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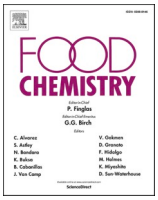

An investigation of extracellular vesicles in bovine colostrum, first milk and milk over the lactation curve

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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.

1. 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

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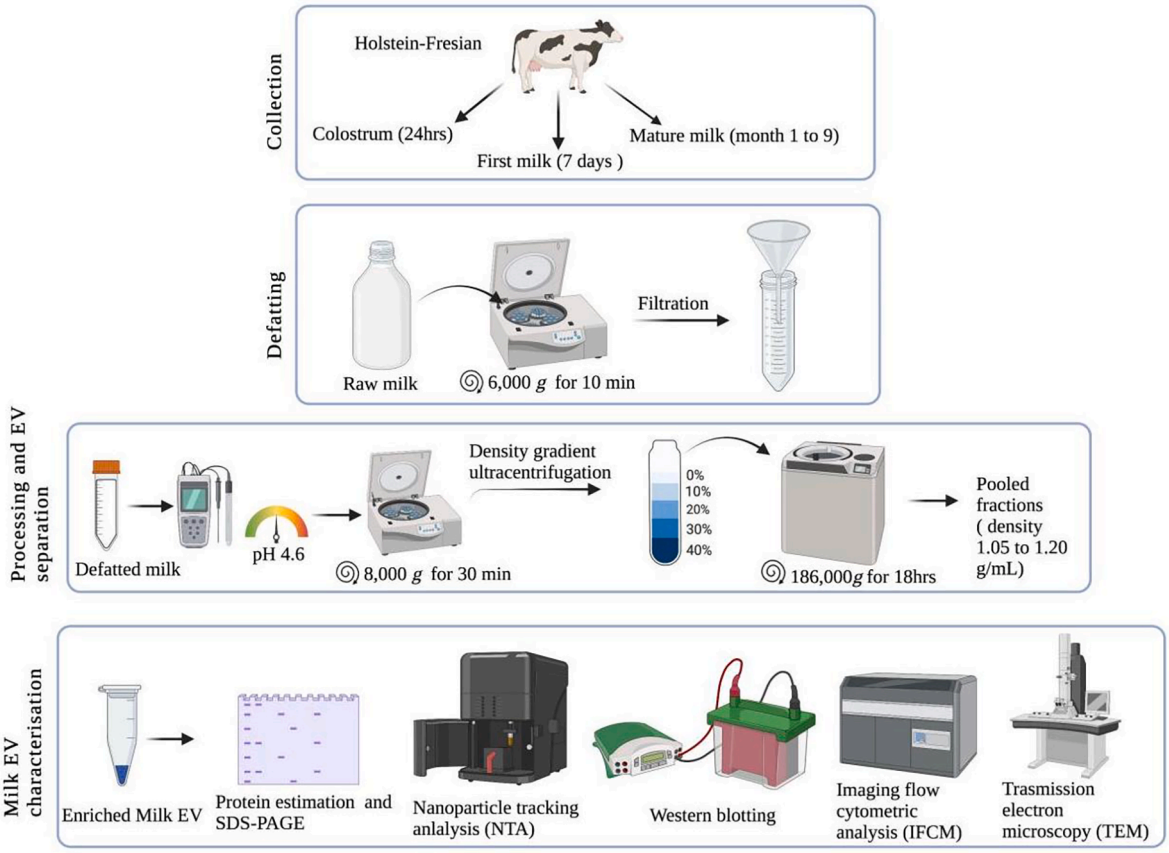

Fig. 1. Flowchart of the methodology used. Methodology including milk collection, pre-treatment and milk EV separation and characterisation from bovine colostrum, first milk and mature milk.

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 (Mukhopadhya et al., 2021a); and then performed comparative quantification and characterisation of the EVs.

2. Materials and methods

2.1. 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×*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).

2.2. Sample processing and EV separation

EVs were separated from COL, FM and MM using a method that we recently developed (Mukhopadhya et al., 2021a; Mukhopadhya 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.

2.3. 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).

