Extracellular Vesicles from Animal Milk

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Contributor: Samanta Mecocci, Massimo Trabalza-Marinucci, Katia Cappelli

Milk represents the main source of nutrition for newborn mammals and serves as the conveyor of maternal messages of a sophisticated signaling system to promote postnatal health. It contains bioactive components that are essential for the development of the newborn immune system such as oligosaccharides, lactoferrin, lysozyme, alpha-lactalbumin, and immunoglobulins. Extracellular vesicles (EVs) were identified to be pivotal in this mother-to-child exchange. EVs are micro- and nanosized structures enclosed in a phospholipidic double-layer membrane that are produced by all cell types. They are released in the extracellular environment and reach close and distant cells. EVs can induce the modulation of biological processes in receiving cells after their absorption through the release of the molecular cargo contained within vesicles. In this way, EVs can also serve through immunomodulant anti-inflammatory, angiogenetic, and pro-regenerative actions depending on the cell of origin and patho/physiological conditions. EVs can be recovered from all biological fluids including milk.

Keywords: milk ; milk-derived EVs ; mEVs ; extracellular vesicles

1. Introduction

Milk represents the main source of nutrition for newborn mammals, but in addition, it delivers a sophisticated signaling system of maternal messages to promote postnatal health. Indeed, it contains bioactive components that are derived from various cell sources (myoepithelium and epithelium, adipose and connective tissues, lymphatic and blood vessels transporting nutritional components, and immune system cells). Milk is essential not only due to its nutritive elements, but also because it plays an important role in the development of the newborn immune system [1]. Recently, the molecular mechanisms underlying the mother-to-child information transfer have been further studied, highlighting the anti-infective and anti-inflammatory properties derived from oligosaccharides, lactoferrin, lysozyme, α-La, and immunoglobulins among the main players also able to shape the microbiota [2]. Moreover, milk is particularly enriched in transcription modulatory elements such as small RNAs [3], which can be found in different milk fractions (cells, lipids, and skim milk) as free molecules or packaged in vesicles [4][5][6]. The immunomodulatory activity of milk has been proven for humans, cows, and donkeys [7], with particular similarities between humans and donkeys regarding the anti-inflammatory properties and the capacity to regulate the balance between pro- and anti-inflammatory cytokines [8][9].

More recently, a pivotal role in these processes has been linked to extracellular vesicles (EVs), which are particular enriched in milk (mEVs), and the signaling molecules they carry [10][11].

EVs are micro- and nanosized structures enclosed in a phospholipidic double-layer membrane that mainly function as message-delivery vectors from producing cells to recipient cells by transferring the transported molecular cargo [12]. These EVs are released into the extracellular environment by virtually all cell types and have been recovered from every biological fluid, including blood, urine, bronchoalveolar lavage fluid, saliva, bile [13][14][15][16], and milk, which is one of the most promising scalable vesicle sources [17]. The regulation in the receiving cells is mediated by a plethora of molecules contained within EVs such as proteins, antigens, lipids, metabolites, RNAs, and DNA fragments [18][19][20]. The complex cargo induces a wide range of functional modulations in the receiving cells, depending on the type of recipient cell and the stimuli that these cells receive [21][22].

2. Milk-Derived EV Isolation: A Critical Point to Overcome

Different EV isolation methods have been developed on the basis of the physical or molecular features of vesicles [14][15]. However, their small size makes this step a real challenge. In fact, a gold-standard method is still missing because a certain degree of bias remains due to the co-isolation of contaminants and protein aggregates. This is particularly true when the vesicles are isolated from complex matrices such as milk, which contains milk fat globules and spherical colloidal aggregates of caseins (casein micelles) that overlap in size with the EVs [23]. In the case of milk, another consideration must be made related to the starting material. It is known that industrialisation processes can affect the

composition of milk in terms of bioactive molecules in different manners related to the applied procedure [24][25]. A great number of mEV evaluations have been made using raw milk, although its consumption is not allowed in some countries due to hygiene-related problems. This could lead to the use of mEVs derived from industrially processed milk, although modifications in mEV numbers and integrity have emerged that indicated the possible alteration of the bioactive component transfer through heat-treated commercial milk [26][27].

A widely used method for EV isolation is based on differential ultracentrifugation (dUC), which allows the sedimentation of the solute through the use of an elevated centrifugation force [28]. In general, some preliminary centrifugation steps are performed before dUC to eliminate cells and cell debris. Generally, 10,000× *g* allows the isolation of apoptotic bodies and larger vesicles; around 35,000× *g* can be used for medium vesicles; and 100,000× *g* is appropriate for smaller EVs. The pellets can be further resuspended for a washing passage to repeat the same ultracentrifugation (UC) step for their recovery [29]. Other than being one of the most time-consuming techniques requiring operator experience and an ultracentrifuge instrument, it is not suitable to isolate vesicles from milk due to the very low mEV purity [30][31][32][33][34]. Centrifuging at such high speeds can damage the EV membranes and shapes and can favour vesicle aggregation and soluble protein co-isolation [34][35][36].

To avoid excessive protein contamination, many strategies can be applied, including the addition of acetic acid, sodium citrate, or ethylendiaminotetracetyc acid (EDTA), which allow for protein precipitation as widely used for mEV isolation [37][38][39][40].

Methods that increase the EV purity and separation of subtypes are based on the floatation on density gradients inserted between dUC steps, which allows for the separation of EVs from non-EV material including proteins or protein–RNA aggregates [41][42][43][44][45]. These density gradients can be continuous (such as when using iodixanol) or discontinuous (when using sucrose); the samples can be loaded for a bottom-up or a top-down migration. The limitations of these methods are similar to those for dUC and are even more pronounced; they are linked to operator skills as well as the time and the instrument required, and also have a low-rate EV recovery.

One of the most used methods for EV isolation is size-exclusion chromatography (SEC) [46], which was first applied to demonstrate the presence of different protein sets between the EV cargo and the solution [47]. It exploits the dimensional features of EVs that constitute the mobile phase, which is made to flow inside the stationary phase of an SEC column consisting of a porous polymer. Extracellular vehicles with larger sizes elute first and travel quicker than the smaller ones, which can precipitate together with protein aggregates. However, the co-isolation of protein aggregates that share the same size range as EVs is a major drawback [48], making the use of SEC alone unfeasible for mEV isolation due to the presence of a great number of proteins in the milk serum.

