative quantification and characterisation of the EVs.

Materials and methods

Collection and defatting of milk samples

Bovine COL, FM and MM were collected from $n = 3$ healthy Holstein-Friesian cows bred in the Dairygold farm Teagasc, Moorepark, Fermoy, Co. Cork, Ireland (for $n = 3$ independent experiments). COL was collected within 24 h post-calving, FM after 7 days, and MM from months 1 to 9 post-calving. All subsequent analyses detailed in the study were performed a minimum of 3 separate times on each of the 3 sets of samples. All samples were defatted at $6,000\times g$ at 4°C for 10 min, the supernatant was filtrated through glass wool, and then immediately stored at -80°C and shipped to Trinity College Dublin for the research to be performed (Fig. 1).

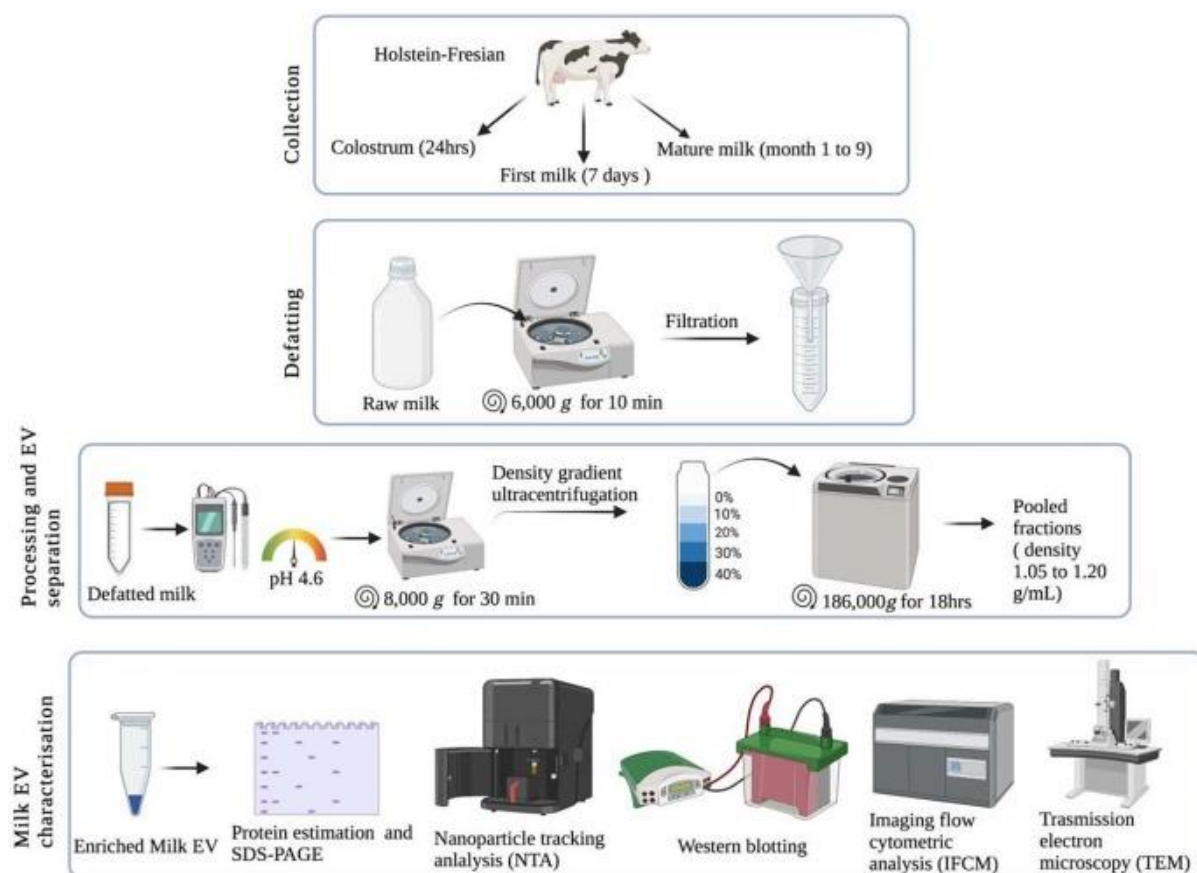


Fig. 1. Flowchart of the methodology used. Methodology including milk collection, pre-treatment and milk EV separation and characterisation from bovine colostrum, first milk and mature milk.