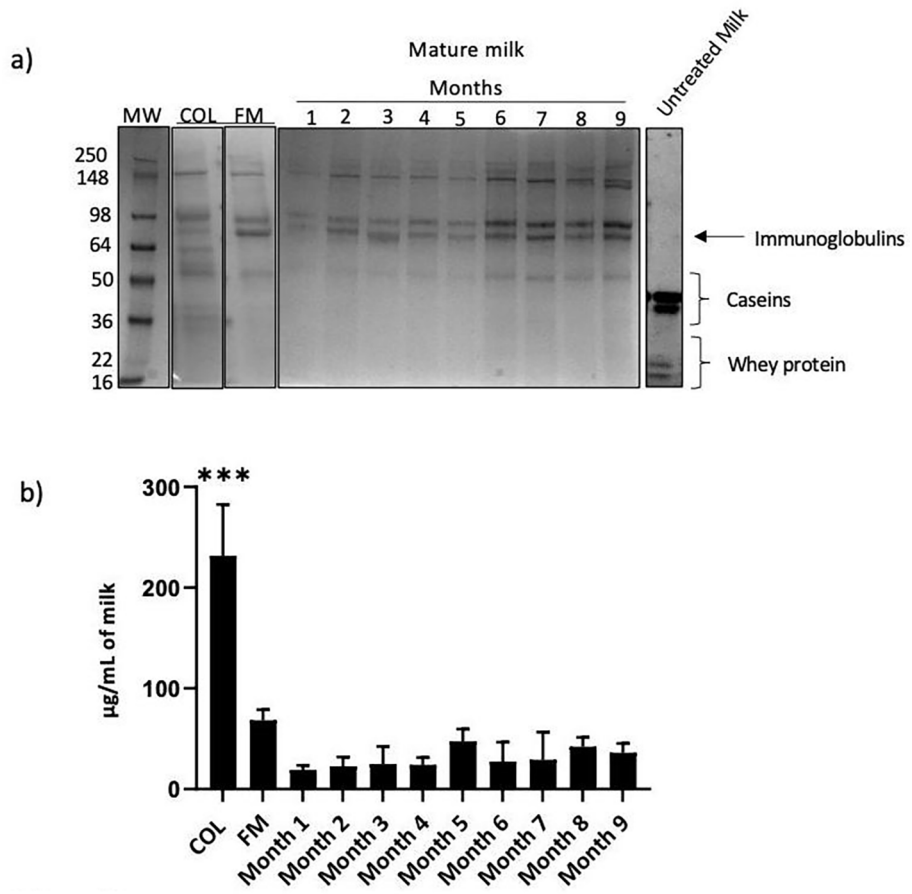

Fig. 2. Milk protein characterisation of bovine colostrum, first milk and mature milk a) Milk protein in EV enriched fraction of colostrum (COL), first milk (FM) and mature milk (MM) were visualised by loading 10 µg of protein on 10% Mini-PROTEAN® TGX™ Precast Gel followed by Coomassie Blue staining. 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 $p < 0.001$.

2.4. 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.

2.5. 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.

2.6. Immunoblotting analysis

Immunoblotting was performed as we previously described (O'Brien et al., 2013). In brief, EVs were lysed using SDS lysis buffer (250 nM 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.

2.7. Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis as we previously described (Mukhopadhya et al., 2021a). In brief, 5 µL of the 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, 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).

2.8. 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 we previously described (Mukhopadhya et al., 2021a). The specific antibodies used in this study were anti-CD63 conjugated with fluorescein