Similarly, ultrafiltration (UF) is an easy method for EV isolation that uses semipermeable membranes characterised by pores [49][50]; however, it does not allow for separating the different EV subpopulations, and soluble proteins are recovered in the pellet together with EVs, which makes this method merely a useful preliminary step for volume reduction without causing EV damage [51][52].

Precipitation-based protocols, including the use of polyethylene glycol (PEG), are easy to use and cheap and have allowed the recovery of large amounts of EVs with an elevated grade of standardisation and scalability [53]. Unfortunately, these methods are characterised by a low grade of purity because all of the soluble particles precipitate, making them unusable for descriptive or functional analysis, particularly for mEVs [54][55].

Another approach to vesicle isolation exploits the surface antigens of EVs through immunoaffinity-based methods, which are generally integrated with analytic tests such as ELISA-like or magnetic isolation [56]. To ensure the recovery of the majority of the vesicles, several commercial kits have implemented the simultaneous use of the tetraspanins CD9, CD63, and CD81 [57] or wide EV-binding molecules such as heparin, heat-shock proteins, phosphatidylserine-binding peptides, or membrane-curvature sensor peptides [58][59][60]. Immunoaffinity isolation is particularly useful in pathological fields such as cancer studies, although it is highly expensive.

Concerning the accuracy and purity when isolating EVs, flow cytometry (FC) is one of the most satisfying methods; it allows for a high-throughput, multiparametric analysis and the separation of single EVs based on their surface composition. However, particles measuring less than 600 nm are not detected due to the limit of the forward/side-scattered (FSC/SSC) light detectors [61]. This is a problem that can be solved by using fluorescently labelled EVs or high-resolution flow cytometers, which allow for EV analysis and sorting [62][63][64]. Although a better standardisation is needed, recent high-resolution FC showed advantages related to the very small sample volume required and the possibility of

evaluating differences in the packaging of biomolecules during biogenesis [65]. Despite their many advantages, applications to real milk have yet to be proven for these methods.

Some promising recently developed methods are based on field-flow fractionation (FFF) and microfluidics. Field-flow fractionation combines the application of a field (thermal energy, centrifugal force, electrostatic force, and cross/tangential flow applied through one or two semipermeable membranes) that allows the separation of the particles into different layers and a longitudinal flow that carries particles through the channel, which leads to their recovery [66]. An upgrade to this technology was developed by Marsh et al. [67] through the incorporation of solubilisation steps and the optimization of time, levels of temperature, and divalent cation chelation, which allowed for providing a pure and scalable production of mEVs [67]. This is an emerging technology for EV separation that is characterised by important desirable features such as a high resolution, high purity, and potential for large-scale production. On the other hand, microfluidic technologies and on-chip biosensors have allowed for high-throughput analyses using a minimal sample volume and reagent consumption in integrated miniaturised devices. The EV separation can be done based on size, surface markers, or new sorting tools such as the application of acoustic, electrophoretic, or electromagnetic fields. Thus, these innovative methods lack a solid standardisation but represent the future of EV isolation [68][69]. Meanwhile, a combined approach using at least two or three consecutive methods is recommended for an appropriate milk-derived EV isolation [70]. It should be considered and remembered that different isolation methods can produce distinct fractions of EVs with different degrees of purity which can be reflected in nonhomogeneous effects in functional studies [71][72].

3. The Theranostic Potential of Animal mEVs

Given the intrinsic beneficial effects of mEVs on gut homeostasis, one of the major promising animal mEV applications is their addition to infant formulae to prevent the development of necrotizing colitis in high-risk infants when breast milk is not available or their use as an adjuvant in IBD. Nevertheless, more studies are needed to clarify their role in cell proliferation, the prevention of fibrosis, angiogenesis, and in cancer treatments, as well as their unwanted side effects. Indeed, Melnik and Schmitz (2019) [73] reported on the potential “dark side” of some molecules contained in pasteurised milk vesicles and highlighted possible adverse effects that the cargo could induce in the receiving cells and tissues by attributing to it a substantial risk for the onset of adulthood chronic metabolic diseases in Western countries [73]. Although this direct cause-and-effect relationship is far from being demonstrated, the benefits appear to be tangible and have a strong potential. A large number of studies in the target therapy field that used EVs as a drug delivery system have recently been published [74]. This application is been driven by the advantages that EVs showed compared to synthetic therapeutic nanocarriers such as liposomes, thereby revealing a wider biodistribution and biocompatibility and a higher internalisation rate [75]. Furthermore, milk can help provide an elevated quantity of vesicles, in addition to being a widely available and inexpensive raw material that is particularly enriched in EVs, and thus represents a promising source of EVs for massive production. These mEV characteristics are ideal for theranostic applications.

Indeed, mEVs can be loaded with chemotherapeutics or other therapeutic molecules such as nucleic acids [76][77][78]. Molecules can be loaded into mEVs through several methods; for example, for RNAs, exploiting molecules contained in mEVs such as GAPDH can help bind lactoferrin that has been electrostatically loaded with small interfering RNAs (siRNAs) [79]. Fluorescently labelled siRNA loaded in mEVs through the lipofection process has been used to demonstrate EV protection and siRNA delivery with promising results [80]. Warren et al. [81] identified cationic chemical transfection as a good method for siRNA loading in mEVs; this turned out to be more efficient than electroporation. Moreover, coating with polyethylene glycol (PEG) increased mEV resistance in the acidic gastric environment and the permeability through the mucin layer, although a high enterocyte uptake and siRNA delivery were also demonstrated for unmodified mEVs [81]. The ultrasonic coating of mEVs with B-cell lymphoma (bcl)-2 siRNA was demonstrated to be effective in reducing cancer cell proliferation and migration through the downregulation of metastatic-related genes both in vitro and in vivo [82]. This method for administering siRNA using mEVs seems to be safe because no toxicity and side effects related to immune response and inflammation were detected after multiple dosings in mice [83]. The miR-31-5p depletion typical of diabetic wounds was recovered by miRNA mEV loading through electroporation, which enhanced wound healing and angiogenesis [84]. Moreover, mEVs were shown to be an efficient shuttle for the oral delivery of locked nucleic-acid-modified antisense oligonucleotides, which are potent RNA and protein modulators whose use in therapeutics was recently proposed [85]. In addition, mEVs can be useful in the delivery of poorly absorbable drugs such as curcumin by improving the stability, solubility, bioavailability, and intestinal uptake through the oral route of administration [86][87]. The anticancer effects of curcumin and resveratrol were amplified by their loading into mEVs, thereby helping to avoid the ATP-binding cassette transporter mechanism of chemoresistance normally used by cancer cells [88]. The potent chemotherapeutic paclitaxel was one of the first to be tested for loading in EVs [89] and was recently used in experiments with mEVs; it was shown that it could efficiently inhibit cancer growth, especially when orally administered instead of being