Sample processing and EV separation

EVs were separated from COL, FM and MM using a method that we recently developed (Mukhopadhyaya et al., 2021a, Mukhopadhyaya et al., 2021b). As represented in Fig. 1, to remove caseins from defatted COL, FM and MM, the pH was dropped to 4.6 using 5 M HCl. It was then centrifuged at 8,000×g at 20 °C for 30 min. Then 2.33 mL of whey was used for density gradient ultracentrifugation following the bottom-up technique using Optiprep iodixanol solution (60%). Ultracentrifugation used an SW Type 32.1 Ti rotor in Optima XPN-100 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 186,000×g for 18 h at 4 °C. Pooled fractions (1.05–1.20 g/mL) were washed twice in sterile PBS at 120,000×g for 2 h at 4 °C. Each resulting pellet was resuspended in 500 µL of 0.22 µm filtered PBS.

SDS-PAGE for protein visualisation

Removal of casein micelles from COL, FM and MM samples was confirmed by performing SDS-PAGE analysis and visualisation of protein bands on a 10% Mini-PROTEAN® TGX™ Precast Gel

(Bio-Rad Laboratories, Cat. #4561034). Specifically, 10 µg of EVs fractions were loaded on the gel, run at 120 V, stained with Coomassie blue (Sigma-Aldrich, Ireland) and visualised using a Chemidoc XRS system (Bio-Rad).

Protein quantification

Bradford assay was performed to determine total protein in samples using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Cat #500–0006). Bovine serum albumin (BSA) standards (0–800 µg/mL) (Sigma-Aldrich, Ireland) were used to generate a standard curve for interpolation of the protein quantities.

Nanoparticle tracking analysis

The concentration and sizes of particles in the EVs fractions were estimated using NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK). Samples were automatically injected into the NTA system under constant flow conditions (flow rate = 50) and a minimum of four × 60-second videos was recorded for each sample. The detection threshold during analysis was selected to ensure that only distinct nano-objects were analysed and any artefacts were removed. Videos of the particles in motion were recorded and analysed using NTA 3.2 software.

Immunoblotting analysis

Immunoblotting was performed as we previously described (O'Brien et al., 2013). In brief, EVs were lysed using SDS lysis buffer (250 mM Tris-HCL, pH 7.4; 2.5% SDS). the lysates (30 µg) were resolved on 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Cat. #4561034) and the protein was transferred onto PVDF membranes (Bio-Rad, Cat. #:1620177). Blots were blocked with 5% (w/v) BSA in PBS containing 0.1% Tween-20 and incubated overnight at 4 °C with primary antibodies against TSG101 (1:1000; Abcam, Cambridge, UK, Cat. #:ab30871), CD9 (1:1000; Abcam, Cat. #:ab92726), CD63 (1:500; Abcam, Cat. #:ab68418), ACTN4 (1:1000, Abcam, Cat. #:ab41525), BTN1A1 (Abcam, Cat. #:ab180605), milk fat globule 1 (MFG1) (1:500, Abcam, Cat. #:ab200649), MUC-1 (Abcam, Cat. #:ab45167) and GRP94 (1:1000, Abcam, Cat. #:ab238126). Secondary antibodies used were anti-mouse (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #: 7076) or anti-rabbit (1:1000 in 5% BSA/PBS-T, Cell Signalling, Cat. #:7–74). SuperSignal West Femto Chemiluminescent Substrate Kit (Fisher Scientific, Cat. # 11859290) was used for detection, imaging was performed using an automated Chemidoc exposure system (Bio-Rad Laboratories), and densitometric analysis was performed using ImageJ. Hs578T mammalian cell lysate (CL) and crude milk were used as controls. Specifically, we used

CL as control for all EVs markers including GRP94; whereas crude milk was used as control for milk fat globule proteins BTN1A1, MFG1 and MUC1.

Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis as we previously described (Mukhopadhyaya et al., 2021a). In brief, 5 μ L of the sample was suspended in 5 μ L of 0.22 μ m-filtered PBS and placed onto carbon-coated grids (Ted-Pella B 300 M, Mason Technology Ltd, Cat. #: 01813-F) for 10 min at RT before fixing with 4% glutaraldehyde (Sigma-Aldrich) and contrasting with 2% phosphotungstic acid (Sigma-Aldrich). The grids were examined at 100 kV using a JEOL JEM-2100 TEM (JOEL, Peabody, USA).

