Review

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Review

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Abstract

Milk is a primary source of vital nutrients and bioactive components fundamental for the growth and development of both newborn animals and humans. Produced by economically significant livestock species (including cattle, buffaloes, goats, sheep and camels) milk is a complex matrix rich in caseins, vitamins, fats and proteins. In addition to its nutritional profile, milk serves as a vehicle for milk-derived extracellular vesicles (mEVs), a specialized class of food-derived EVs (fEVs) that exert pleiotropic effects aligned with the One Health concept, relating animal health, human nutrition, and ecosystem stability. mEVs offer unique advantages, such as high biocompatibility and gastrointestinal stability, rendering them also potential therapeutic tools, as drug delivery systems. However, challenges remain regarding the standardization of mEVs and the variability of their molecular cargo. This review provides a comparative analysis of mEVs across diverse species, including bovines, water buffaloes, yaks, camels, goats, pigs, horses, donkeys, and humans, with a focus on their unique functional profiles. Indeed, a critical issue in mEVs research is the isolation process: recommendations to minimize contamination from milk fat globules and casein micelles (which can cover EV signals) are given. Finally, current detection methods and instrumentation, with a specific focus on advancing Flow Cytometry (FC) approaches are discussed. Key insights include the use of Conventional FC (with fluorescence triggering, the necessity of rigorous controls and calibration, and the utility of Bead-Based Assays to overcome resolution limits) and of Imaging Flow Cytometry (IFC). In both technical approaches, the application of different EVs generic fluorescent markers and the strategic selection of tetraspanins (i.e., CD9, CD63, CD81), is mandatory, emphasizing the importance of selecting appropriate antibody clones or considering cross-reactivity when targeting these antigens across different mammalian species.

Keywords: milk EVs (mEVs); mammalian milk EVs; Conventional and Imaging Flow Cytometric detection; EV fluorescent markers; mAb cross reactivity; tetraspanin detection; mEVs as drug delivery systems; mEVs as food-derived EVs

1. Introduction

Milk is the primary source of vital nutrients and bioactive components essential for the growth and development of both newborn animals and humans. It is produced by several livestock species of significant economic importance, notably cattle, water buffaloes, goats, sheep and camels. Together with its derivatives, milk is a complex matrix and a rich source of nutritional, growth and immunological factors, including casein, vitamins, fats, carbohydrates, proteins and extracellular vesicles (EVs) [1–3].

Small EVs were first isolated from bovine milk in 1973, when they were described as membrane-derived vesicles approximately 100 nm in diameter, originating from the apical plasma membrane of mammary epithelial cells [4].

Since that initial discovery of bovine milk-derived EVs (mEVs), several studies have confirmed the presence of mEVs across a wide range of domestic animal species, including goats [5], water buffaloes [6], donkeys [7], pigs [8], horses [9], camels [10] and yaks [11].

EVs are a heterogeneous group of membrane-enclosed vesicles, including exosomes, microvesicles and apoptotic bodies, which differ in size and biogenesis and cellular origins. Exosomes typically range from 30 to 150 nm, microvesicles from 100 to 1000 nm and apoptotic bodies from 50 to 5000 nm [12,13]. Moreover, exosomes have an endocytic origin and are released from multivesicular bodies, which fuse with the plasma membrane, leading to the release of intraluminal vesicles into the extracellular microenvironment. In contrast, microvesicles are formed by the outward budding of the cell surface membrane, whereas apoptotic bodies are shed from the membrane of cells undergoing apoptosis [14,15].

Beyond size and morphology these categories differ significantly in their molecular cargo [16] reflecting specific biogenesis pathways and specialized physiological functions [17,18]. The overlapping physical characteristics between exosomes and microvesicles coupled with the lack of specific markers that differentiate them, have made it challenging to study these two populations individually. Therefore, the Minimal Information for Studies of Extracellular Vesicles (MISEV2023) guidelines, published by the International Society for Extracellular Vesicles (ISEV), recommends using the generic term “extracellular vesicle”, unless authors can identify reliable subcellular markers in their experimental models. Alternatively, EV subtypes can be referred to using operational terms such as small EVs (<200 nm) and large EVs (>200 nm) [19].

EVs carry a diverse array of biomolecules, including proteins, lipids, metabolites and genetic material such as DNA, mRNA and non-coding RNAs (ncRNAs). This molecular cargo is shielded from degradation by extracellular proteases and nucleases by the vesicle’s robust lipid bilayer [20]. Their cargo enables EVs to mediate intercellular communication and influence various physiological and pathological processes.

The biocompatibility of EVs and the protection they confer to their cargo allow these molecules to overcome biological barriers and adverse environments, such as the gastrointestinal (GI) tract, without damage, thus maintaining their functionality [21]. Cargo is packaged by the parent cell in a regulated manner, and emerging evidence suggests that both the concentration of released EVs and the specific composition of their cargo vary according to the physiological or activation state of the originating cell [22]. The biological significance of EVs lies in their role as mediators of intercellular communication, capable of modulating the functions of recipient cells [23]. This interaction, whith recipient cells, can occur locally or at distant systemic sites, involves complex ligand-receptor signaling at the cell surface or the direct fusion of the vesicle with the plasma membrane. In most instances, EVs are internalized via endocytic pathways, leading to the integration of EV membrane components into the recipient cell and the release of cargo into the cytoplasm or nucleus. This transfer can activate new signaling pathways and elicit significant functional alterations in the target cell [24,25].

1. Pleiotropic effects of mEVs Within the One Health Framework

The One Health paradigm acknowledges the intrinsic interconnectedness of human, animal, and environmental health. The study of milk-derived extracellular vesicles (mEVs) provides a compelling illustration of this interconnectedness, as these vesicles act as cross-species biological messengers. Over the past decades, studies on EVs have become increasingly significant in life sciences, due to their essential functions in both physiological and disease conditions. Almost all cells secrete different types of EVs, which can have a variety of effects. Recent studies have shown that mEVs appear to survive digestion and harsh conditions, for this reason may help to protect or improve the intestinal barrier integrity or reduce inflammation in gut models [26–28]. mEVs also appear to have immunomodulatory properties as they can carry immunoregulatory miRNAs and can modulate macrophage differentiation, cytokine production and inflammatory responses [1,29]. Beyond the immune system, mEVs have demonstrated significant osteogenic potential. For instance, the study conducted by Go et al. (2021) [30] demonstrated how mEVs promote the differentiation and proliferation of Saos-2 cells by increasing the expression of the key osteogenic transcription factors RUNX2 and Osterix. A further study confirmed that mEVs could be used as anti-osteoporotic agents due to their ability to increase the number of osteocytes [30–32].

From a OneHealth perspective, the therapeutic potential of mEVs extends to veterinary applications. Recent findings suggest that mEVs from diverse sources, including colostrum, mature milk and milk from clinical mastitis, can counteract oxidative stress and ferroptosis in bovine mammary epithelial cells infected with *Klebsiella pneumoniae*, thereby providing a protective effect on mammary tissues [33].

2. Advantages Offered by mEVs as Potential Therapeutic Tool

mEVs have gained significant attention due to their unique advantages, including their stability within the GI tract, biocompatibility, safety, and their substantial potential as vehicles for oral drug delivery [34,35]. The oral route is generally considered the most preferable method for drug administration; however, a significant number of therapeutic agents, including poorly water-soluble small molecules and macromolecular biologics such as peptides, exhibit low oral bioavailability. Although parenteral administration can overcome these limitations by enabling rapid systemic delivery of unstable compounds, it remains an invasive procedure and is often associated with pain at the injection site and limited reversibility of drug effects [36,37].

mEVs are naturally designed for oral transmission, remaining stable under gastric and enzymatic stress and reaching the intestine intact and functional [38,39]. It is hypothesised that the interaction with the gut environment may result in the modulation of the gut-liver axis, an intricate networking link which is established through blood circulation, the portal vein and the bile duct. The close interconnection between the gut microbiome, the intestine and the liver suggests that modulating the gut barrier and the gut microbiome could represent a novel approach for the treatment of metabolic disorders. Consistently, mEVs exhibit greater mechanical rigidity and a more compact structure to maintain stability under lower pH conditions [40,41]. Following exposure to various conditions ranging from the mouth to the colon, mEVs remain intact, whereas the integrity of other synthetic nanoparticles, such as liposomes, is compromised [42]. Beyond the gut, it has been reported that orally administered mEVs reach other organs, including liver, spleen, kidneys, pancreas, ovaries, lungs, heart and brain, as indicated by various fluorophore labels [43,44]. Dysbiosis is a common pathological characteristic of gut disorders. According to a recent study, microvesicles exert therapeutic effects on gut disorders, such as inflammatory bowel disease, by regulating the abundance of gut microbiota and the excretion of bacterial EVs. For instance, the oral administration of mEVs has been demonstrated to regulate intestinal immune homeostasis and restore gut microbiota, thereby alleviating ulcerative colitis. mEVs have been shown to promote the growth of intestinal epithelial cells, support intestinal development and protect epithelial cells from oxidative stress–induced death. Furthermore, mEVs have been demonstrated to modulate intestinal stem cell activity, by increasing the expression of a key marker of these stem cells. These activities are critical for maintaining intestinal epithelial homeostasis, a continuous process that is finely orchestrated by the balance between stem cell proliferation, migration and differentiation, ensuring proper renewal and functional integrity of the gut lining [45,46]. Furthermore, milk-derived antimicrobial peptides (MAPs) found in EVs reduce infection risk in wounds by inhibiting the proliferation of pathogen strains such as *E. coli* and *S.* aureus [47].

3. Milk EVs as Food-Derived EVs (fEVs)

In recent years, food-derived EVs (fEVs) have received more attention due to emerging evidence of the correlation between diet and gut health, as well as the unique features of fEVs, such as their resistance to the gastric environment. Among those fEVs we can quote mEVs, which can survive in an ex vivo system that mimics different segments of the GI tract despite the harsh conditions [40]. mEVs have been proposed to modulate immune cells associated with the oral and gut mucosa while also supporting epithelial cell function, which is crucial for maintaining intestinal homeostasis [48]. Oral delivery is favored for its convenience, non-invasiveness and cost-effectiveness. Furthermore, in addition to improving patient compliance, the oral route is significant from a physiological perspective. One of the most promising strategies for overcoming the challenges associated with oral delivery is the use of nanoparticulate systems. EVs possess the ability to encapsulate both hydrophilic and hydrophobic molecules and pass through biological barriers via membrane-associated proteins [49–51].