administered by intraperitoneal injection, and showed no toxicity to normal cells [77][90]. One study showed that the sublingual administration of mEVs loaded with the antidiabetic drug liraglutide reduced blood glucose in diabetic mice; however, the same effect was not detected when administered via oral gavage [91]. These differences could be due to the efficiency of the drug loading into vesicles because the liraglutide–mEVs prepared with the extrusion method showed 2.45 times the drug load compared to the mEVs prepared via direct incubation, although the method was superior to sonication, freeze–thaw cycle, saponin-assisted, and electroporation methods [92]. Loading features could be influenced by the species of origin when producing mEVs, as well as by the consequent functional efficiency. In a comparative study of cow, buffalo, and goat mEVs loaded with the chemotherapeutic doxorubicin through three different methods, goat mEVs showed a higher loading capacity across all of the methods compared to the other two species; this was reflected in their efficacy in inducing cell apoptosis in cancer cells in vitro [93]. Doxorubicin-loaded-mEV target therapy was previously tested through the induction of hyaluronan expression onto the phospholipid bilayer that is a ligand for the CD44 receptor, thus allowing for CD44-overexpressing, cell-specific drug delivery [94]. Indeed, mEVs have been evaluated for drug loading and its effects in several molecules and pathologies, especially cancer, with promising and encouraging results in terms of efficacy and safety; the drug power was amplified but the side effects were reduced by the action of the mEVs being more targeted [95][96][97][98][99].

The use of EVs as theranostics, especially for human clinical applications, has rapidly grown in recent years. The ISEV held a workshop in 2018 on “EVs in Clinical Theranostic” to discuss technical issues and standardisation in order to increase the power of EVs in clinical practice [100]. Concerning mEVs and animal clinical science, particularly regarding their use as biomarkers of pathological conditions, the research is in its infancy because the first study on the topic appeared just four years ago and only a few studies have been carried out to date. The mEV RNA contents in the mastitis of cows were the first studied; Cai et al. [101] identified 18 differentially expressed miRNAs in the mEVs of healthy and infected animals whose targets participated in immune system processes and inflammation pathways, and the results indicated miR-223 and miR-142-5p as potential candidate biomarkers of mastitis [101]. Later, two other miRNAs were shown to play important roles in the response to infection with *Staphylococcus aureus*, miR-378, and miR-185, and were found to be particularly abundant and highly differentially expressed in mastitic milk compared to normal milk [102]. A specific packaging of another type of small RNAs—the circRNAs—was highlighted by Ma et al. [103] in response to bacterial infections because the presence of *Staphylococcus aureus* induced a different set of circRNAs in mEVs, most of which were implicated in immune functions [103]. In the case of subclinical mastitis, in which the symptoms are masked, the identification of biomarkers is crucial but more complex. Indeed, the molecules produced by cells, even those contained in mEVs, can vary between infection stages and according to other environmental variables. For this reason, Saenz-de-Juano and collaborators [104] evaluated the changes in mEV size and concentrations using tunable resistive pulse sensing (TRPS) and the miRNA contents during three consecutive days of sampling by comparing the differences between mEVs from naturally infected udder quarters, their healthy adjacent quarters, and quarters from uninfected udders [104]. Chronic subclinical mastitis did not show differences in the mEV number and size compared to healthy udders; the miRNA contents remained the same regardless of the health status of the quarter during the three sampling days. Only individual-cow changes were observed, which confirmed miR-223-3p as the most expressed miRNA in all of the chronic subclinical mastitis quarters [104]. In addition, bovine leukemia virus (BLV) infection was investigated in order to highlight potential mEV biomarkers for preventing the spread of the virus and thereby reducing economic losses for farmers. Indeed, the mRNA and protein expression profiles of mEVs were found to be modified by BLV infections; their monitoring can be useful in evaluating the clinical stages of the infection [105][106]. In particular, the concentrations of eight mRNAs were increased in the mEVs of infected cattle by Hiraoka and collaborators, which suggested a combined evaluation of the expression profiles of these genes to identify early BLV infections [107]. On the contrary, miR-424-5p was found to be significantly upregulated in the mEVs of infected animals relative to healthy ones; this was also validated in the re-qPCR of a cohort of animals selected ad hoc, thus identifying it as a valuable biomarker to identify high-risk animals for BLV transmission [108]. Recent work suggested the use of mEVs as biomarkers in heat-stressed cows; several differentially expressed miRNAs were detected whose targets were implicated in apoptosis, autophagy, and the p38 MAPK pathways, thus possibly regulating heat-stress resistance in dairy cows [109].

Nevertheless, associating mEV cargo variations with specific pathological conditions is a challenge that needs further investigation due to the interchange between the metabolic imbalance and infectious disease/stressful stimuli that can occur in the mammary gland.