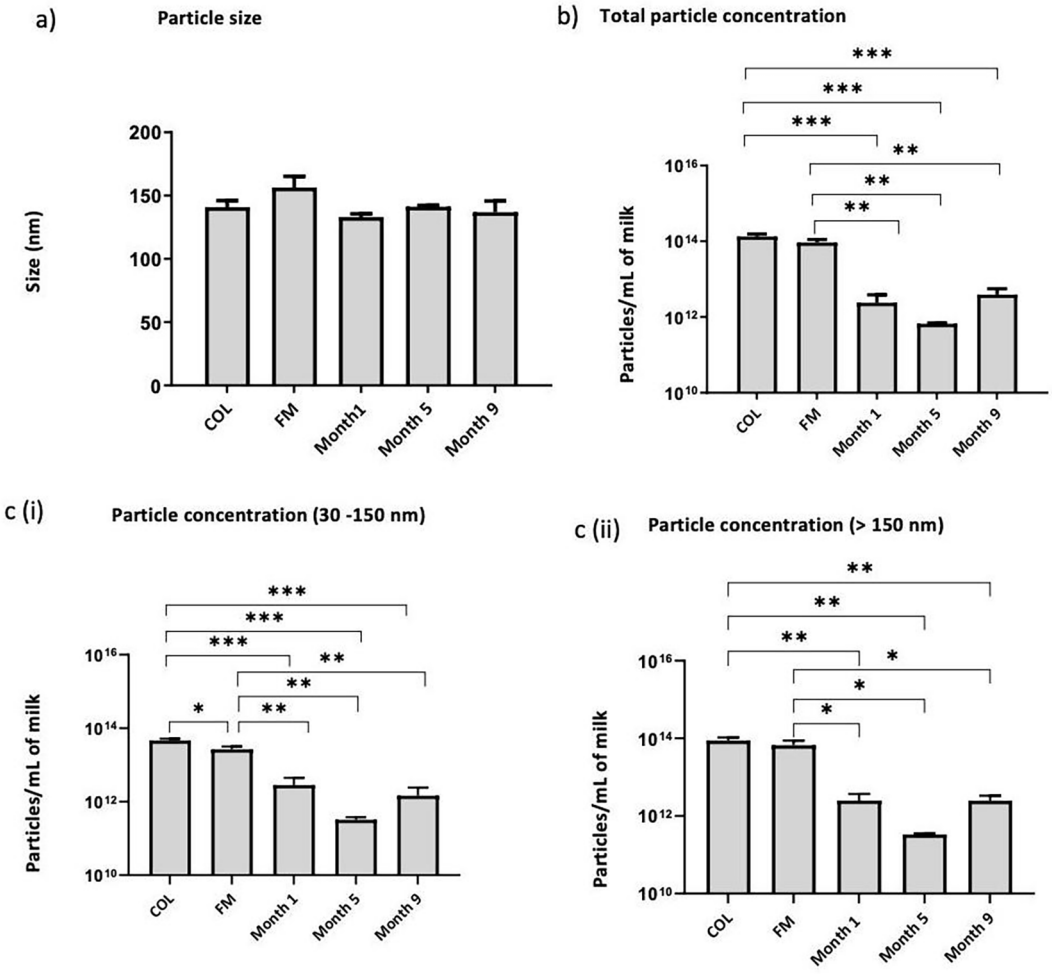

Fig. 3. Particle sizes and concentration from colostrum, first milk and mature milk EVs NTA analysis of particle sizes and concentration involved a minimum of four × 60-second videos recorded for each sample. a) Comparison of particle size in colostrum, first milk and mature milk. b) Total particle concentration 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 ± SEM, the significant p-value is reported; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

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 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 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).

2.9. Data analysis

Data presented in this study are the means of ≥ 3 experiments ± 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).

3. Result

3.1. 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. 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 ($p < 0.001$).

3.2. 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

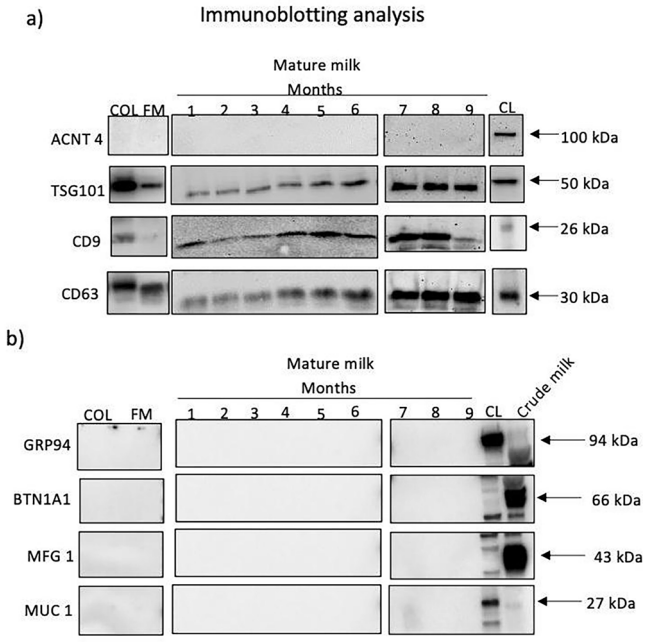

Fig. 4. Detection of EV specific markers by immunoblotting. Immunoblotting analysis of EVs separated from colostrum, first milk, and mature milk was performed by loading 30 μg of protein for all the EVs samples and CL (cell lysate). a) Analysis of EVs-associated positive markers TSG101, CD63, CD9 and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

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 heterogenous pool, COL ($p < 0.001$) and FM ($p < 0.01$) had significantly greater amounts of EVs when compared to MM. Specifically, COL had $1.3x10^{14} \pm 2.2x10^{13}$ particles/mL and FM had $9.3x10^{13} \pm 1.8x10^{13}$ particles/mL, whereas the corresponding values for MM particle concentration month 1 was $2.3x10^{12} \pm 1.5x10^{12}$ particles/mL, month 5 was $6.6x10^{11} \pm 3.0x10^{10}$ particles/mL and month 9 was $3.9x10^{12} \pm 1.6x10^{12}$ 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.

3.3. 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 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.