Attention has been given to mEVs, particularly the subfraction of milk exosomes (mEX), which contain proteins, lipids, messenger RNAs (mRNAs), microRNAs (miRNAs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) [52].

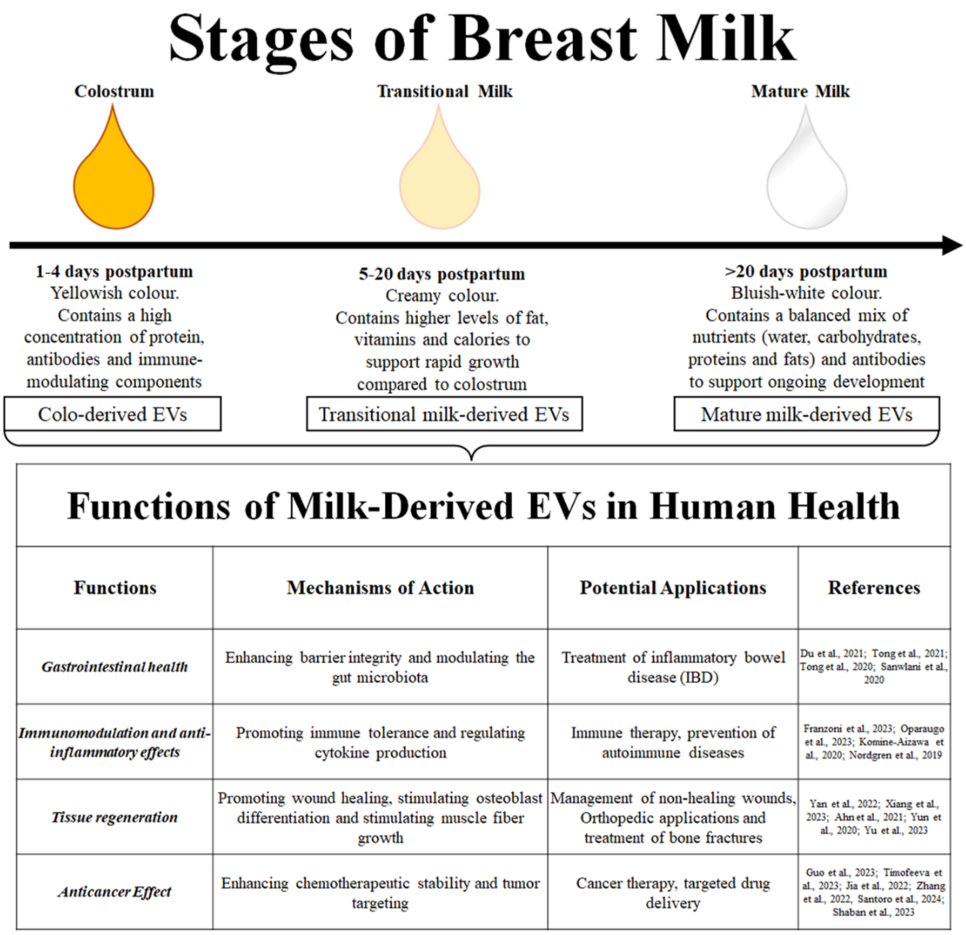
A recent study on porcine mEVs found that miRNAs transported by the vesicles protected the intestine from lipopolysaccharide (LPS)-induced injury by inhibiting the NF-κB and p53 pathways. Specifically, miR-148, the most abundant microRNA in mEVs, has been shown to inhibit the NF-κB signalling pathway and suppress colitis and colitis-associated tumorigenesis [38,53].

Milk production is typically divided into three stages: colostrum (produced immediately after birth), transitional milk (produced from the second to the fifteenth day after calving) and mature milk (produced one month after calving) [54,55] (Figure 1).

Colo EVs contain a rich mixture of bioactive components, including growth factors, proteins, mi-RNAs, lipids, etc., conferring nutritional and immune health benefits. This establishes a complex signaling system between mother and child, supporting postnatal health. The bioactive components transferred through milk are essential for the development of newborns [2].

Moreover, EVs in colostrum outnumber those in mature milk and have a higher protein concentration as well as a stronger effect on inhibiting the expression of genes linked to apoptosis and pro-inflammatory cytokines. Other functions in which colostrum EVs are involved are wound healing (through the stimulation of fibroblast proliferation and migration) and the treatment of different conditions such as mastitis, a prevalent disease characterized by inflammation and damage in mammary glands caused primarily by bacterial infection [56].

In addition, the characteristics of EVs derived from colostrum and mature milk differ in their capacity to mediate the function of a small intestine epithelial cell line (IEC-6 cells). This is crucial, as these cells play a vital role in forming the intestinal barrier and providing host defense against pathogens. These recent findings emphasize the potential of EVs from colostrum and mature milk to influence the health of various organs and systems, including gut health, skin and immune system [57]. From previous considerations, it is evident that several safety concerns exist when considering mEVs for human therapeutic use or as a nutritional supplement, similar to those associated with other cell-cultured milk technologies [58]. Primarily, the proteins carried within the mEVs could potentially elicit an immune response or allergic reactions in human consumers. Furthermore, there is a risk of contaminant enrichment, where residues from infections or drugs (e.g., antimicrobials) present in cattle milk samples could be concentrated within the vesicles themselves. Finally, since rare studies suggest a potential risk of increasing metastasis in certain cancer treatments, the specific mechanisms and long-term effects of mEVs in the human body demand rigorous preclinical and clinical assessment [1].



Functions	Mechanisms of Action	Potential Applications	References
Gastrointestinal health	Enhancing barrier integrity and modulating the gut microbiota	Treatment of inflammatory bowel disease (IBD)	Du et al., 2021; Tong et al., 2021; Tong et al., 2020; Sanwlani et al., 2020
Immunomodulation and anti-inflammatory effects	Promoting immune tolerance and regulating cytokine production	Immune therapy, prevention of autoimmune diseases	Franzoni et al., 2023; Oparaugo et al., 2023; Komine-Aizawa et al., 2020; Nordgren et al., 2019
Tissue regeneration	Promoting wound healing, stimulating osteoblast differentiation and stimulating muscle fiber growth	Management of non-healing wounds, Orthopedic applications and treatment of bone fractures	Yan et al., 2022; Xiang et al., 2023; Ahn et al., 2021; Yun et al., 2020; Yu et al., 2023
Anticancer Effect	Enhancing chemotherapeutic stability and tumor targeting	Cancer therapy, targeted drug delivery	Guo et al., 2023; Timofeeva et al., 2023; Jia et al., 2022; Zhang et al., 2022, Santoro et al., 2024; Shaban et al., 2023

Figure 1. Scheme of the different productions over the lactation curve: features of the products and their specific EVs content.

4. Reported Issues for Cattle mEVs and Their Cargo

Beyond safety concerns related to the general health status of dairy animals, a major issue in mEV research concerns the impact of infectious and inflammatory diseases on milk composition and, consequently, on EV abundance and cargo. While this aspect has been more extensively investigated in cattle, similar mechanisms are expected to occur in other dairy species such as goats and sheep, although the available literature is more limited. In cattle, both clinical and subclinical mastitis are well known to profoundly alter milk yield, protein and lipid composition, somatic cell content and inflammatory mediators, all of which are factors that may directly influence EV biogenesis and molecular cargo. Accordingly, several studies have reported significant changes in the concentration and miRNA composition of bovine milk EVs in animals affected by mastitis or chronic viral infections, such as enzootic bovine leukosis, suggesting that mEV profiles reflect the inflammatory and immunological status of the mammary gland [59,60]. Comparable disease-related alterations are also documented in small ruminants. In dairy sheep, mastitis—both clinical and subclinical—induces marked changes in milk protein fractions, fat globule structure and immune cell infiltration, which are known to affect the secretion of bioactive milk components [61]. Similarly, in goats, infectious diseases such as caprine arthritis encephalitis (CAE) and intramammary infections have been shown to modify milk yield, casein composition and immune-related factors, potentially impacting the release and cargo of milk EVs [62]. Although direct evidence linking these conditions to mEV composition in goats and sheep is still scarce, the strong parallels with bovine mastitis strongly suggest that health-related variability of mEVs is a general phenomenon across dairy species rather than a cattle-specific issue. The limited number of omics-based studies available for sheep and goat milk EVs likely underestimates the magnitude of this problem. Comparative analyses of milk EVs across species have already demonstrated that transcriptomic and miRNA cargoes are sensitive to source-related biological variables and immune status [7], supporting the hypothesis that disease-driven modulation of EV cargo may represent a conserved response among lactating mammals. Taken together, these observations indicate that animal health status constitutes a critical confounding factor in the interpretation of cattle mEV data and should be considered with equal importance in other dairy species. Failure to account for inflammatory or infectious conditions may therefore contribute to the high variability reported in milk EV studies and complicate the identification of biologically meaningful and transferable EV-associated signatures.

In addition to health-related factors, breed-related variability represents a major biological source of heterogeneity in cattle milk-derived extracellular vesicles. Dairy cattle have undergone intense genetic selection for divergent production traits, resulting in breeds that differ markedly in milk yield, fat and protein content, metabolic efficiency and immune responsiveness. Such breed-specific physiological and metabolic differences are expected to influence not only bulk milk composition but also EV biogenesis, release dynamics and cargo selection.

Evidence supporting this concept is emerging from miRNomics and transcriptomic studies, which indicate that milk EV-associated miRNA and mRNA profiles vary according to genetic background. Breed-dependent differences in milk miRNA expression have been reported in cattle and other livestock species, with implications for immune regulation, metabolism and mammary gland function [63]. Although cattle provide the most evident example due to the high number of specialized dairy breeds and the availability of molecular data, similar intra-species variability is likely to occur in other dairy animals such as goats and sheep, where fewer studies are currently available. This suggests that breed-related variability is a general issue in milk EV research rather than a species-specific anomaly.