Imaging flow cytometry analysis

The imaging flow cytometry (IFCM) analysis of intact/non-lysed EVs separated from COL, FM and MM samples was performed as we previously described (Mukhopadhyaya et al., 2021a). The specific antibodies used in this study were anti-CD63 conjugated with fluorescein isothiocyanate (FITC) CD63-FITC, 1:150, Cat. #:353006, Biolegend) and CD9-PE (1:1500, Cat. #:312106, Biolegend). EVs were incubated with the antibodies for 45 min at RT in the dark and washed using a 300 kDa filter (Nanosep, Cat. #:516–8531). Samples were resuspended in 50 μ L IFCM buffer and acquired within 2 h on the ImageStream X Mk II imaging flow cytometer (Amnis/Luminex, Seattle, USA) at 60x magnification and low flow rate and EVs-free IFCM buffer. Unstained EVs, single-stained controls, and fluorescence minus one (FMO) controls were run in parallel. EVs were gated as SSC-low versus fluorescence, then as non-detectable brightfield (fluorescence vs Raw Max Pixel Brightfield channel). Gated EVs were confirmed in the IDEAS Image Gallery. Data analysis was performed using IDEAS software v6.2 (Amnis/Luminex, Seattle, USA).

Data analysis

Data presented in this study are the means of ≥ 3 experiments $\pm$ SEM from separate samples of $n = 3$ cows. Graph Pad Prism 9 software was used to perform statistics analysis of all data. Unpaired two-tailed Student's *t*-test was used to compare two groups and one or two-way ANOVA was used to compare more than two groups. Differences in the value of $p < 0.05$ were considered statistically significant. Shapiro-Wilk test was done to evaluate the distribution of our data (Suppl. Fig. S5(a)). All relevant EVs-related information was submitted to the EV-TRACK knowledgebase and assigned EV-TRACK ID: EV220299 (Van Deun et al., 2017).

Result

Casein removal analysis by SDS-PAGE and protein quantification

Casein from COL, FM and MM was removed by isoelectric precipitation at pH 4.6. As shown in Fig. 2a (Suppl. Fig. S2a) we observed that acid treatment of milk efficiently removed most, if not all, of the casein, as evident when compared to untreated milk. The total protein quantification in the EVs samples, performed by Bradford assay (Fig. 2b), showed COL EVs samples had a very high protein concentration compared to FM and MM (p < 0.001).