References

1. Cacho, N.T.; Lawrence, R.M. Innate Immunity and Breast Milk. Front. Immunol. 2017, 8, 584.

2. Morrin, S.T.; Buck, R.H.; Farrow, M.; Hickey, R.M. Milk-Derived Anti-Infectives and Their Potential to Combat Bacterial and Viral Infection. J. Funct. Foods 2021, 81, 104442.
3. Andreas, N.J.; Kampmann, B.; Mehring Le-Doare, K. Human Breast Milk: A Review on Its Composition and Bioactivity. Early Hum. Dev. 2015, 91, 629–635.
4. Tomé-Carneiro, J.; Fernández-Alonso, N.; Tomás-Zapico, C.; Visioli, F.; Iglesias-Gutierrez, E.; Dávalos, A. Breast Milk MicroRNAs Harsh Journey towards Potential Effects in Infant Development and Maturation. Lipid Encapsulation Can Help. Pharmacol. Res. 2018, 132, 21–32.
5. Zeng, B.; Chen, T.; Luo, J.; Xie, M.; Wei, L.; Xi, Q.; Sun, J.; Zhang, Y. Exploration of Long Non-Coding RNAs and Circular RNAs in Porcine Milk Exosomes. Front. Genet. 2020, 11, 652.
6. Rubio, M.; Bustamante, M.; Hernandez-Ferrer, C.; Fernandez-Orth, D.; Pantano, L.; Sarria, Y.; Piqué-Borras, M.; Vellve, K.; Agramunt, S.; Carreras, R.; et al. Circulating MiRNAs, IsomiRs and Small RNA Clusters in Human Plasma and Breast Milk. PLoS ONE 2018, 13, e0193527.
7. Trinchese, G.; Cavaliere, G.; De Filippo, C.; Aceto, S.; Prisco, M.; Chun, J.T.; Penna, E.; Negri, R.; Muredda, L.; Demurtas, A.; et al. Human Milk and Donkey Milk, Compared to Cow Milk, Reduce Inflammatory Mediators and Modulate Glucose and Lipid Metabolism, Acting on Mitochondrial Function and Oleylethanolamide Levels in Rat Skeletal Muscle. Front. Physiol. 2018, 9, 32.
8. Quintanilha, B.; Reis, B.; Duarte, G.; Cozzolino, S.; Rogero, M. Nutrimiromics: Role of MicroRNAs and Nutrition in Modulating Inflammation and Chronic Diseases. Nutrients 2017, 9, 1168.
9. Chiofalo, B.; Dugo, P.; Bonaccorsi, I.L.; Mondello, L. Comparison of Major Lipid Components in Human and Donkey Milk: New Perspectives for a Hypoallergenic Diet in Humans. Immunopharmacol. Immunotoxicol. 2011, 33, 633–644.
10. Melnik, B.C.; Schmitz, G. MicroRNAs: Milk's Epigenetic Regulators. Best Pract. Res. Clin. Endocrinol. Metab. 2017, 31, 427–442.
11. Zonneveld, M.I.; van Herwijnen, M.J.C.; Fernandez-Gutierrez, M.M.; Giovanazzi, A.; de Groot, A.M.; Kleinjan, M.; van Capel, T.M.M.; Sijts, A.J.A.M.; Taams, L.S.; Garssen, J.; et al. Human Milk Extracellular Vesicles Target Nodes in Interconnected Signalling Pathways That Enhance Oral Epithelial Barrier Function and Dampen Immune Responses. J. Extracell. Vesicles 2021, 10, e12071.
12. Zhang, Y.; Liu, Y.; Liu, H.; Tang, W.H. Exosomes: Biogenesis, Biologic Function and Clinical Potential. Cell Biosci. 2019, 9, 19.
13. Ogawa, Y.; Miura, Y.; Harazono, A.; Kanai-Azuma, M.; Akimoto, Y.; Kawakami, H.; Yamaguchi, T.; Toda, T.; Endo, T.; Tsubuki, M.; et al. Proteomic Analysis of Two Types of Exosomes in Human Whole Saliva. Biol. Pharm. Bull. 2011, 34, 13–23.
14. Masyuk, A.I.; Huang, B.Q.; Ward, C.J.; Gradilone, S.A.; Banales, J.M.; Masyuk, T.V.; Radtke, B.; Splinter, P.L.; LaRusso, N.F. Biliary Exosomes Influence Cholangiocyte Regulatory Mechanisms and Proliferation through Interaction with Primary Cilia. Am. J. Physiol. Gastrointest. Liver Physiol. 2010, 299, G990–G999.
15. Admyre, C.; Grunewald, J.; Thyberg, J.; Gripenbäck, S.; Tornling, G.; Eklund, A.; Scheynius, A.; Gabrielsson, S. Exosomes with Major Histocompatibility Complex Class II and Co-Stimulatory Molecules Are Present in Human BAL Fluid. Eur. Respir. J. 2003, 22, 578–583.
16. Dear, J.W.; Street, J.M.; Bailey, M.A. Urinary Exosomes: A Reservoir for Biomarker Discovery and Potential Mediators of Intrarenal Signalling. Proteomics 2013, 13, 1572–1580.
17. Admyre, C.; Johansson, S.M.; Qazi, K.R.; Filen, J.-J.; Lahesmaa, R.; Norman, M.; Neve, E.P.A.; Scheynius, A.; Gabrielsson, S. Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. J. Immunol. 2007, 179, 1969–1978.
18. Kalluri, R.; LeBleu, V.S. The Biology, Function, and Biomedical Applications of Exosomes. Science 2020, 367, eaau6977.
19. EL Andaloussi, S.; Mäger, I.; Breakefield, X.O.; Wood, M.J.A. Extracellular Vesicles: Biology and Emerging Therapeutic Opportunities. Nat. Rev. Drug Discov. 2013, 12, 347–357.
20. Malkin, E.Z.; Bratman, S.V. Bioactive DNA from Extracellular Vesicles and Particles. Cell Death Dis. 2020, 11, 584.
21. Lucchetti, D.; Ricciardi Tenore, C.; Colella, F.; Sgambato, A. Extracellular Vesicles and Cancer: A Focus on Metabolism, Cytokines, and Immunity. Cancers 2020, 12, 171.
22. Capomaccio, S.; Cappelli, K.; Bazzucchi, C.; Coletti, M.; Gialletti, R.; Moriconi, F.; Passamonti, F.; Pepe, M.; Petrini, S.; Mecocci, S.; et al. Equine Adipose-Derived Mesenchymal Stromal Cells Release Extracellular Vesicles Enclosing Different Subsets of Small RNAs. Stem Cells Int. 2019, 2019, 4957806.

23. Hu, Y.; Thaler, J.; Nieuwland, R. Extracellular Vesicles in Human Milk. Pharmaceuticals 2021, 14, 1050.

24. Shome, S.; Jernigan, R.L.; Beitz, D.C.; Clark, S.; Testroet, E.D. Non-Coding RNA in Raw and Commercially Processed Milk and Putative Targets Related to Growth and Immune-Response. BMC Genom. 2021, 22, 749.

25. Zhao, Z.; Yu, S.; Xu, M.; Li, P. Effects of Microwave on Extracellular Vesicles and MicroRNA in Milk. J. Dairy Sci. 2018, 101, 2932–2940.

26. Kleinjan, M.; van Herwijnen, M.J.; Libregts, S.F.; van Neerven, R.J.; Feitsma, A.L.; Wauben, M.H. Regular Industrial Processing of Bovine Milk Impacts the Integrity and Molecular Composition of Extracellular Vesicles. J. Nutr. 2021, 151, 1416–1425.

27. Yu, S.; Zhao, Z.; Sun, L.; Li, P. Fermentation Results in Quantitative Changes in Milk-Derived Exosomes and Different Effects on Cell Growth and Survival. J. Agric. Food Chem. 2017, 65, 1220–1228.