Environmental and management-related factors further amplify this complexity. Diet composition, feeding strategies and nutrient availability are known to profoundly affect milk yield, lipid and protein fractions and the metabolic status of lactating animals. These factors have also been shown to modulate EV-associated miRNA profiles, supporting a direct link between nutrition and EV cargo composition. Environmental stressors, including heat stress, housing conditions and seasonal variations, represent additional confounding variables. Heat stress in dairy cattle has been shown to alter milk exosomal miRNA expression patterns, particularly those involved in inflammation, stress response and metabolic regulation [64]. Given the increasing impact of climate change on livestock systems, environment-driven modulation of mEV cargo is likely to become an increasingly relevant issue, especially when comparing studies conducted under different geographical and climatic conditions.

Understanding how genetic selection, environmental pressures and animal welfare shape mEV composition is therefore essential not only for basic biology but also for the safe and effective exploitation of milk EVs in nutraceutical, functional food and therapeutic applications. Failure to account for this multi-layered variability may limit the translational value and reproducibility of milk EV studies.

Besides the described safety issues descending from animal Health/Disease Status (i.e., Bovine Leukemia Virus or mastitis), additional key issues associated with cattle mEVs involve variability in EV content, challenges with isolation and scalability, concerns regarding their use as therapeutics, and potential impacts of industrial processing. The molecular cargo (proteins, lipids, and nucleic acids like miRNAs and mRNAs) and quantity of EVs in cattle milk are highly variable and influenced by numerous factors [65] which can introduce variability if the milk source is not carefully controlled. Indeed, different cattle breeds produce milk with varying compositions, affecting the inherent EV characteristics. Finally, the stage of lactation, the cow's feeding regime and general environmental/management practices all impact the features and composition of mEVs (Table 1).

4.1. Bovine mEVs

Bovine milk has gained increasing scientific attention due to the presence of a high number of EVs. These vesicles have been reported to enhance cognitive function and support bone health. Moreover, these EVs have the ability to protect cells from oxidative stress; hence, they can be used as a promising tool in regenerative medicine [66]. Studies have also shown that such vesicles can reduce intestinal inflammation and support the gut microbiome [67,68]. Bovine mEVs can reduce inflammation by regulating nutrient metabolites, such as increasing lipid anti-inflammatory metabolites and decreasing fecal amino acids; hence, they can be used to treat colitis [69]. Research findings highlighted that miRNAs derived from EVs are highly conserved in human, bovine, and caprine milk. Moreover, microRNAs such as miR-30a-5p, miR-22-3p, and miR-26a that have a major role in regulating immune function are commonly present in the colostrum and mature milk of cows and caprines [70]. Data suggested that cow mEVs contain TGFβ and miR-148a that can regulate chondrocyte homeostasis and prevent cartilage damage; hence, they can be used as a therapeutic option for osteoarthritis [71]. Furthermore, investigations have shown that oral delivery of bovine mEVs mitigates arthritis [72]. Experimental evidence suggested that bovine mEVs are highly biocompatible, as they can be easily taken by cells without producing cytotoxic effects; hence, they can be considered as promising drug delivery carriers for various therapeutic applications [35].

4.2. Water Buffalo mEVs

Water buffalo (Bubalus bubalis) milk is characterised by higher levels of macronutrients, particularly fat (6–8%) and protein (4–5%), compared with cow's milk [73,74]. Furthermore, the concentration of calcium, an important milk mineral, is approximately 1.5 times higher than in cow's milk [75]. Buffalo milk also contains antioxidant and anti-inflammatory compounds, such as delta-valerobetaine and biliverdin, alongside pentasaccharides and gangliosides, which are usually absent in cow's milk [73]. In addition, specific probiotic species such as Lactobacillus, Streptococcus, Lactococcus, and Enterococcus are abundantly found in buffalo's milk, which highlights its probiotic benefits [76]. Remarkably, buffalo milk contains a significant number of EVs. These EVs contain 96% of miRNA, which could help in immune regulation, blood vessel development and epigenetic regulations [6]. Furthermore, a protein analysis conducted by Joshi et al. (2024) [77] on EVs derived from three buffalo milk samples identified 331 common proteins. The biological functions of these proteins were related to immunity, cell cycle regulation and metabolism. Significantly, the identification of 114 novel proteins in buffalo mEVs, when compared with bovine mEVs, suggests a crucial role in muscle development [77]. In 2023 Samuel et al. [3] isolated and characterized EVs from cow, buffalo, sheep and goat milk, which collectively account for 99% of global milk consumption. Comparative proteomic analysis of mEVs from these sources revealed that those from buffalo milk contain proteins potentially involved in immune regulation. Moreover, the study also determined the anti-cancer effects of buffalo mEVs, with data showing that these EVs induced a higher rate of cell death in colon cancer cells [3]. Further investigations highlighted that miRNAs obtained from EVs of buffalo milk can be involved in immune response and metabolism. A comparison of exosome miRNA profiling in buffalo milk with that of other samples revealed a total of 32 upregulated miRNAs. These miRNAs could be involved in metabolism, while the 16 downregulated miRNAs are anticipated to be involved in the immune response [78]. The size of buffalo mEVs was within the range of 50–200 nm. Furthermore, after isolating EVs from buffalo milk, plasma and urine, a comparative analysis showed that immune-related miRNAs (such as miR-21, miR-500, miR-125b, miR-155, and miR-27b) were abundant in the mEVs. Moreover, miR-21 and miR-500 exhibited high stability in milk samples compared to the buffalo's plasma and urine [79].

4.3. Yak mEVs

Yaks (*Bos grunniens*) living in high-altitude regions exhibit strong resistance to hypoxia and possess a high metabolic capacity. Exosomes present in yak milk have been shown to activate hypoxia-inducible factor (HIF) signaling pathways, which promote the survival of intestinal epithelial cells (IECs) under conditions of oxygen deficiency. This mechanism plays a role in the yaks' greater hypoxia tolerance compared with Holstein cows. Yak mEVs exhibited significantly higher expression of the proteins TSG101, CD63, and Hsp70 than cow milk-derived exosomes. Furthermore, yak milk-derived exosomes were found to be more effective than cow milk-derived exosomes in promoting the growth of IEC-6 cells under hypoxic conditions. Specifically, when the exosome concentration was between 200 and 240 ng/**μ**L under hypoxic conditions, yak milk-derived exosomes significantly increased IEC-6 cell survival post-treatment more effectively than cow milk-derived exosomes [80,81].

4.4. Goat mEVs

Goat mEVs have attained much scientific attention owing to their unique properties and therapeutic potential to treat various diseases. Studies showed that also goat mEVs possess immune-regulating properties and hence could be applied in treating autoimmune illnesses and cancer [82]. Of note, experimental evidence revealed that goat mEVs possess higher loading capacity in comparison to cow and buffalo mEVs [83]. Moreover, EVs derived from goat milk can be applied as novel gene therapy vehicles, as they may impact many metabolic pathways in the cells [53,84]. Investigations revealed that goat mEVs have potent antiviral activity and they can significantly lower the infection caused by the dengue virus [85]. Since miRNAs play an important role in regulating the expression of many different genes in humans, thereby influencing cell differentiation and proliferation [86], a complete set of miRNAs was investigated in goat mEVs and the content was then compared and validated against miRNA EV content from cow milk. A total of 295 miRNAs were identified. Notably, goat milk samples contain a greater number of identified beta-miRNAs than cow milk. Moreover, miR-148a, miR-21-5p, miR-26a, and miR-30a-5p were found to be common between the two species [7,87].

4.5. Camel mEVs

Camel milk has attracted scientific attention because of its distinctive composition and properties. Due to its anticancer, antibacterial, antidiabetic and immune-regulatory properties, it can be used as a biopharmaceutical agent. Interestingly, camel milk components can enhance reactive oxygen species (ROS) in cancer cells but reduce ROS in healthy cells. Its anticancer and immune-regulatory properties could be due to the lactoferrin, and kappa casein mRNAs found within camel mEVs [88]. Furthermore, proteomic analysis revealed that camel mEVs were rich in proteins involved in EVs synthesis and secretion processes, including intracellular protein transport, translation and cell-to-cell adhesion [89]. In addition, investigations revealed that camel mEVs help in the reduction of colon damage caused by hypoxia; alongside this, they also enhance beneficial microbiota such as Lactobacillus and Bifidobacterium and reduce Enterobacteriaceae, suggesting their potential benefits as nutraceuticals and dietary supplements, especially for people residing in high altitudes [90]. Experimental evidence suggested that the oral administration of camel mEVs significantly reduced diet-induced obesity, caused by consuming a fat-rich diet, by increasing thermogenesis and regulating lipid metabolism. These unique vesicles decrease body fat percentage and lipid accumulation while simultaneously lowering serum levels of triglycerides, free fatty acids and cholesterol, thus providing a potential therapeutic option for treating obesity [91]. Camel mEVs can be utilized as novel carriers to transport curcumin, a polyphenol with chemo preventive and tumor-suppressive properties, in lung cancer. In vitro antiproliferative assays demonstrated that curcumin delivered via camel mEVs significantly increased the cytotoxic effect against both drug-sensitive and taxol-resistant lung cancer cells when compared to free curcumin. These investigations suggest that camel mEVs represent an efficient carrier for the therapeutic delivery of curcumin [92]. Studies have shown that camel milk and its derived EVs reduce kidney damage and fibrosis while promoting oxidative balance in rats. Therefore, these vesicles can represent a promising therapeutic approach for the future treatment of diabetic nephropathy [93].