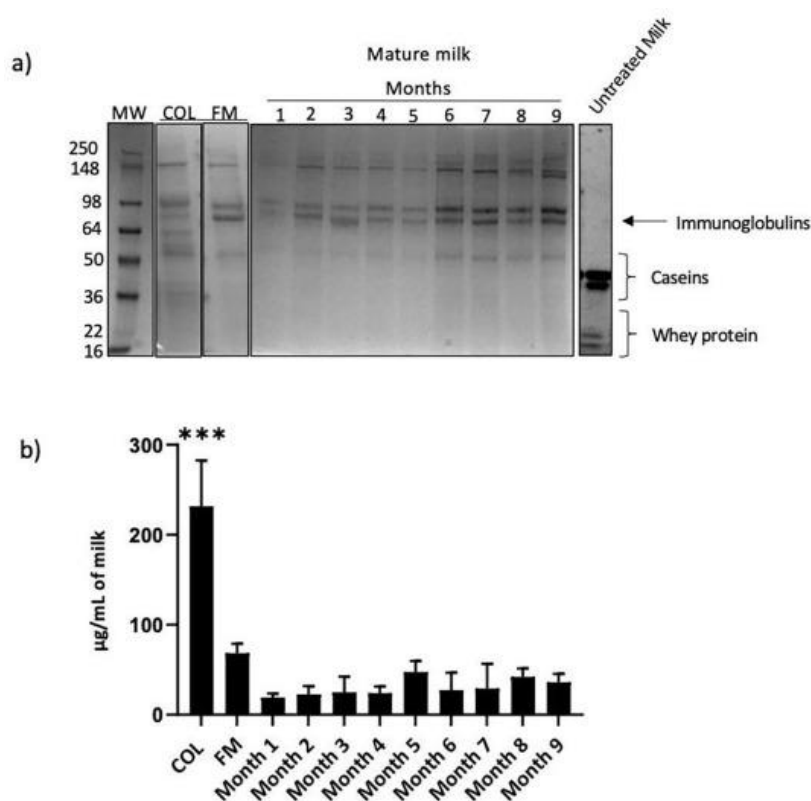


Fig. 2. Milk protein characterisation of bovine colostrum, first milk and mature milk a) Milk protein in EV enriched fraction of colostrum (COL), first milk (FM) and mature milk (MM) were visualised by loading 10 µg of protein on 10% Mini-PROTEAN® TGX™ Precast Gel followed by Coomassie Blue staining. Molecular weights (MW) standard is reported to represent milk proteins presented in all milk EV samples. b) Protein concentration was assessed by Bradford assay and reported in µg/mL of milk used. Each bar represents 3 biological repeats, statistical analysis using one-way ANOVA test was performed, error bars represent standard error of mean (±SEM), *** indicates p < 0.001.

Particles concentration and size measurement by nanoparticle tracking analysis

NTA estimated particle sizes of COL EVs and FM EVs enriched fractions were 140 ± 5.1 nm and 156 ± 8.8 nm, respectively, while EVs in MM month 1 were 133 ± 2.6 nm, month 5 were

141 ± 1.0 nm and in month 9 were 136 ± 8.8 nm. Overall, no significant differences were observed in particle size among the samples analysed (Fig. 3a; Suppl. Figure S1). Total particles/EVs concentration, as well as concentration of particles/EVs sized 30–150 nm (size range of small EVs/sEVs), and concentration of particles/EVs sized > 150 nm (size range representing medium and large EVs) from COL, FM and MM samples are presented in Fig. 3b and Fig. 3c. Firstly considering the overall heterogeneous pool, COL ($p < 0.001$) and FM ($p < 0.01$) had significantly greater amounts of EVs when compared to MM. Specifically, COL had $1.3 \times 10^{14} \pm 2.2 \times 10^{13}$ particles/mL and FM had $9.3 \times 10^{13} \pm 1.8 \times 10^{13}$ particles/mL, whereas the corresponding values for MM particle concentration month 1 was $2.3 \times 10^{12} \pm 1.5 \times 10^{12}$ particles/mL, month 5 was $6.6 \times 10^{11} \pm 3.0 \times 10^{10}$ particles/mL and month 9 was $3.9 \times 10^{12} \pm 1.6 \times 10^{12}$ particles/mL (Fig. 3b). Then considering the EVs based on size distribution, a similar trend was observed in that COL had significantly higher concentration of sEVs (Fig. 3c(i)) compared to FM ($p < 0.05$) and MM ($p < 0.001$). Likewise, FM had significantly ($p < 0.01$) higher concentration of particles compared to MM. For those sized > 150 nm Fig. 3c(ii), COL had a significantly ($p < 0.01$) higher concentration of particles compared to MM and FM had significantly ($p < 0.05$) higher concentration compared to MM. However, no significant differences were observed between COL and FM. Overall, COL and FM had similar amounts of particles/EVs with COL typically having highest concentrations of particles considered to represent EVs. Thus, both COL and FM were substantially more enriched in particles/EVs compared to MM. Once MM was being produced, however, the particle/EVs concentrations did not differ significantly over the 9 months of the lactation curve.

An additional comparison of bovine milk EVs throughout the lactation curve was done by comparing particles/EVs separated from a pool of many cows, known as bulk milk. As illustrated and detailed in Suppl. Figure S3, the particle/EVs concentrations in bulk milk were similar to mature milk and significantly less than that in both COL or FM.