28. Gardiner, C.; Vizio, D.D.; Sahoo, S.; Théry, C.; Witwer, K.W.; Wauben, M.; Hill, A.F. Techniques Used for the Isolation and Characterization of Extracellular Vesicles: Results of a Worldwide Survey. J. Extracell. Vesicles 2016, 5, 32945.

29. Théry, C.; Amigorena, S.; Raposo, G.; Clayton, A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. Curr. Protoc. Cell Biol. 2006, 30, 3–22.

30. Momen-Heravi, F.; Balaj, L.; Alian, S.; Trachtenberg, A.J.; Hochberg, F.H.; Skog, J.; Kuo, W.P. Impact of Biofluid Viscosity on Size and Sedimentation Efficiency of the Isolated Microvesicles. Front. Physiol. 2012, 3, 162.

31. Livshits, M.A.; Khomyakova, E.; Evtushenko, E.G.; Lazarev, V.N.; Kulemin, N.A.; Semina, S.E.; Generozov, E.V.; Govorun, V.M. Isolation of Exosomes by Differential Centrifugation: Theoretical Analysis of a Commonly Used Protocol. Sci. Rep. 2015, 5, 17319.

32. Taylor, D.D.; Shah, S. Methods of Isolating Extracellular Vesicles Impact Down-Stream Analyses of Their Cargoes. Methods 2015, 87, 3–10.

33. Cvjetkovic, A.; Lötvall, J.; Lässer, C. The Influence of Rotor Type and Centrifugation Time on the Yield and Purity of Extracellular Vesicles. J. Extracell. Vesicles 2014, 3, 23111.

34. Baranyai, T.; Herczeg, K.; Onódi, Z.; Voszka, I.; Módos, K.; Marton, N.; Nagy, G.; Mäger, I.; Wood, M.J.; El Andaloussi, S.; et al. Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. PLoS ONE 2015, 10, e0145686.

35. Linares, R.; Tan, S.; Gounou, C.; Arraud, N.; Brisson, A.R. High-Speed Centrifugation Induces Aggregation of Extracellular Vesicles. J. Extracell. Vesicles 2015, 4, 29509.

36. van der Pol, E.; Böing, A.N.; Harrison, P.; Sturk, A.; Nieuwland, R. Classification, Functions, and Clinical Relevance of Extracellular Vesicles. Pharm. Rev. 2012, 64, 676–705.

37. Somiya, M.; Yoshioka, Y.; Ochiya, T. Biocompatibility of Highly Purified Bovine Milk-Derived Extracellular Vesicles. J. Extracell. Vesicles 2018, 7, 1440132.

38. Vaswani, K.; Koh, Y.Q.; Almughlliq, F.B.; Peiris, H.N.; Mitchell, M.D. A Method for the Isolation and Enrichment of Purified Bovine Milk Exosomes. Reprod. Biol. 2017, 17, 341–348.

39. Kusuma, R.J.; Manca, S.; Friemel, T.; Sukreet, S.; Nguyen, C.; Zempleni, J. Human Vascular Endothelial Cells Transport Foreign Exosomes from Cow's Milk by Endocytosis. Am. J. Physiol. Cell Physiol. 2016, 310, C800–C807.

40. Benmoussa, A.; Michel, S.; Gilbert, C.; Provost, P. Isolating Multiple Extracellular Vesicles Subsets, Including Exosomes and Membrane Vesicles, from Bovine Milk Using Sodium Citrate and Differential Ultracentrifugation. Bio. Protoc. 2020, 10, e3636.

41. Willms, E.; Johansson, H.J.; Mäger, I.; Lee, Y.; Blomberg, K.E.M.; Sadik, M.; Alaarg, A.; Smith, C.I.E.; Lehtiö, J.; EL Andaloussi, S.; et al. Cells Release Subpopulations of Exosomes with Distinct Molecular and Biological Properties. Sci. Rep. 2016, 6, 22519.

42. Webber, J.; Clayton, A. How Pure Are Your Vesicles? J. Extracell. Vesicles 2013, 2, 19861.

43. Bobrie, A.; Colombo, M.; Krumeich, S.; Raposo, G.; Théry, C. Diverse Subpopulations of Vesicles Secreted by Different Intracellular Mechanisms Are Present in Exosome Preparations Obtained by Differential Ultracentrifugation. J. Extracell. Vesicles 2012, 1, 18397.

44. Aalberts, M.; van Dissel-Emiliani, F.M.F.; van Adrichem, N.P.H.; van Wijnen, M.; Wauben, M.H.M.; Stout, T.A.E.; Stoorvogel, W. Identification of Distinct Populations of Prostasomes That Differentially Express Prostate Stem Cell Antigen, Annexin A1, and GLIPR2 in Humans1. Biol. Reprod. 2012, 86, 1–8.

45. Jeppesen, D.K.; Hvam, M.L.; Primdahl-Bengtson, B.; Boysen, A.T.; Whitehead, B.; Dyrskjøt, L.; Ørntoft, T.F.; Howard, K.A.; Ostenfeld, M.S. Comparative Analysis of Discrete Exosome Fractions Obtained by Differential Centrifugation. J. Extracell. Vesicles 2014, 3, 25011.

46. Böing, A.N.; van der Pol, E.; Grootemaat, A.E.; Coumans, F.A.W.; Sturk, A.; Nieuwland, R. Single-Step Isolation of Extracellular Vesicles by Size-Exclusion Chromatography. J. Extracell. Vesicles 2014, 3, 23430.

47. Pan, B.-T.; Johnstone, R.M. Fate of the Transferrin Receptor during Maturation of Sheep Reticulocytes in Vitro: Selective Externalization of the Receptor. Cell 1983, 33, 967–978.

48. Liangsupree, T.; Multia, E.; Riekkola, M.-L. Modern Isolation and Separation Techniques for Extracellular Vesicles. J. Chromatogr. A 2021, 1636, 461773.

49. Xu, R.; Simpson, R.J.; Greening, D.W. A Protocol for Isolation and Proteomic Characterization of Distinct Extracellular Vesicle Subtypes by Sequential Centrifugal Ultrafiltration. In Exosomes and Microvesicles; Hill, A.F., Ed.; Methods in Molecular Biology; Springer: New York, NY, USA, 2017; Volume 1545, pp. 91–116. ISBN 978-1-4939-6726-1.

50. Xu, R.; Greening, D.W.; Rai, A.; Ji, H.; Simpson, R.J. Highly-Purified Exosomes and Shed Microvesicles Isolated from the Human Colon Cancer Cell Line LIM1863 by Sequential Centrifugal Ultrafiltration Are Biochemically and Functionally Distinct. Methods 2015, 87, 11–25.