4.6. Porcine mEVs

Porcine mEVs offer several health benefits, particularly concerning gut health and intestinal immunity. In vivo investigations in mice and piglets revealed that porcine mEVs enhanced the proportions of intestinal secretory immunoglobulin A (SIgA), which plays a significant role in gut-related immunity and mucosal homeostasis. Interestingly, an important circular RNA (circ-XPO4) present in porcine mEVs was found to enhance intestinal immunity. This enhancement is mediated by circ-XPO4 promoting the expression of the polymeric immunoglobulin receptor (pIgR) through the suppression of miR-221-5p in intestinal cells [94]. Ávila G. et al. (2025) [95] investigated the effect of porcine mEVs on the proteome of porcine peripheral blood mononuclear cells (PBMC). Gene Ontology (GO) enrichment analysis revealed that porcine mEVs treated cells were enriched in innate immunity-related proteins, i.e., TLR2, APOE, CD36 and MFGE8, compared to the control. In vitro experiments demonstrated that monocytes could internalize porcine mEVs, regulating immune functions by decreasing their phagocytic capacity and increasing their oxidative activity. Thus, these vesicles can act as immune regulators [95]. Interestingly, porcine mEVs can also be internalized by macrophages to deliver immune-relevant miRNAs. Moreover, such EVs promoted macrophage polarization toward the M2-like phenotype, consequently inducing anti-inflammatory effects [96]. Experimental evidence suggested that porcine mEVs play an important role in intestinal tract development. Data reported that these vesicles enhanced proliferation of intestinal epithelial (IPEC-J2) cells by upregulating genes such as CDX2, IGF-1R and PCNA, which are mainly involved in intestinal proliferation, consequently promoting digestive tract development [97]. Furthermore, researchers investigated mRNA and protein from porcine milk-derived exosomes for the first time. Transcriptomic analysis revealed a total of 16,304 mRNA; among these, 2,409 were newly identified mRNAs, and some of them were involved in metabolisms and signaling pathways. Proteomic analysis identified 639 proteins in total; most of them were mainly residing in cytoplasm and have a more specific role in immunity, while some of the proteins were tissue specific. Clusters of Orthologous Groups (COG) analysis revealed that many of the identified mRNAs and proteins were associated with cell cycle control and cell division [98]. Investigations suggested that porcine mEVs contain a high amount of miRNAs, which can transfer information from mother pig milk to piglets [99]. Around 1081 known and 2311 novel miRNAs were identified from pig mEVs. These miRNAs were known to be involved in cell signaling and development of the immune system [100].

4.7. Horse mEVs

Experimental evidence suggested that horse mEVs contain around 5-8 different types of major proteins, such as CD81, CD63 receptors, beta-lactoglobulin and lactadherin, actin, butyrophilin, lactoferrin and xanthine dehydrogenase [101]. Research findings suggested that mare’s mEVs can be used as efficient drug delivery vehicles; investigations highlighted that EVs loaded with quercetin efficiently improved β-galactosidase activity and cell viability in doxorubicin-treated cells and reduced damage to the myocardium, kidneys and liver in aged model animals [9].

4.8. Donkey mEVs

Caria et al. (2025) [102] performed detailed characterization and proteomic analysis of EVs derived from donkey colostrum and mature donkey milk and found that EV constituents derived from both milks were involved in tissue repair and immunity. In particular, the proteins present in EVs from donkey colostrum play a role in defense and regeneration [96]. Donkey mEVs have shown anti-inflammatory properties, as they contain the amino acid asparagine, which can reduce intestinal injuries caused by bacterial lipopolysaccharides (LPS). These EVs also possess strong antioxidant properties due to the presence of glutathione [103]. Detailed characterization of mRNA and small

RNA content of donkey mEVs also highlighted their immunomodulatory and anti-inflammatory potential; the role of donkey mEVs in lipid metabolism and reducing oxidative stress was also reported [7]. Liu et al. 2025 [104] carried out proteomic analysis of EVs from donkey colostrum and mature milk. Investigations revealed the presence of immune-associated proteins in colostrum; these proteins exhibited important roles in autophagy and lysosome pathways, while EV proteins derived from mature milk were involved in the nutritional metabolism pathways [104].

Table 1. Summary of Animal sources for breast milk/colostrum EVs: their diameter and concentrations reported in specific references.

Typology of breast milk	Animal Origins	Diameter	Concentration	References
Colostrum	Holstein cows	149.6 (range of 88.5–239.0 nm)	4.3×10^{11} particles	**[56]**
	Bovine (Holstein cows), caprine (Saanen breed)	NA	NA	**[70]**
	Donkey (Amiata breed)	153 ± 4.4 nm for DC-EVs	7.3×10^{10} ($+9.3 \times 10^{9}$)	**[102]**
	Bamei pigs and Landrace pigs	sizes ranged from 50 to 100 nm	12.5×10^{9} for Bamei colostrum and $17{,}5 \times 10^{9}$ fo Landrace pigs	**[100]**
	Bovine	140 ± 5.1 nm	$1.3 \times 10^{14} \pm 2.2 \times 10^{13}$ particles/mL	**[105]**
	Bovine (Piemontese cows)	177.4 ± 2.4 nm	1.02×10^{12} ($\pm 4.88 \times 10^{10}$)	**[106]**
	Bovine	140 ± 5.1 nm	$1.3 \times 10^{14} \pm 2.2 \times 10^{13}$ particles/mL	**[54]**
	Buffalo	81 ± 26 nm	318,539	**[6]**
	Ewe and goat	146.5 nm for eve colostrum and 153.9 nm for goat colostrum	6.6×10^{12}, for eve colostrum and 2.5×10^{12} for goat colostrum	**[107]**
	Porcine	140 nm	sEV 15×10^{10}	**[96]**
Transitional Milk or First Milk	Bovine	156 ± 8.8 nm	$9.3 \times 10^{13} \pm 1.8 \times 10^{13}$ particles/mL	**[54]**
	Porcine	150 nm	sEV 9×10^{10}	**[96]**
Mature Milk	Bovine	155.1 ± 16 nm	$2.7 \times 10^{10} \pm 3 \times 10^{9}$ particles mL−1	**[108]**
	Bovine	MM month 1 were 133 ± 2.6 nm, month 5 were 141 ± 1.0 nm and in month 9 were 136 ± 8.8 nm.	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	**[54]**
	Buffalo	60 ± 13 nm	70,232	**[6]**

Cow, Donkey and Goat	142.7 ± 2.9 nm for cow, 150.5 ± 3.2 for donkey, and 124.1 ± 2.3 for goat	1.22×10^{12} (± 3.63×10^{10}) for cow; 3.51×10^{11} (± 1.22×10^{10}) for donkey; and 7.39×10^{11} (± 1.57×10^{10}) for goat	**[7]**
Cows (Bos taurus) and yaks (Bos grunniens)	112.4 ± 48.6 nm	NA	**[80]**
Bovine Leukemia Virus-infected and uninfected cattle	milk sEV from BLV-infected and uninfected cattle were 145.6 nm and 145.7 nm, respectively	milk sEV from BLV-infected and uninfected cattle were 1,2 x 10^{10} and 4 x 10^{10}, respectively	**[109]**
Enzootic Bovine Leukosis-infected and uninfected cattle	approximately 100 nm	NA	**[110]**
Murrah Buffaloes	range of 30–150 nm	NA	**[77]**
BLV-uninfected healthy and EBL Holstein dairy cattle	100–150 nm	NA	**[60]**
Cow, Buffalo, Sheep and Goat	115, 105, 135 and 105 nm for the Cow, Buffalo, Goat and Sheep samples, respectively.	9 x 10^6, 11 x 10^6, 4.8 x 10^6 and 4.2 x 10^6 for the Cow, Buffalo, Goat and Sheep samples, respectively.	**[3]**
Donkey (Amiata breed)	143.4 ± 8.2 nm for MDM-EVs	3.7×10^{10} (+5.9×10^9)	**[102]**
Bamei pigs and Landrace pigs	sizes ranged from 50 to 100 nm	11x10^9 for Bamei colostrum and 12,5 x 10^9 fo Landrace pigs	**[111]**
Bovine and Human	the size varied within the 30- to 200-nm range	NA	**[42]**
Camelus (C.) dromedarius, C. bactrianus and hybrids	25-170 nm	9.49 x 10^8 - 4.18 x 10^{10}	**[89]**
Porcine (Landrace pigs)	146.9 nm	NA	**[94]**
Porcine	152 nm	sEV 4.8 x 10^{10}	**[96]**
Mare Horse	different isolation methods: SEC (110 ± 8 nm), TEIK (118 ± 13 nm), and IP (131.5 ± 16 nm)	NA	**[9]**

4.9. Human mEVs

Human milk EVs act as natural nanocarriers, facilitating maternal-infant communication and delivering various functional "cargo" that remain largely intact through the infant's digestive system

[47]. Human milk EVs origin recently propelled several research groups [112] to deeply investigate mucus diffusivity, endocytic mechanisms of epithelial uptake and the composition of human milk EV subpopulation transported across the intestinal epithelium, impacting the intestinal absorption of human milk EVs. Human mEVs contain specific miRNAs that are associated with the promotion of infant development and can potentially prevent certain diseases [113]. Clinical and epidemiological studies have confirmed the beneficial effects of human milk feeding (compared to infant formula), showing that it prevents both early and long-term diseases. Examples include necrotizing enterocolitis, neonatal sepsis, respiratory and GI tract infections, obesity, diabetes mellitus, allergic diseases and malignancies [114–116]. Human mEVs contribute to both short-term immunity and long-term developmental outcomes through a variety of mechanisms: i) mEVs help modulate the infant's developing immune system, promoting a balanced response, reducing excessive inflammation and potentially aiding in the management of conditions such as inflammatory bowel disease and colorectal cancer [45] ii) EVs play a significant role in the maturation and integrity of the infant's gut. They promote the expression of tight junction proteins (like Claudin-1) and increase stem cell proliferation, making the gut lining stronger and less "leaky" [117], supporting gut barrier function and help establish a beneficial gut microbiota, particularly in preterm infants. iii) The components within human milk EVs exhibit antiviral effects against various pathogens, including HIV-1, rotavirus, and human cytomegalovirus (CMV) [118]. In detail, mEVs were demonstrated to promote the uptake of human immunodeficiency virus (HIV)-1 by macrophages and inhibited uptake by T cells [119]. Binding of EVs to antigen-presenting cells inhibited HIV infection of both dendritic cells and CD4+ T cells, suggesting mEVs as a novel protective factor against vertical transmission of HIV-1, having the ability to attach to DC-SIGN receptors [120]; iv) mEVs contain miRNAs that can contribute to the infant's cognitive development and potentially leading to improved neurodevelopmental outcomes observed in breastfed children. Wijenayake and coworkers investigated the uptake of MEVs by human microglia cells in vitro and explored the functional outcomes of human mEV uptake, finding EVs were taken up and localized in baseline and primed microglia. Indeed, this was the first evidence of mEV uptake by a brain macrophage, suggesting a potential role in regulating epigenetic machinery and neuroimmune modulation [121]. However, also bovine EVs (bEVs) were shown to pass the blood brain barrier (BBB) of the pups, altering gene expression and promoting neuronal growth in the brain [122,123]. Based on the mentioned ONE HEALTH approach, such findings seem to promote the concept of using EVs derived from human milk in infant feeding formulas to promote neurological function, however this raises the ethical aspects of using human milk to derive EVs. v) Finally, recent research suggests that human milk EVs can enhance skeletal muscle growth and function [124] by impacting specific signaling pathways involved in muscle development. Parry et al. 2019 [125] found that mEVs orchestrate intricate and context-dependent effects on skeletal muscle growth and maturation, although the investigation was for bovine EVs on mice [125,126]. Table 2 summarizes diameter, concentration and references with detailed pleiotropic effects, of mEVs for the main, relevant reason that, human research provides a wide range of reagents that can be used to explore various areas with effective, reliable application: the latter is an aspect that is far from obvious in the veterinary/One Health field and finding suitable reagents requires additional tests and time.