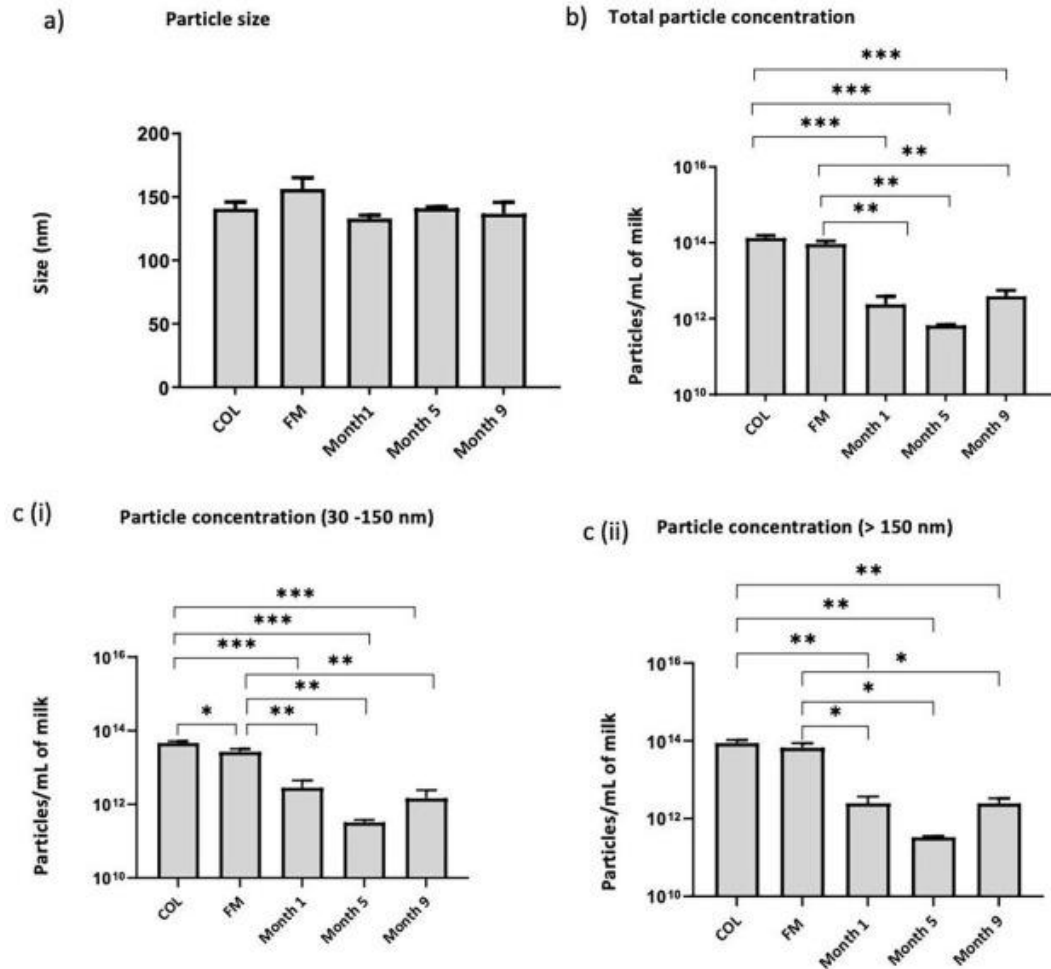


Fig. 3. Particle sizes and concentration from colostrum, first milk and mature milk EVs NTA analysis of particle sizes and concentration involved a minimum of four $\times$ 60-second videos recorded for each sample. a) Comparison of particle size in colostrum, first milk and mature milk. b) Total particle concentration comparison of colostrum, first milk and mature milk EV represented by months 1, 5 and 9; c (i) Comparison of particle concentration within 30–150 nm of colostrum, first milk and mature milk EV samples. c (ii) Comparison of particle concentration over 150 nm of colostrum, first milk and mature milk EV samples. Three replicates of each sample were analysed by NTA independently, data analysed by one-way ANOVA test and presented as mean bars $\pm$ SEM, the significant p-value is reported; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Immunoblotting analysis using protein markers associated with EVs

To confirm the presence of EVs in COL, FM and MM, immunoblotting analysis was performed on lysed samples for three EVs positive markers (TSG101, CD9 and CD63) and one negative marker (ACNT4). All samples were found to be positive for TSG101, CD9 and CD63, whereas no signal was detected for ACTN4 in any samples (Fig. 4a; Suppl. Fig. S2b(i)). Likewise, GRP94, known to be associated to large vesicles (Théry et al., 2018), was not detected in any of the samples analysed. Additionally, we investigated markers associated to milk fat globules, BTN1A1, MFG1

and MUC-1. These were not detected in any of the milk EV samples, confirming the enrichment of EVs in our samples and the absence of other vesicles and MFGs (Fig. 4b; Suppl. Fig. S2b(ii)). Overall, what was observed by immunoblotting, where 30 µg of protein was loaded for all samples, was that all 3 EVs-positive markers evaluated were detected, and the amounts detected were quite reproducible between separate experiments. Although immunoblotting analysis of lysed EVs is qualitative not quantitative, no substantial differences were evident between COL, FM, and MM. Thus, from this point onwards, months 1, 5 and 9 were selected to represent the MM throughout the lactation period.