51. Welton, J.L.; Webber, J.P.; Botos, L.-A.; Jones, M.; Clayton, A. Ready-Made Chromatography Columns for Extracellular Vesicle Isolation from Plasma. J. Extracell. Vesicles 2015, 4, v4.27269.

52. Musante, L.; Tataruch, D.; Gu, D.; Benito-Martin, A.; Calzaferri, G.; Aherne, S.; Holthofer, H. A Simplified Method to Recover Urinary Vesicles for Clinical Applications and Sample Banking. Sci. Rep. 2015, 4, 7532.

53. Gámez-Valero, A.; Monguió-Tortajada, M.; Carreras-Planella, L.; Franquesa, M.; Beyer, K.; Borràs, F.E. Size-Exclusion Chromatography-Based Isolation Minimally Alters Extracellular Vesicles' Characteristics Compared to Precipitating Agents. Sci. Rep. 2016, 6, 33641.

54. Konoshenko, M.Y.; Lekchnov, E.A.; Vlassov, A.V.; Laktionov, P.P. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. Biomed. Res. Int. 2018, 2018, 8545347.

55. Lobb, R.J.; Becker, M.; Wen Wen, S.; Wong, C.S.F.; Wiegmans, A.P.; Leimgruber, A.; Möller, A. Optimized Exosome Isolation Protocol for Cell Culture Supernatant and Human Plasma. J. Extracell. Vesicles 2015, 4, 27031.

56. Koliha, N.; Wiencek, Y.; Heider, U.; Jüngst, C.; Kladt, N.; Krauthäuser, S.; Johnston, I.C.D.; Bosio, A.; Schauss, A.; Wild, S. A Novel Multiplex Bead-Based Platform Highlights the Diversity of Extracellular Vesicles. J. Extracell. Vesicles 2016, 5, 29975.

57. Wiklander, O.P.B.; Bostancioglu, R.B.; Welsh, J.A.; Zickler, A.M.; Murke, F.; Corso, G.; Felldin, U.; Hagey, D.W.; Evertsson, B.; Liang, X.-M.; et al. Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures. Front. Immunol. 2018, 9, 1326.

58. Balaj, L.; Atai, N.A.; Chen, W.; Mu, D.; Tannous, B.A.; Breakefield, X.O.; Skog, J.; Maguire, C.A. Heparin Affinity Purification of Extracellular Vesicles. Sci. Rep. 2015, 5, 10266.

59. Ghosh, A.; Davey, M.; Chute, I.C.; Griffiths, S.G.; Lewis, S.; Chacko, S.; Barnett, D.; Crapoulet, N.; Fournier, S.; Joy, A.; et al. Rapid Isolation of Extracellular Vesicles from Cell Culture and Biological Fluids Using a Synthetic Peptide with Specific Affinity for Heat Shock Proteins. PLoS ONE 2014, 9, e110443.

60. Nakai, W.; Yoshida, T.; Diez, D.; Miyatake, Y.; Nishibu, T.; Imawaka, N.; Naruse, K.; Sadamura, Y.; Hanayama, R. A Novel Affinity-Based Method for the Isolation of Highly Purified Extracellular Vesicles. Sci. Rep. 2016, 6, 33935.

61. Welsh, J.A.; Holloway, J.A.; Wilkinson, J.S.; Englyst, N.A. Extracellular Vesicle Flow Cytometry Analysis and Standardization. Front. Cell Dev. Biol. 2017, 5, 78.

62. Kormelink, T.G.; Arkesteijn, G.J.A.; Nauwelaers, F.A.; van den Engh, G.; Nolte-'t Hoen, E.N.M.; Wauben, M.H.M. Prerequisites for the Analysis and Sorting of Extracellular Vesicle Subpopulations by High-Resolution Flow Cytometry. Cytom. Part A 2016, 89, 135–147.

63. Higginbotham, J.N.; Zhang, Q.; Jeppesen, D.K.; Scott, A.M.; Manning, H.C.; Ochieng, J.; Franklin, J.L.; Coffey, R.J. Identification and Characterization of EGF Receptor in Individual Exosomes by Fluorescence-Activated Vesicle Sorting. J. Extracell. Vesicles 2016, 5, 29254.

64. Morales-Kastresana, A.; Telford, B.; Musich, T.A.; McKinnon, K.; Clayborne, C.; Braig, Z.; Rosner, A.; Demberg, T.; Watson, D.C.; Karpova, T.S.; et al. Labeling Extracellular Vesicles for Nanoscale Flow Cytometry. Sci. Rep. 2017, 7, 1878.

65. Maia, J.; Batista, S.; Couto, N.; Gregório, A.C.; Bodo, C.; Elzanowska, J.; Strano Moraes, M.C.; Costa-Silva, B. Employing Flow Cytometry to Extracellular Vesicles Sample Microvolume Analysis and Quality Control. Front. Cell Dev. Biol. 2020, 8, 593750.

66. Giddings, J.C.; Yang, F.J.F.; Myers, M.N. Flow-Field-Flow Fractionation: A Versatile New Separation Method. Science 1976, 193, 1244–1245.

67. Marsh, S.R.; Pridham, K.J.; Jourdan, J.; Gourdie, R.G. Novel Protocols for Scalable Production of High Quality Purified Small Extracellular Vesicles from Bovine Milk. Nanotheranostics 2021, 5, 488–498.

68. Gholizadeh, S.; Shehata Draz, M.; Zarghooni, M.; Sanati-Nezhad, A.; Ghavami, S.; Shafiee, H.; Akbari, M. Microfluidic Approaches for Isolation, Detection, and Characterization of Extracellular Vesicles: Current Status and Future Directions. Biosens. Bioelectron. 2017, 91, 588–605.

69. Guo, S.-C.; Tao, S.-C.; Dawn, H. Microfluidics-Based on-a-Chip Systems for Isolating and Analysing Extracellular Vesicles. J. Extracell. Vesicles 2018, 7, 1508271.

70. Morozumi, M. Comparison of Isolation Methods Using Commercially Available Kits for Obtaining Extracellular Vesicles from Cow Milk. J. Dairy Sci. 2021, 104, 6463–6471.

71. Sukreet, S. Isolation of Extracellular Vesicles from Byproducts of Cheesemaking by Tangential Flow Filtration Yields Heterogeneous Fractions of Nanoparticles. J. Dairy Sci. 2021, 104, 16.