3.4. Presence of EV surface markers detected by imaging flow cytometric analysis

Quantitative characterisation of single intact COL, FM and MM EVs

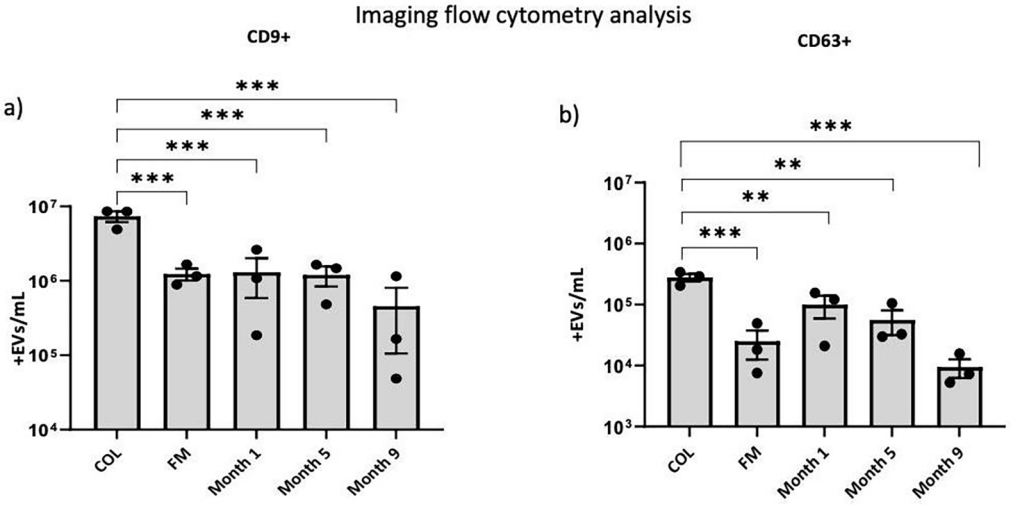

Fig. 5. Imaging flow cytometry analysis of EV surface marker. Number of positive events (particles) in colostrum, first milk and mature milk analysed by imaging flow cytometry (IFCM). Events were gated for low side scatter channel (SSC) signals and no signals on bright-field, based on EV size and events positive for these gating was acquired as objects (+EVs) in low SSC. Data presented as mean and individual values ± 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 ± SEM. One-way ANOVA tests were performed and significance is indicated as ** $p < 0.01$, *** $p < 0.001$.

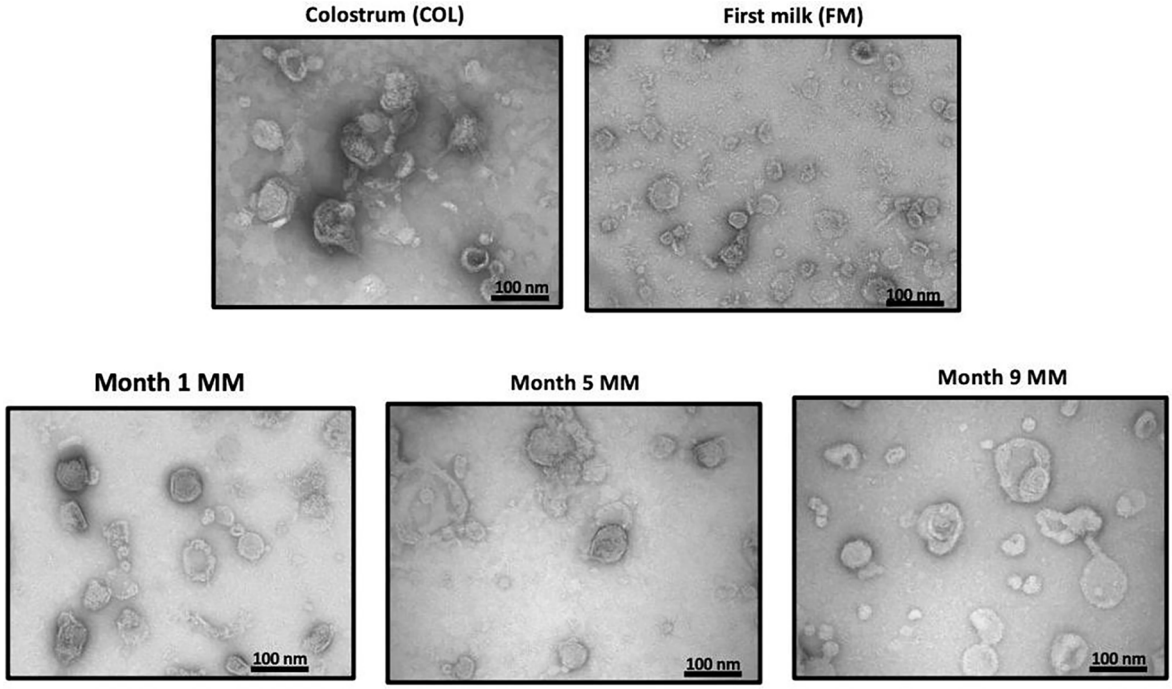

Fig. 6. Transmission electron microscopy analysis of EV morphology Representative transmission electron microscopy analysis of colostrum, first milk and mature milk EV samples. Scale bar = 100 nm.

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.

3.5. *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. 6).

4. 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 (Mukhopadhya 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 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 Samuel et al (2017) and 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 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 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.

5. 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134029.

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