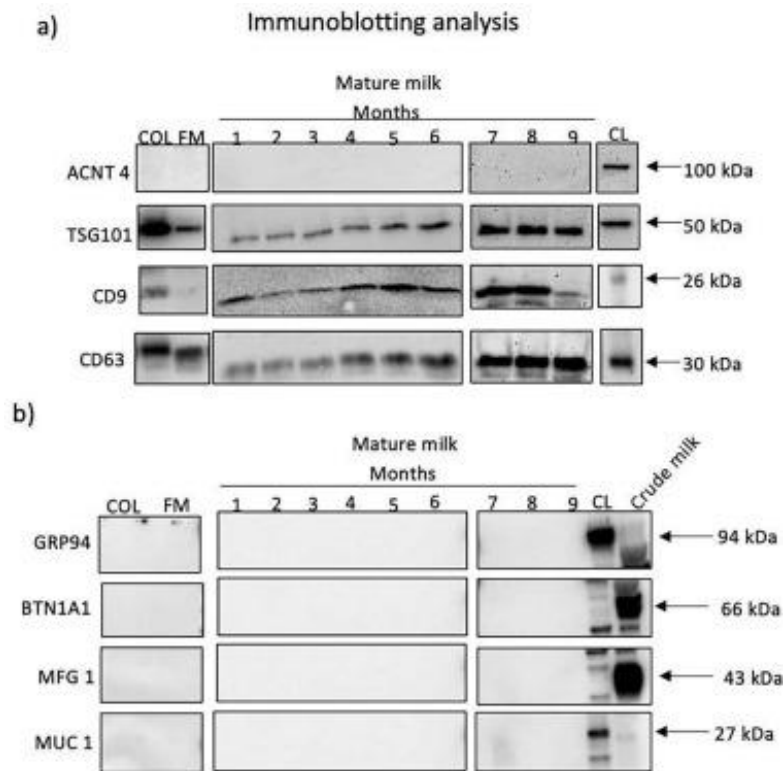


Fig. 4. Detection of EV specific markers by immunoblotting. Immunoblotting analysis of EVs separated from colostrum, first milk, and mature milk was performed by loading 30 µg of protein for all the EVs samples and CL (cell lysate). a) Analysis of EVs-associated positive markers TSG101, CD63, CD9 and negative marker ACTN4. b) Analysis of three milk fat globules markers BTN1A1, MUC1, MFG1 and one microvesicles marker GRP94.

Presence of EV surface markers detected by imaging flow cytometric analysis

Quantitative characterisation of single intact COL, FM and MM EVs samples were performed by IFCM. Here, EVs were analysed based on the presence of EVs surface markers CD9 and CD63. COL was found to have significantly higher amounts of CD9+ and CD63+ particles compared to

FM and MM (Fig. 5a&b; Suppl. Figure S4). Throughout the remainder of the lactation curve no significant differences were evident.