Table 2. Summary of the specific Human source (among Kingdom Animalia) for breast milk/colostrum EVs: their diameter and concentrations reported in precise references.

Typology of breast milk	Animal Origins	Diameter	Concentration	References
Colostrum	Human	NA	NA	**[70]**
	Human	258.8 nm (Mode: 196.1 ± 7.4 nm)	4.96 x 10^{12} ± 3.21 x 10^{10} particles/ml	**[127]**
	Human	150.2 nm for human colostrum	2.1 × 10^{13} for human colostrum	**[107]**

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	Human	c.a. 50nm	NA	**[128]**
Transitional Milk or First Milk	Human	188 nm for term and 161 nm for preterm human transitional milk	6 x 10^6 for term and 8 x 10^6 for preterm human transitional milk	**[48]**
	Human	10 to 210 nm	NA	**[129]**
Mature Milk	Human	103,8 nm (96-116,6 nm) for Fresh milk, 104,7 nm (101,7-113,3 nm) for Frozen milk	3.85 x 10^{11} (2.11 - 4.45 x 10^{11}) for Fresh milk, 3.93 x 10^{11} (2.23 - 5.02 x 10^{11}) for Frozen milk	**[130]**
	Human	163.5 nm ± 83.5	1.5 x 10^{11} ± 3.4 x 10^{10} particles/mL	**[131]**
	Human	150.4 ± 69.3 nm	7.08 × 10^8/ml	**[132]**
	Human	the size varied within 30- to 600-nm range	NA	**[42]**
	Human	149.7 ± 20.7 nm	2.18 ± 1.53 × 10^{11}	**[133]**
	Human	c.a. 50nm	NA	**[119]**
	Human	EVs pellet with sizes ranging from 100 to 200 nm, smaller vesicle-like particles in the supernatant ≤ 50 nm	8.18 × 10^{10} particles/mL	**[122]**

5. Isolation Methods: Recommendations to Minimize Caseins and Fat Globules

The use of EVs is not without its drawbacks, one of which is the complexity of the isolation process, which can be difficult to achieve. Different preparation methods can impact the concentration and purity of EVs in different ways. Consequently, selecting a suitable preparation technique is vital for ensuring the quality of research. A variety of methods have currently been developed, each with its own set of advantages and limitations [134]. Among the isolation methods approved by the Minimal Information for Studies of Extracellular Vesicles (MISEV), we have ultracentrifugation, density gradient centrifugation, exclusion chromatography, ultrafiltration, immunomagnetic separation, polymer precipitation, microfluidic platform, asymmetric flow field-flow fractionation, and anion exchange chromatography [135,136]. A significant disparity exists among the diverse methodologies employed for the isolation of EVs, primarily characterized by the distinct separation criteria that underpin each approach. For example, ultracentrifugation uses a size- and density-based separation criterion, polymer precipitation uses a solubility-based separation criterion and ultrafiltration uses a size-based separation criterion. Filtration, ultracentrifugation and affinity separation are the most common isolation methods. Various peptides, proteins, lipids and cell debris contaminants are present in the source samples, some of which are similar to EVs in structure and composition, whereas some interact with EVs, preventing extraction [137,138].

Several studies have investigated the issue of contaminants, particularly fat globules and casein micelles, during the purification of EVs from milk samples. In this context, milk represents a particularly challenging biological matrix, as it is a complex colloidal system in which non-vesicular particles such as milk fat globules and casein micelles overlap with EVs in both size and buoyant density, thereby representing major sources of co-isolated contaminants [2]. In human milk, caseins (primarily the alpha, beta and kappa-isoforms) constitute a major portion of the protein content, accounting for approximately 3.6 g/L or about 40% of total protein [139]. These proteins interact with colloidal calcium phosphate to form supramolecular assemblies known as casein micelles [140]. The size of casein micelles, typically ranging from 100 to 600 nm [141], overlaps with that of EVs, which span from <200 nm for small EVs to 200–1,000 nm for larger vesicles. Their buoyant density (around 1.06 g/mL) also closely matches the density range of EVs (1.08–1.19 g/mL) [139]. Because of these similarities, casein micelles frequently co-isolate with human milk EVs (HmEVs), especially smaller

EV subpopulations, regardless of the purification technique employed. Consequently, removing or disaggregating casein micelles prior to HmEV isolation is critical for improving vesicle purity.

The aforementioned principle also applies to bovine milk as it contains other colloidal structures with milk EVs, such as milk-fat globules and casein micelles. Casein is the major milk protein and comprises 80% of total protein content in bovine milk in contrast to 35% in human breast milk [142]. Furthermore, milk samples have a markedly different casein profile, with alpha-casein comprising more than half of the total casein fraction [143]. In contrast, human milk is dominated by beta-casein, with approximate proportions at two weeks postpartum of 15% alpha-, 55% beta-, and 30% kappa-casein [144]. Alpha- and beta-casein also exhibit distinct biochemical characteristics: alpha-casein is more hydrophilic, whereas beta-casein has greater hydrophobicity [145].

These biochemical differences may influence casein–EV interactions and contribute to species-specific challenges in milk EV isolation and downstream molecular characterization. Casein separation is an important step in obtaining pure milk EVs. Cetinkaya et al. (2024) [146] demonstrated that the combined use of chymosin and EDTA is highly effective and should be considered a preferred method for removing casein micelles from human milk samples. Several studies have systematically compared different strategies to eliminate casein micelles, particularly in the context of EV isolation from bovine and porcine milk, highlighting that pre-treatment choice has a direct impact on EV purity and molecular yield. Rahman et al., 2019 [109] evaluated the effects of acidification on milk-derived EVs with those of standard ultracentrifugation (UC) demonstrating that the acidification to pH 4.6 using HCl treatment significantly enhances the isolation of bovine mEVs by effectively precipitating casein micelle. The authors concluded that acidification represents a rapid, cost-effective, and robust alternative to standard ultracentrifugation alone, providing high-purity vesicles suitable for advanced downstream applications such as RNA sequencing and functional assays [109]. Morphological analysis via TEM confirmed that acidified EVs retain their integrity, exhibiting the characteristic cup-shaped structure and typical size range (30–200 nm). Furthermore, the samples were found to be enriched significantly in exosomal markers (CD63, CD81) and RNA. The researchers concluded that acidification could represent a rapid, cost-effective, and superior alternative to standard UC, providing high-purity vesicles for advanced downstream applications like RNA sequencing and functional assays [146]. Wang et al. (2024) [147] evaluated several pretreatment methods to overcome the common problem of casein contamination during the isolation of porcine mEVs. Their results showed that hydrochloric acid treatment (HA) before ultracentrifugation effectively removes caseins and other protein complexes. Compared to conventional ultracentrifugation (UC) alone or other chemical treatments, the pretreatment with HA followed by UC produced mEVs with significantly higher purity and fewer non-vesicular protein contaminants. Furthermore, the HA/UC method resulted in a much higher concentration of miRNA (specifically miR-148a-3p) per milligram of protein.

Importantly, mEVs isolated using HA/UC also demonstrated superior biological performance in vitro, including enhanced cell proliferation and anti-inflammatory effects, reinforcing the functional relevance of effective casein depletion strategies [147].

In addition to chemical and enzymatic pre-treatments, recent studies have shown that the technological history of milk samples, including homogenization and pasteurization, can substantially influence both EV integrity and the extent of co-isolated milk proteins and lipids. In particular, homogenization has been associated with increased carryover of non-vesicular milk components, further supporting the need for stringent pre-treatment and cleanup steps when working with processed milk [148]

Finally, growing evidence supports the use of orthogonal isolation workflows combining pre-treatment steps aimed at reducing casein and fat globules with downstream size-exclusion chromatography or density-based separation, as these approaches improve the analytical quality and reproducibility of milk EV preparations compared with single-step ultracentrifugation [149,150].

6. Detection Methods: Hints on the Most Commonly Employed Instruments

The investigation of EVs presents significant hurdles for the scientific community. Researchers studying EVs, focusing on their functional, physical and biochemical attributes, frequently encounter issues with reproducibility. There are a variety of methods that can be used to detect EVs (Figure 2), such as nanoparticle tracking (NTA), Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), Transmission Electron microscopy (TEM), Dynamic Light Scattering (DLS), Mass Spectrometry (MS), Polymerase Chain Reaction (PCR), Flow Cytometry (FC), Western Blot (WB) and Total Internal Reflection Fluorescence (TIRF) [151,152]. Each of these methods is able to obtain different information about the samples analyzed.