72. Wijenayake, S.; Eisha, S.; Tawhidi, Z.; Pitino, M.A.; Steele, M.A.; Fleming, A.S.; McGowan, P.O. Comparison of Methods for Pre-Processing, Exosome Isolation, and RNA Extraction in Unpasteurized Bovine and Human Milk. PLoS ONE 2021, 16, e0257633.

73. Melnik, B.C.; Schmitz, G. Exosomes of Pasteurized Milk: Potential Pathogens of Western Diseases. J. Transl. Med. 2019, 17, 3.

74. Munir, J.; Ngu, A.; Wang, H.; Ramirez, D.M.O.; Zempleni, J. Review: Milk Small Extracellular Vesicles for Use in the Delivery of Therapeutics. Pharm. Res. 2022.

75. Roerig, J.; Schiller, L.; Kalwa, H.; Hause, G.; Vissiennon, C.; Hacker, M.C.; Wölk, C.; Schulz-Siegmund, M. A Focus on Critical Aspects of Uptake and Transport of Milk-Derived Extracellular Vesicles across the Caco-2 Intestinal Barrier Model. Eur. J. Pharm. Biopharm. 2021, 166, 61–74.

76. del Pozo-Acebo, L.; López de las Hazas, M.-C.; Tomé-Carneiro, J.; Gil-Cabrerizo, P.; San-Cristobal, R.; Busto, R.; García-Ruiz, A.; Dávalos, A. Bovine Milk-Derived Exosomes as a Drug Delivery Vehicle for MiRNA-Based Therapy. Int. J. Mol. Sci. 2021, 22, 1105.

77. Agrawal, A.K.; Aqil, F.; Jeyabalan, J.; Spencer, W.A.; Beck, J.; Gachuki, B.W.; Alhakeem, S.S.; Oben, K.; Munagala, R.; Bondada, S.; et al. Milk-Derived Exosomes for Oral Delivery of Paclitaxel. Nanomed. Nanotechnol. Biol. Med. 2017, 13, 1627–1636.

78. Aqil, F.; Munagala, R.; Jeyabalan, J.; Agrawal, A.K.; Kyakulaga, A.-H.; Wilcher, S.A.; Gupta, R.C. Milk Exosomes-Natural Nanoparticles for SiRNA Delivery. Cancer Lett. 2019, 449, 186–195.

79. Shandilya, S.; Rani, P.; Onteru, S.K.; Singh, D. Natural Ligand-Receptor Mediated Loading of SiRNA in Milk Derived Exosomes. J. Biotechnol. 2020, 318, 1–9.

80. Shandilya, S.; Rani, P.; Onteru, S.K.; Singh, D. Small Interfering RNA in Milk Exosomes Is Resistant to Digestion and Crosses the Intestinal Barrier In Vitro. J. Agric. Food Chem. 2017, 65, 9506–9513.

81. Warren, M.R.; Zhang, C.; Vedadghavami, A.; Bokvist, K.; Dhal, P.K.; Bajpayee, A.G. Milk Exosomes with Enhanced Mucus Penetrability for Oral Delivery of SiRNA. Biomater. Sci. 2021, 9, 4260–4277.

82. Tao, H.; Xu, H.; Zuo, L.; Li, C.; Qiao, G.; Guo, M.; Zheng, L.; Leitgeb, M.; Lin, X. Exosomes-Coated Bcl-2 SiRNA Inhibits the Growth of Digestive System Tumors Both in Vitro and in Vivo. Int. J. Biol. Macromol. 2020, 161, 470–480.

83. Matsuda, A.; Moirangthem, A.; Angom, R.S.; Ishiguro, K.; Driscoll, J.; Yan, I.K.; Mukhopadhyay, D.; Patel, T. Safety of Bovine Milk Derived Extracellular Vesicles Used for Delivery of RNA Therapeutics in Zebrafish and Mice. J. Appl. Toxicol. 2020, 40, 706–718.

84. Yan, C.; Chen, J.; Wang, C.; Yuan, M.; Kang, Y.; Wu, Z.; Li, W.; Zhang, G.; Machens, H.-G.; Rinkevich, Y.; et al. Milk Exosomes-Mediated MiR-31-5p Delivery Accelerates Diabetic Wound Healing through Promoting Angiogenesis. Drug Deliv. 2022, 29, 214–228.

85. Grossen, P.; Portmann, M.; Koller, E.; Duschmalé, M.; Minz, T.; Sewing, S.; Pandya, N.J.; van Geijtenbeek, S.K.; Ducret, A.; Kusznir, E.-A.; et al. Evaluation of Bovine Milk Extracellular Vesicles for the Delivery of Locked Nucleic Acid Antisense Oligonucleotides. Eur. J. Pharm. Biopharm. 2021, 158, 198–210.

86. Carobolante, G.; Mantaj, J.; Ferrari, E.; Vllasaliu, D. Cow Milk and Intestinal Epithelial Cell-Derived Extracellular Vesicles as Systems for Enhancing Oral Drug Delivery. Pharmaceutics 2020, 12, 226.

87. Vashisht, M.; Rani, P.; Onteru, S.K.; Singh, D. Curcumin Encapsulated in Milk Exosomes Resists Human Digestion and Possesses Enhanced Intestinal Permeability in Vitro. Appl. Biochem. Biotechnol. 2017, 183, 993–1007.

88. González-Sarrías, A.; Iglesias-Aguirre, C.E.; Cortés-Martín, A.; Vallejo, F.; Cattivelli, A.; del Pozo-Acebo, L.; Del Saz, A.; López de las Hazas, M.C.; Dávalos, A.; Espín, J.C. Milk-Derived Exosomes as Nanocarriers to Deliver Curcumin and

Resveratrol in Breast Tissue and Enhance Their Anticancer Activity. Int. J. Mol. Sci. 2022, 23, 2860.