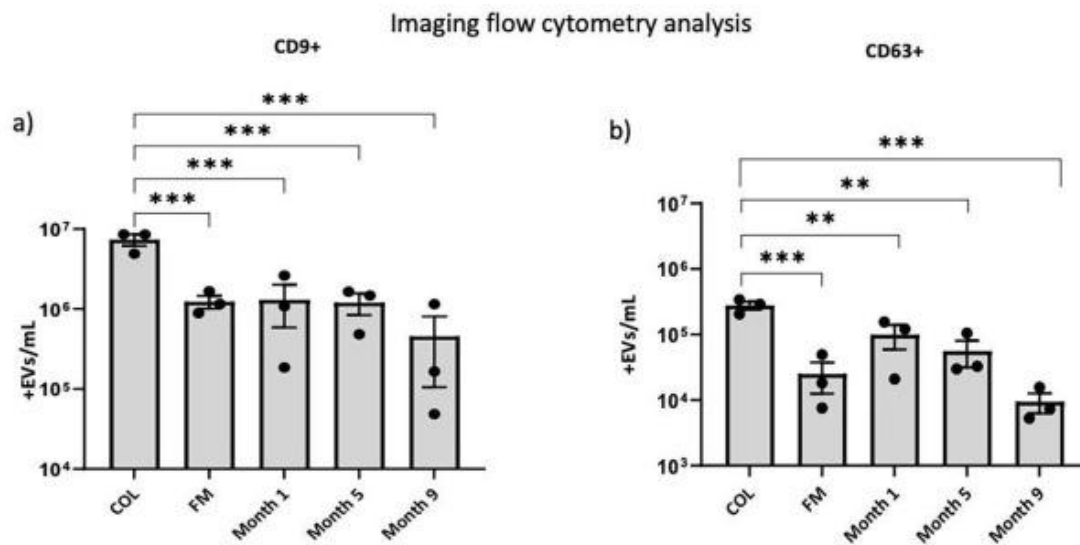


Fig. 5. Imaging flow cytometry analysis of EV surface marker. Number of positive events (particles) in colostrum, first milk and mature milk analysed by imaging flow cytometry (IFCM). Events were gated for low side scatter channel (SSC) signals and no signals on brightfield, based on EV size and events positive for these gating was acquired as objects (+EVs) in low SSC. Data presented as mean and individual values $\pm$ SEM. The number of positive b) CD9 and c) CD63 in colostrum, first milk and mature milk. The IDEAS software was used to analyse the data. Data is presented as means and individual values $\pm$ SEM. One-way ANOVA tests were performed and significance is indicated as ** $p < 0.01$, *** $p < 0.001$.

Transmission electron microscopy analysis of bovine milk EVs

The structure of EVs separated from COL, FM, and MM, and as analysed by TEM, were of vesicular morphology with intact membranes, with no substantial differences observed between sample preparations (representative micrographs presented in Fig. 6).

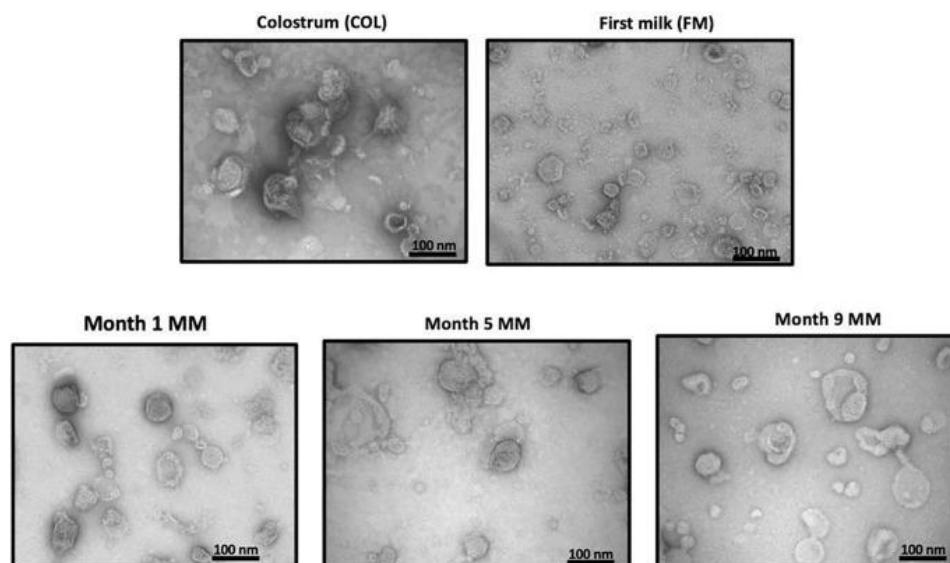


Fig. 6. Transmission electron microscopy analysis of EV morphology. Representative transmission electron microscopy analysis of colostrum, first milk and mature milk EV samples. Scale bar = 100 nm.

Discussion

In the dairy industry, milk yield is one of the most important factors to determine the profitability of dairy cows (VandeHaar et al., 2006, Hutjens, 2016). Therefore, we aimed to collect and characterise EVs from bovine COL, FM, and MM, to investigate their similarities and differences over the lactation curve. Of note, some previous studies have explored milk EVs from different species at distinct stage of lactation, but EVs separation and characterisation throughout the entire lactation cycle was lacking. For instances, Ferreira et al. (2021) separated porcine milk vesicles, that they termed ‘exosomes’, from colostrum at day 0 and mature milk but at days 7–14 post-partum only. Similarly, Lin et al. (2020) separated vesicles that they termed ‘exosomes’ from porcine and bovine milk, but only within a few days of parturition. Furthermore, in both studies the fundamental characterisation of the “exosomes” was very limited. Thus, while arguably all studies have their merits, it is our understanding that the research we present here is the first longitudinal study of EVs from colostrum, first milk, and mature milk spanning the whole of the lactation curve from exactly the same donors. Furthermore, these EVs were collected by a method accepted to remove most non-EVs components (*i.e.* ultracentrifugation on a density gradient) and there were subsequently extensively characterised by orthogonal methods, in keeping with MISEV2018 guidelines (Théry et al., 2018). Taking this approach, here we observed that although abundant intact EVs are present at all the stages of the lactation curve, the EVs concentration is significantly higher in COL and FM, decreasing in month 1 of MM to a level that is then maintained throughout the