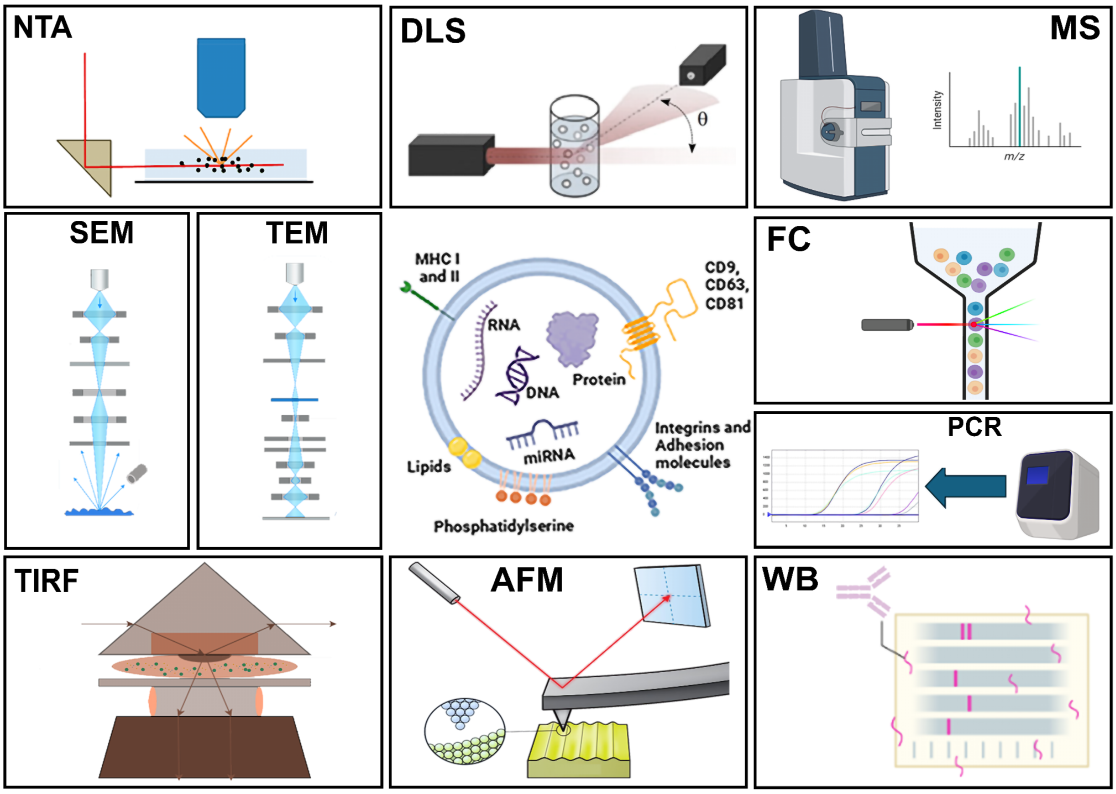


Figure 2. Representation of the different techniques involved in the evaluation of EVs: Nanoparticle Tracking Analysis (NTA), Dynamic Light Scattering (DLS), Mass Spectrometry (MS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Flow Cytometry (FC), Polymerase Chain Reaction (PCR), Total Internal Reflection Fluorescence (TIRF), Atomic Force Microscopy (AFM) and Western Blotting (WB).

The NTA process involves two steps. First, particles in suspension are illuminated with a laser beam. Then, the scattered light is recorded using a light microscope. The mean square displacement of each particle is determined by tracking the Brownian motion of each particle. The total number of particles is then used to estimate the concentration of particles. NTA is a very valid method for measuring EVs' size and concentration, along with TEM, a high-resolution imaging technique that visualizes the ultrastructure of very small particles, including EVs, at the nanometer scale [153]. The techniques that were mentioned included ELISA, WB and FC, which are used to detect specific proteins, such as EVs markers.

MS is a powerful analytical technique that ionizes compounds and sorts them based on their mass-to-charge ratio. MS has been instrumental in the proteomic analysis of EVs, with advances in chromatography-coupled MS enhancing the identification of new EV protein biomarkers. The proteomic study of EVs usually involves three steps: isolating and purifying EVs; identifying proteins through MS; and analyzing the data in detail [156,157]. SEM focuses on surface morphology by utilizing secondary electron signals. A focused electron beam scans the sample surface, inducing the emission of secondary electrons, which are then collected by a specialized detector. These electrical signals then generate an image on a screen [158].

In TEM, an image is created by electron interference when the electron beam crosses the sample. Since the wavelength of the electron beam is shorter than the wavelength of visible light by three orders of magnitude, the images are recorded with resolution of 1 nm. Unfortunately, benefits from high resolution can be easily outweighed by disadvantages related to the measurement conditions and sample preparation. The specimens analyzed by TEM have to be fixed and dehydrated before the measurement. Additionally, the image acquisition is carried out under vacuum conditions. Nonetheless, electron microscopy is utilized for EVs visualization, and the obtained images are then used for diameter determination of the studied vesicles [159,160].

The PCR is widely recognised for its ability to detect nucleic acids, including analysing the varied compositions of nucleic acids found within EVs. Initial research utilizing techniques such as RT-qPCR and microarrays verified the presence of different types of RNA, including mRNA, miRNA and long non-coding RNA (lncRNA), within EVs [161].

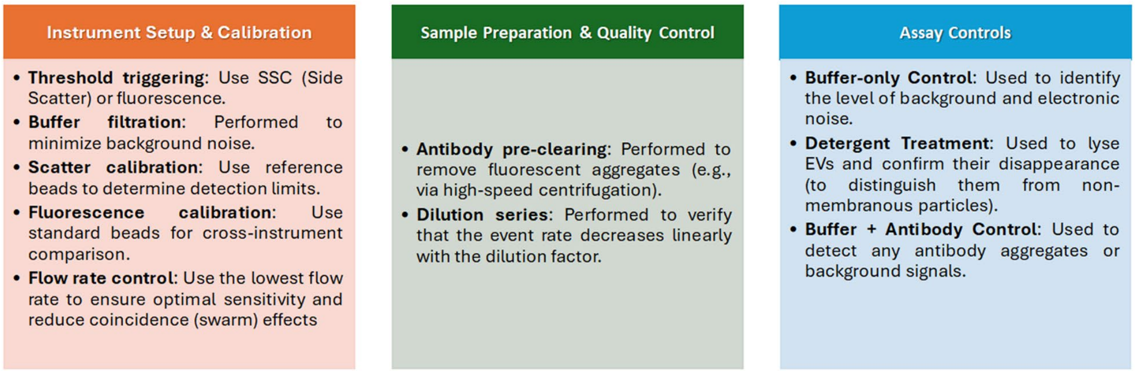
TIRF analysis is a sophisticated method of observing single molecules or nanoparticles. It involves monitoring their fluorescence following excitation by total internal reflection. This method enables accurate quantification through the counting of fluorescence spots and the measurement of their intensity [162].

AFM is a technique that detects and records the interactions between a probing tip and a sample's surface. A key feature of AFM is its ability to measure samples in their native state with minimal preparation. In this case, the EVs first had to be immobilised on a freshly cleaved mica surface and then scanned. AFM enables the acquisition of real 3D images of surface topography with very high resolution; however, for imaging to be successful, all vesicles must be attached to atomically flat surfaces such as mica [163].

WB is fundamental to the biochemical sciences for profiling EV proteins. The process involves treating purified EVs with a buffer containing denaturants and protease inhibitors, followed by protein separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins are then transferred to a cellulose membrane, where they can be detected using specific antibodies and enhanced chemiluminescence [164,165].

7. Insights into Flow Cytometry Approaches

Flow Cytometry (FC) is a technique that offers a multiparametric technology capable of identifying single EVs and measuring their cellular origin. It is typically employed in the characterization of EVs for biomarker expression. Flow Cytometers measure fluorescence and light scattering signals originating from thousands of single particles per second in a fluid stream [166]. FC allows the analysis and sorting of larger EVs and, through bead-based approaches, the capture of EVs labelled with fluorescent antibodies [135]. Different strategies, able to overcome several challenges, are available for EV detection by Conventional FC: Fluorescence Triggering: Triggering the detection system on a bright fluorescence signal (e.g., from a potent membrane dye like lactadherin or a highly expressed antibody) can detect smaller EVs than using light scattering alone [167,168]. The Mechanism of Detection for Lactadherin (also known as MFG-E8) is based on C1 and C2 domains (contained in the molecule) that specifically interact with phosphatidylserine in a calcium-independent manner. For detection purposes, lactadherin is typically conjugated with a fluorophore, such as fluorescein isothiocyanate (FITC). The ISEV provides guidelines and works toward standardizing these measurements [169]. However, all the other EV fluorescent markers can be used as the guide-parameter [167]; and these approaches, sometimes referred to as fluorescence-triggered flow cytometry (FT-FC) or single-EV flow cytometry (vFC), have become a standard strategy for rigorous and reproducible EV analysis. Although the points of attention are clearly indicated by the MISEV guidelines [170], here we report that extreme care must be taken to remove unbound dyes or dye aggregates, which can be mistakenly counted as EVs. Controls and Calibration: Rigorous controls (buffer-only, unstained, detergent lysis, serial dilutions) and the use of reference beads, particularly those that mimic EV refractive index like hollow organsilica beads (HOBs), are essential for reliable and reproducible data. A foundational set of recommendations was initially established in 2014, known as the Minimal Information for the Study of EVs (MISEV) [13], and was subsequently updated [171]. These guidelines should be followed in all aspects of EV research, from sample collection and preparation through different technical approaches and final data reporting. The MIFlowCyt-EV standard provides specifications for the appropriate experimental design, reagents, protocols and panels for EV characterization, by FC. The FC analysis of EV presented several challenges (due to micro-sized particles) that are now gradually being resolved. Details are given in the following publications [172–174] and summarized in Figure 3. These controls serve as an indispensable reference point, facilitating comparisons across all other experimental samples and controls concerning critical parameters such as the event rate, signal intensity, and the development of appropriate gating strategies and to set thresholds and properly define the boundaries separating true positive signals from instrumental or sample-related background noise.



Instrument Setup & Calibration

- **Threshold triggering**: Use SSC (Side Scatter) or fluorescence.
- **Buffer filtration**: Performed to minimize background noise.
- **Scatter calibration**: Use reference beads to determine detection limits.
- **Fluorescence calibration**: Use standard beads for cross-instrument comparison.
- **Flow rate control**: Use the lowest flow rate to ensure optimal sensitivity and reduce coincidence (swarm) effects

Sample Preparation & Quality Control

- **Antibody pre-clearing**: Performed to remove fluorescent aggregates (e.g., via high-speed centrifugation).
- **Dilution series**: Performed to verify that the event rate decreases linearly with the dilution factor.

Assay Controls

- **Buffer-only Control**: Used to identify the level of background and electronic noise.
- **Detergent Treatment**: Used to lyse EVs and confirm their disappearance (to distinguish them from non-membranous particles).
- **Buffer + Antibody Control**: Used to detect any antibody aggregates or background signals.

Figure 3. Schematic representation of Standardized Framework for Single EV Detection and Characterization by Flow Cytometry: details reported by MISEV guidelines.