89. Pascucci, L.; Coccè, V.; Bonomi, A.; Ami, D.; Ceccarelli, P.; Ciusani, E.; Viganò, L.; Locatelli, A.; Sisto, F.; Doglia, S.M.; et al. Paclitaxel Is Incorporated by Mesenchymal Stromal Cells and Released in Exosomes That Inhibit in Vitro Tumor Growth: A New Approach for Drug Delivery. J. Control. Release 2014, 192, 262–270.
90. Chen, J.; Cao, F.; Cao, Y.; Wei, S.; Zhu, X.; Xing, W. Targeted Therapy of Lung Adenocarcinoma by the Nanoplatform Based on Milk Exosomes Loaded with Paclitaxel. J. Biomed. Nanotechnol. 2022, 18, 1075–1083.
91. Xu, M.; Chen, G.; Dong, Y.; Yang, J.; Liu, Y.; Song, H.; Song, H.; Wang, Y. Liraglutide-Loaded Milk Exosomes Lower Blood Glucose When Given by Sublingual Route. ChemMedChem 2022, 17, e202100758.
92. Shi, Y.; Guo, S.; Liang, Y.; Liu, L.; Wang, A.; Sun, K.; Li, Y. Construction and Evaluation of Liraglutide Delivery System Based on Milk Exosomes: A New Idea for Oral Peptide Delivery. Curr. Pharm. Biotechnol. 2022, 23, 1072–1079.
93. Ahmed, F.; Tamma, M.; Pathigadapa, U.; Reddanna, P.; Yenuganti, V.R. Drug Loading and Functional Efficacy of Cow, Buffalo, and Goat Milk-Derived Exosomes: A Comparative Study. Mol. Pharm. 2022, 19, 763–774.
94. Li, D.; Yao, S.; Zhou, Z.; Shi, J.; Huang, Z.; Wu, Z. Hyaluronan Decoration of Milk Exosomes Directs Tumor-Specific Delivery of Doxorubicin. Carbohydr. Res. 2020, 493, 108032.
95. Pullan, J.; Dailey, K.; Bhallamudi, S.; Feng, L.; Alhalhooly, L.; Froberg, J.; Osborn, J.; Sarkar, K.; Molden, T.; Sathish, V.; et al. Modified Bovine Milk Exosomes for Doxorubicin Delivery to Triple-Negative Breast Cancer Cells. ACS Appl. Bio Mater. 2022, 5, 2163–2175.
96. Go, G.; Park, H.J.; Lee, J.H.; Yun, C.W.; Lee, S.H. Inhibitory Effect of Oxaliplatin-Loaded Engineered Milk Extracellular Vesicles on Tumor Progression. Anticancer Res. 2022, 42, 857.
97. Mudd, A.M.; Gu, T.; Munagala, R.; Jeyabalan, J.; Egilmez, N.K.; Gupta, R.C. Chemoprevention of Colorectal Cancer by Anthocyanidins and Mitigation of Metabolic Shifts Induced by Dysbiosis of the Gut Microbiome. Cancer Prev. Res. 2020, 13, 41–52.
98. Ogunnaike, M.; Wang, H.; Zempleni, J. Bovine Mammary Alveolar MAC-T Cells Afford a Tool for Studies of Bovine Milk Exosomes in Drug Delivery. Int. J. Pharm. 2021, 610, 121263.
99. Luo, S.; Sun, X.; Huang, M.; Ma, Q.; Du, L.; Cui, Y. Enhanced Neuroprotective Effects of Epicatechin Gallate Encapsulated by Bovine Milk-Derived Exosomes against Parkinson's Disease through Antiapoptosis and Antimitophagy. J. Agric. Food Chem. 2021, 69, 5134–5143.
100. Soekmadji, C.; Li, B.; Huang, Y.; Wang, H.; An, T.; Liu, C.; Pan, W.; Chen, J.; Cheung, L.; Falcon-Perez, J.M.; et al. The Future of Extracellular Vesicles as Theranostics—An ISEV Meeting Report. J. Extracell. Vesicles 2020, 9, 1809766.
101. Cai, M.; He, H.; Jia, X.; Chen, S.; Wang, J.; Shi, Y.; Liu, B.; Xiao, W.; Lai, S. Genome-Wide MicroRNA Profiling of Bovine Milk-Derived Exosomes Infected with Staphylococcus Aureus. Cell Stress Chaperones 2018, 23, 663–672.
102. Ma, S.; Tong, C.; Ibeagha-Awemu, E.M.; Zhao, X. Identification and Characterization of Differentially Expressed Exosomal MicroRNAs in Bovine Milk Infected with Staphylococcus Aureus. BMC Genom. 2019, 20, 934.
103. Ma, S.; Niu, M.; Hao, Z.; Liu, M.; Tong, C.; Zhao, X. Selective Packaged Circular RNAs in Milk Extracellular Vesicles during Staphylococcus Aureus Infection May Have Potential against Bacterial Infection. RNA Biol. 2021, 18, 818–831.
104. Saenz-de-Juano, M.D.; Silvestrelli, G.; Bauersachs, S.; Ulbrich, S.E. Determining Extracellular Vesicles Properties and MiRNA Cargo Variability in Bovine Milk from Healthy Cows and Cows Undergoing Subclinical Mastitis. BMC Genom. 2022, 23, 189.
105. Ishikawa, H.; Rahman, M.d.M.; Yamauchi, M.; Takashima, S.; Wakihara, Y.; Kamatari, Y.O.; Shimizu, K.; Okada, A.; Inoshima, Y. MRNA Profile in Milk Extracellular Vesicles from Bovine Leukemia Virus-Infected Cattle. Viruses 2020, 12, 669.
106. Rahman, M.d.M.; Badr, Y.; Kamatari, Y.O.; Kitamura, Y.; Shimizu, K.; Okada, A.; Inoshima, Y. Data on Proteomic Analysis of Milk Extracellular Vesicles from Bovine Leukemia Virus-Infected Cattle. Data Brief 2020, 33, 106510.
107. Hiraoka, M.; Takashima, S.; Wakihara, Y.; Kamatari, Y.O.; Shimizu, K.; Okada, A.; Inoshima, Y. Identification of Potential MRNA Biomarkers in Milk Small Extracellular Vesicles of Enzootic Bovine Leukosis Cattle. Viruses 2022, 14, 1022.
108. Nakanishi, R.; Takashima, S.; Wakihara, Y.; Kamatari, Y.O.; Kitamura, Y.; Shimizu, K.; Okada, A.; Inoshima, Y. Comparing MicroRNA in Milk Small Extracellular Vesicles among Healthy Cattle and Cattle at High Risk for Bovine Leukemia Virus Transmission. J. Dairy Sci. 2022, 105, 5370–5380.
109. Wang, Y.; Fang, J.; Zeng, H.-F.; Zhong, J.-F.; Li, H.-X.; Chen, K.-L. Identification and Bioinformatics Analysis of Differentially Expressed Milk Exosomal MicroRNAs in Milk Exosomes of Heat-Stressed Holstein Cows. Funct. Integr. Genom. 2022, 22, 77–87.