remainder of the 9 months mature milk period. COL was particularly enriched in sEVs. Separation of EVs from milk is challenging due to the biological complexity of this matrix, thus previously we developed a combination of acid pre-treatment before performing EVs separation to remove casein and then separated relatively pure EVs that are suitable for characterisation (Mukhopadhyaya et al., 2021a). Thus, based on that success here we followed the same casein removal and EVs separation methods for COL, FM and MM from months 1 to 9, to represent the entire bovine lactation curve. Following separation of EV enriched fractions, SDS-PAGE analysis indicated efficient casein removal. This is important as many milk EVs studies do not remove the very highly abundant casein micelles which have a size of approximately 150 nm (falling well within the size range of EVs) and so they substantially confuse the interpretation of milk EVs data. In fact, to our knowledge, this is the first ever study in which isoelectric precipitation was performed to remove casein micelles from bovine COL (as well as FM and MM across the lactation curve) and separate pure EVs-enriched fractions post ultracentrifugation. As indicated in the Results Section, NTA analysis suggested significantly lower particles/EVs concentration in MM, compared to COL and FM. While Samuel et al., 2017, Hata et al., 2010 reported abundant EVs in both bovine COL and MM, in those studies no acid pre-treatment was performed prior to remove casein micelles. Also, those studies did not extend to comparing each of COL EVs and FM EVs and MM EVs, as we report here. Here, in addition to establishing that both COL and FM are enriched in EVs compared to MM, we discovered that this then remains steady in the following months in MM throughout the lactation curve. Further characterisation of COL, FM and MM samples was performed by immunoblotting for EVs-specific protein markers. In line with the MISEV2018 guidelines (Théry et al., 2018), three positive EVs markers (TSG101 and two tetraspanins CD9 and CD63), one negative marker (ACTN4) and one microvesicles marker (GRP94) were evaluated. COL, FM, and MM EVs were positive for TSG101, CD9 and CD63, and all were negative for ACTN4 and GRP94, which supports the presence of EVs in all samples. In the context of work by others, Yun et al., 2020, Ross et al., 2021 reported the presence of EVs in bovine COL. However, again no pre-treatment was used to remove casein micelles which makes EVs quantification and characterisation challenging and potentially more unreliable. Additionally, here we also used markers for milk fat globules (MFGs) such as BTN1A1, MUC- 1 and MFG1 which were not detected in any of our milk EVs samples. Therefore, following our optimised protocol, when applied in this study we successfully removed most of the non-EVs components to achieve higher purity during EV separation. Imaging flow cytometry technology was used to quantify EVs surface markers CD9 and CD63 on intact intact

EVs. Supporting what was observed by the cruder NTA analysis, COL had highest quantities of particles/EVs [CD9+ and CD63+] compared to FM and MM, confirming that bovine COL is highly enriched in EVs. Additionally, CD9+ and CD63+ EVs did not differ significantly between the MM months. Furthermore, the presence of EVs in all milk samples was further validated by qualitative TEM analysis. All COL, FM, and MM samples analysed had typical vesicular morphology *i.e.*, round with intact membranes and were heterogenous in size.

Conclusion

This first longitudinal study of EVs in COL, FM and MM showed all sources to be rich in vesicles carrying typical EVs markers, intact membranes, and with expected heterogeneity in sizes. As we hypothesised, COL has substantially more EVs than MM. It is particularly enriched in small EVs. No significant differences were observed between EVs in months 1–9 of MM. Altogether, although COL is a particularly rich sources of EVs, mature milk throughout the lactation curve is also an abundant source of intact EVs. Thus, if a very rich source of EVs is required, we would suggest COL as an optimal source. However, for scalable sources -such as would be required if EVs are to be used as natural, cheap, safe drug delivery vehicles- there is reason to be optimistic that milk collected at any stage of the lactation curve would be suitable for this purpose.

Acknowledgements

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