8. Bead-Based Assays Multiplex

A common and broadly useful approach is to capture EVs onto larger, commercially available beads that are well within the detection range of conventional flow cytometers. This method allows for the phenotyping of EVs using specific antibodies, though it analyzes bulk populations rather than single EVs. Briefly, microbeads are coated with specific antibodies (e.g., anti-CD9, CD63, or CD81) or streptavidin (for biotinylated EVs) and are incubated with the sample to collect EVs [175]. Then, Captured EVs are stained with fluorescently conjugated antibodies targeting surface proteins.and it is possible to perform the FC Readout: The flow cytometer detects the fluorescence of the entire bead-EV complex. A higher fluorescent signal (as MFI) indicates a greater abundance of the target protein on the captured EVs. Different kits are commercially available belonging to: Multiplex Bead Assays (with fluorescently barcoded beads to simultaneously detect several different surface markers in a single sample [130]; Bead-conjugated EV Assay Detected (with high-affinity biotin-streptavidin interactions to capture EVs on 5 μm polystyrene beads for sensitive high-throughput clinical analysis). Furthermore, MISEV furnished details also for the bead-based EV detection [170] and this technique is largely employed [176].

9. Imaging Flow Cytometry (IFC)

IFC integrates the high-throughput capabilities of conventional flow cytometry, specifically the quantification of light scatter and multi-parametric fluorescence, with the morphological insights of high-resolution microscopy. Unlike traditional systems, IFC operates through a triggerless acquisition mechanism, characterized by zero dead time and continuous monitoring of the sample core, which effectively precludes coincidence-related data loss. The architectural features of IFC stem largely from the utilization of Charge-Coupled Device (CCD) sensors rather than Photomultiplier Tubes (PMTs). CCDs offer several distinct analytical advantages: 1) performant Signal-to-Noise Ratio which significantly reduces electronic noise floor, 2) enhanced Linear Dynamic Range that guarantees greater flexibility in quantifying varying signal intensities, 3) high quantum efficiency that takes to an optimal sensitivity for detecting low-abundance photons. IFC employs Time Delay Integration (TDI) for CCD readout: by facilitating integration periods in the millisecond range, as opposed to the microsecond scales typical of PMT-based systems, TDI maximizes signal collection without incurring a readout noise penalty. Furthermore, every particle traversing the focal plane is recorded. This eliminates the requirement for a hardware trigger and minimizes the background interference Moreover, while coincidence events are inherently mitigated by the continuous flow architecture, the presence of multiple objects within a single frame does not compromise data quality. Such events are readily distinguished through automated image-based deconvolution or spatial filtering, allowing for the precise exclusion of artifacts from the final analytical dataset [177–180]. IFC methodologies have facilitated the longitudinal tracking of intracellular EV uptake and, more recently, the assessment of EV docking kinetics on the cell surface [181]. Nevertheless, the simultaneous profiling of multiple surface antigens on individual vesicles via IFC still presents substantial technical hurdles.

10. EV Generic Fluorescent Markers

This term refers to various non-antibody-based fluorescent dyes and kits used in biological research to label and track EVs. Researchers use a variety of dyes to label different components of EVs, such as membranes or proteins, in fact although antibody labelling of EV markers is a performing strategy for EV detection by many platforms, there are no protein targets for antibody labelling that are presented on the surface of majority of EVs in quantities adequate to yield successful labelling and detection across all platforms [182]. Furthermore, Loconte and coworkers have shown that the method used for EV labelling influences the detection of the different types of EV interactions with the recipient cells [183], underlining the importance of the choice of the right tracer (or even better, of the best combination of more than one tracer in EV internalization studies). Here a useful list of the most employed fluorescent probes is reported, with the main advantages and disadvantages, citing different literature sources, on the basis of which the readers can deepen the features of the single probe:

10.1. Membrane Dyes for Single EVs Detection with and w/o EV Separation

1. LCD (Lipophilic Cationic Dye) (LCD and FITC-conjugated phalloidin kit, BD Biosciences), and vFRed™ (Cellarcus Biosciences) belong to the group of dyes allowing for direct detection, sizing (with beads), and characterization (co-staining with antibodies) in complex samples like plasma, milk or other body fluids. Membrane labeling with LCD is generally compatible with subsequent immunophenotyping, indeed, protocols utilizing LCD have been shown to provide repeatable and standardized counts of circulating EV sub-phenotypes [184]. LCD is lipophilic and positively charged, allowing it to intercalate into and "probe" the lipid bilayers of membrane-bearing structures like EVs, of note, when used with dyes like phalloidin (which binds to F-actin), LCD can distinguish intact EVs from damaged vesicles or cell debris. vFRed™ is a next-generation, far-red fluorescent lipophilic dye specifically engineered for the high-resolution labeling of EVs, including exosomes and microvesicles. The labeling is achieved through the spontaneous insertion of the dye's hydrophobic aliphatic chains into the phospholipid bilayer of the EVs [171,185]. The AcoDyes (Acoerela) are a patented series of highly water-soluble, fluorogenic membrane dyes from the company Acoerela designed for accurately tracking EVs both in vitro and in vivo. In fact, detection via flow cytometry can be performed without needing ultracentrifugation (UC). Specific dyes like Aco-490, Aco-430 and Aco-600 bind directly to EV membranes, differentiating true EVs from background noise: this depends on their chemistry, inducing the "light on" mechanism, i.e., different emission profiles for bound and unbound dyes. Therefore, all these stainings can be used to label and detect EVs

isolated by different techniques, besides the possibility to be employed for detection in biofluids, reducing the need for extensive enrichment steps that might alter vesicle morphology or count.

10.2. Membrane Dye for Single EVs Detection After EV Separation

2. These lipophilic dyes incorporate into the lipid bilayer of the EVs; indeed, we want to underline that these dyes are employed on sec-purified, ultracentrifuged or differentially centrifuged EVs. Chen and coworkers found that PKH67 and PKH26 could maximally label ~60%–80% of EVs isolated from the conditioned cell culture medium [186]. Both PKH dyes are widely used but can form aggregates that interfere with detection, requiring careful use at optimal concentrations. Indeed, a density gradient centrifugation step (to remove unbound dye) is required by the manufacturer for PKH67 protocol, although this step is inevitably associated with increased material loss during preparation. DiI and di-8-ANEPPS: These molecules can provide efficient and uniform labeling of EVs. Fluorescent dyes such as di-4-ANEPPS and di-8-ANEPPS are highly sensitive fluorescent dyes displaying consistent potentiometric responses in a wide variety of systems [187,188]. While they have similar spectral properties, di-8-ANEPPS is less water-soluble and more stable in the membrane than di-4-ANEPPS due to its longer hydrophobic carbon tails [189]. di-8-ANEPPS and high concentration of DiI could achieve efficient and uniform labelling of EVs with nearly 100% labelling efficiency for di-8-ANEPPS and 70%–100% for DiI in EVs isolated from the conditioned cell culture medium [186]. MemGlow and CellMask: These probes reveal a bright and sensitive staining of EV membranes with minimal aggregation, although MemGlow showed an affinity to VLDLs. In a recent study, employing nanoFCM, these dyes (CellMask Deep Red, MemGlow 488 and 640) were compared with the following probes: ExoBrite 490/515 (Biotium); ExoBrite 640/660 (Biotium); CellTracker Deep Red (CTDR, Thermofisher). EV dilution and staining protocol is detailed by Brealey J and coworkers [182]. ExoBrite™ True EV Membrane Stains are offered as alternatives to traditional dyes and are noted for minimal background aggregation and high-resolution imaging performance, even by the spectral flow cytometry apparatus [190]. Furthermore, in imaging Flow Cytometry, a novel lipid dye called Exoria (Exopharm Limited), was recently applied [191]. Finally, Calcein AM, Calcein Violet and CFSE are additional generic markers for flow cytometry detection of EVs in cell uptake studies [192]. Membrane labeling with CFSE [5-(and-6)-carboxyfluorescein diacetate succinimidyl ester] normally is performed at 37 °C, ensuring optimal conditions for enzyme activity and thus the turnover to the fluorescent variant CFSE. However, Ender and coworkers (2020) incubated EVs with 40 µM CFSE for 10 min at 4 °C or room temperature, finding the best yield of intact CFSE+ EVs. In mEVs (from both bovine and human origin) the protein-binding dye CFSE was incubated at 37 °C for 2 hours, at 40 µM concentration, and the free dye was removed by ultrafiltration at 2000g for 30 min. Usually, this kind of labelling is carried out for cellular uptake experiments. De Rond et al. (2018) [193], reported an overview of the properties of different markers for plasma EVs, including calcein violet and lactadherin, which have not been used as generic markers previously of their work [185], concluding that none of the generic markers detected all and only EVs. For milk EV specific cellular uptake experiments (and stability), Calcein-AM dye (Thermo Scientific) or CTDR were used following a well-established protocol, at 10 µM concentration [194].

11. Tetraspanins CD9, CD63 and CD81 for Detecting mEVs

The organization of membrane microdomains, termed tetraspanin-enriched microdomains (TEMs), is performed by tetraspanins, a protein superfamily, which form clusters and interact with a large variety of transmembrane and cytosolic signaling proteins [195]. CD9, CD63 and CD81 are among the tetraspanins that have a broad tissue distribution, while others such as CD37 and CD53, are restricted to particular tissues. Exosomes have been described as highly enriched in tetraspanins and tetraspanins have been proposed as possible exosomal markers. Tetraspanins can interact with various receptors and signaling molecules at the membrane. Consequently, they may be involved in the attachment of exosomes to target cells and their absorption, or in antigen presentation in response to the immune system [196,197]. Research performed on the human cell line HEK293 analyzed the proteins most highly enriched in exosomes, identifying CD9, CD63 and CD81 as the most prevalent. These proteins have therefore been utilized for exosome capture and detection. CD63 and CD81 are the most frequently identified proteins in exosomes and are considered classical markers [195,198]. As tetraspanins are expressed on the surface of EVs, they are extensively utilized in the flow cytometric analysis of said EVs. In immunoassays developed for EVs phenotyping, the tetraspanins CD9, CD63 and CD81 are commonly used as bona fide EV-associated markers for total EV detection. These tetraspanins play a key role in EV formation, cargo selection/sorting, and EV release and uptake. Furthermore, the presence of specific combinations of CD9, CD63 and/or CD81 on EVs can provide information about their particular biogenesis pathway [199]. CD9 is a member of the tetraspanin family of proteins, which coordinate lateral interactions with other membrane proteins, particularly integrins, on the cell surface plays a key role in essential cellular functions in many immune and endothelial cells. These functions include intracellular signaling, cell stimulation and proliferation and cell viability. Subsequently, it became associated with numerous cellular processes, including motility, proliferation, differentiation, fusion and adhesion [200,201]. Some studies, such as those by Suzuki et al. (2009), [202] have demonstrated that, in a murine model, CD9 negatively regulates LPS-induced macrophage activation, and that knocking it out increases infiltration of macrophages into the lungs [202]. Another study by Wang et al. (2002) [203] showed that murine CD9 is expressed on all peritoneal macrophages but is downregulated upon activation by IFNγ [203]. Therefore, CD9 expression may be an indicator of antigen-presenting cell (APC) subsets with a higher capacity for T cell activation [200]. Milburn J.V. et al. 2021 [200] conducted a study with the aim of using two novel monoclonal antibodies to detect CD9 expression on porcine leukocytes. CD9 was found to be expressed on monocytes, indicating their role as antigen-presenting cells (APCs). The study demonstrated that CD9 is uniformly expressed on porcine monocytes, which are APC precursors, as well as on a distinct population of CD9+ porcine B cells. Therefore, it is possible that a CD9+ phenotype also represents a subset of porcine B cells with enhanced antigen presentation or T cell activation function. However, CD81 is the most widely used and informative marker, particularly for milk-derived EVs in all species [199].

12. Antibody Availability and Cross-Reactivity: Critical Issues in One Health EV Flow Cytometry

Monoclonal antibodies are essential tools for many molecular immunology investigations and are particularly useful when used in combination with techniques such as epitope mapping and molecular modelling. This combination enables the antigenic profiling and visualisation of macromolecular surfaces [204]. The remainder of the molecule is specific to a single epitope, and each antibody is the product of a single B cell clone. Therefore, an antibody with unique specificity that is derived from a single B cell clone is known as a monoclonal antibody. A wide range of commercially available bovine mAb clones is available for use in FC and many of these have been validated as cross-reactive with Water Buffalo leukocyte antigens. Commonly used T-cell markers include MM1A for CD3, CC8 and CACT138A for CD4 and CC63 and CC58 for CD8α and CD8β. B-cell identification largely relies on CD21 clones (CC21 and CC51) as well as anti-CD79a. Activation or co-stimulatory molecules, such as CD28 and CD80, are detected using clones (CC219, CC220 and IL-A159). Additional markers, such as CD205 (IL-A114, CC98), and endothelial CD31 (e.g., CO.3E-1D4), further expand the panel. These bovine mAb have been shown to be suitable for buffalo immunophenotyping: a recent study validated eight monoclonal antibodies, including anti-CD3ε, CD16, CD18, CD45R0, CD79a and CD172a as reliably cross-reactive with buffalo leukocytes [205]. The foundational work of Davis et al. (2001) [206] identified 138 cross-reactive clones among over two hundred tested, targeting a broad spectrum of buffalo molecules, such as CD2, CD3, CD4, CD5, CD6, CD8, CD11b, CD11c, CD18, CD21, CD25, CD29, CD44, CD62L, CD45R, CD79 and MHC I/II [198]. Subsequent studies have further confirmed robust cross-reactivity for markers including CD14, CD16, CD163 and CD172a [207]. These findings demonstrate that bovine mAb are a reliable and accessible toolkit for buffalo FC, provided clone-specific titration is performed on buffalo cells to optimize staining quality. Among the monoclonal antibodies that cross-react with cattle, CD63, CD9 and CD81 have been identified. Several proteins have been conventionally used to mark microvesicle bodies and exosomes, they include members of the tetraspanin family (e.g., CD9, CD63, and CD81), components of the ESCRT complex, and members of the Rab family [208].

12.1. CD9 Clones

The mAb MM2/57 clone is a mouse IgG2b anti-CD9 antibody. MM2/57 recognizes a conserved CD9 epitope and is cross-reactive with bovine CD9. This makes it suitable for applications such as FC, immunohistochemistry and Western blotting in multiple species. CD9 is a tetraspanin involved in adhesion, migration and signaling expressed on various bovine cell types and bovine-specific anti-CD9 antibodies confirm that it is detectable and biologically relevant in cattle. Although suppliers have documented the cross-reactivity of MM2/57 with bovine tissue, peer-reviewed studies using this specific clone in cattle are limited. However, according to the literature, MM2/57 and two other anti-human CD9 mAb clones (LT86A and RHIA) are cross reactive with cattle leukocytes and a study conducted by Sopp P et al. (2007) [209] revealed that more than twenty mAbs reacted with bovine cells, exhibiting a consistent staining pattern indicative of recognition of the bovine homologue of the human CD antigen. This included mAbs such as CD9 [209].

12.2. CD81 Clones

the research team led by Weiskirchen aimed to present an efficient protocol to isolate EVs from bovine and human milk. The authors aimed to demonstrate that the protocol yields EV preparations that express canonical exosomal marker proteins such as CD81 in both bovine and human milk samples. Since bovine and human CD81 share 94% sequence identity, it has been demonstrated that this antibody also reacts with bovine CD81. Several well-characterized clones exist for bovine CD9, most notably IVA50, a mouse mAb raised against bovine thrombocytes that are validated for use in both FC and WB. It recognizes a ~24kDa tetraspanin in bovine cells.

12.3. CD63 Clones

For bovine CD63, the most commonly used clone is CC25, a mouse mAb that binds the bovine homologue of human CD63. Bio-Rad reports that this clone is effective in both FC and WB and it has been employed in studies of lysosomes and EVs in cattle. In contrast, clone H5C6 (anti-CD63) is widely used in human immunology however, there is no documented, validated cross-reactivity with bovine CD63. Overall, while there is a solid toolkit of anti-bovine tetraspanin antibodies, for water buffalo (*Bubalus bubalis*) the options are more limited and would likely require the validation of these bovine clones for cross-reactivity [210].

A bibliometric analysis was performed to identify the main patterns, trends, and perspectives on milk EVs. These analyses allow us to identify the main themes, trends and gaps in literature.

The search of documents on Web of Science as reference database was built on specific keywords, namely “milk EVs” or “Yak Milk EVs”, or “Horse Milk EVs”, or “Buffalo Milk EVs”, or “Bovine Milk EVs”, or “Porcine Milk EVs”, or “Caprine Milk EVs”, or “Goat Milk EVs” or “Camel Milk EVs” and “Flow Cytometry” or “Fluorescence Detection” or “EV Tracers”, and “Tetraspanins” or “CD9”, or “CD81” or “CD63”, or “cross reactivity”, and “Gut health”, or “Immune Cell”, or “Bone Health”, or “Neuronal Development”.

A keywords co-occurrence analysis was performed via VOSviewer software (1.6.20 version) to generate network maps of the main research topics (i.e., clusters) and trends in keywords (Figure 4 and Figure 5).

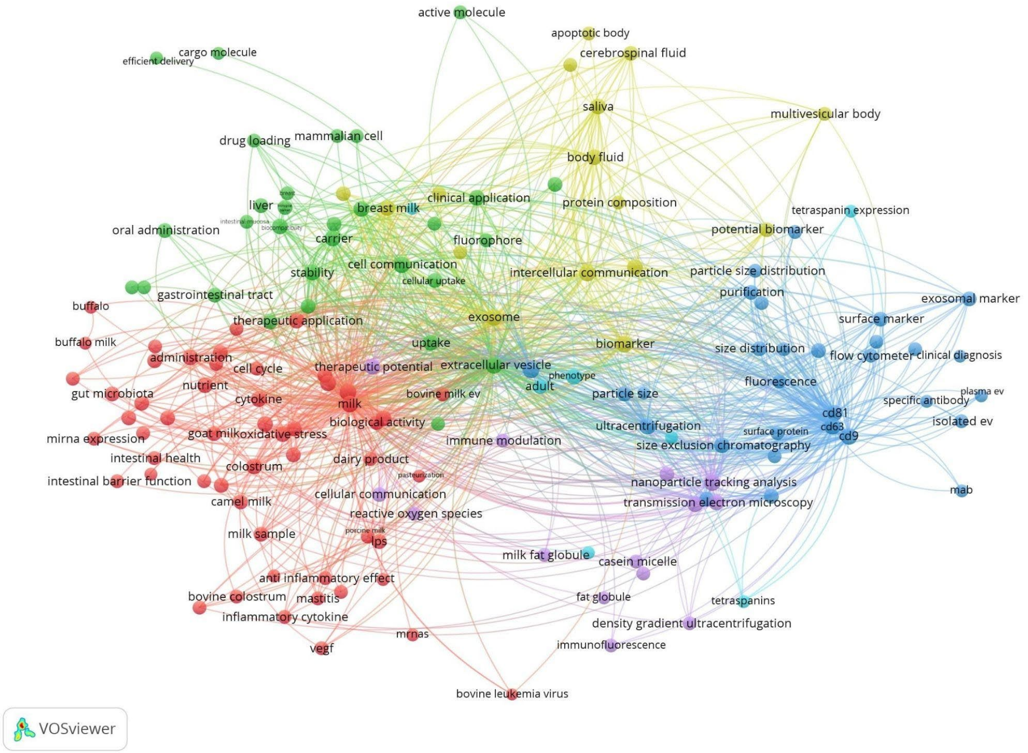


Figure 4. Keyword co-occurrence network generated through bibliometric analysis using VOSviewer: the figure illustrates the network of keywords extracted from selected publications in which the nodes represent keywords and their size is proportional to their frequency of occurrence. Links between nodes indicate co-occurrence relationships. Indeed, different colours denote thematic clusters that were automatically identified by the software. These clusters highlight the following: the lime cluster where terms are linked to EV characterization and biogenesis; the blue cluster where terms are related to tetraspanins and technical methods for isolating/analyzing EVs; the red cluster in which terms are related to inflammation, intestinal health, oxidative stress and milk-derived components; the green cluster in which terms are associated with mEVs, gastrointestinal tract uptake and delivery applications and, finally, the purple cluster where terms are associated with milk proteins and structural components.

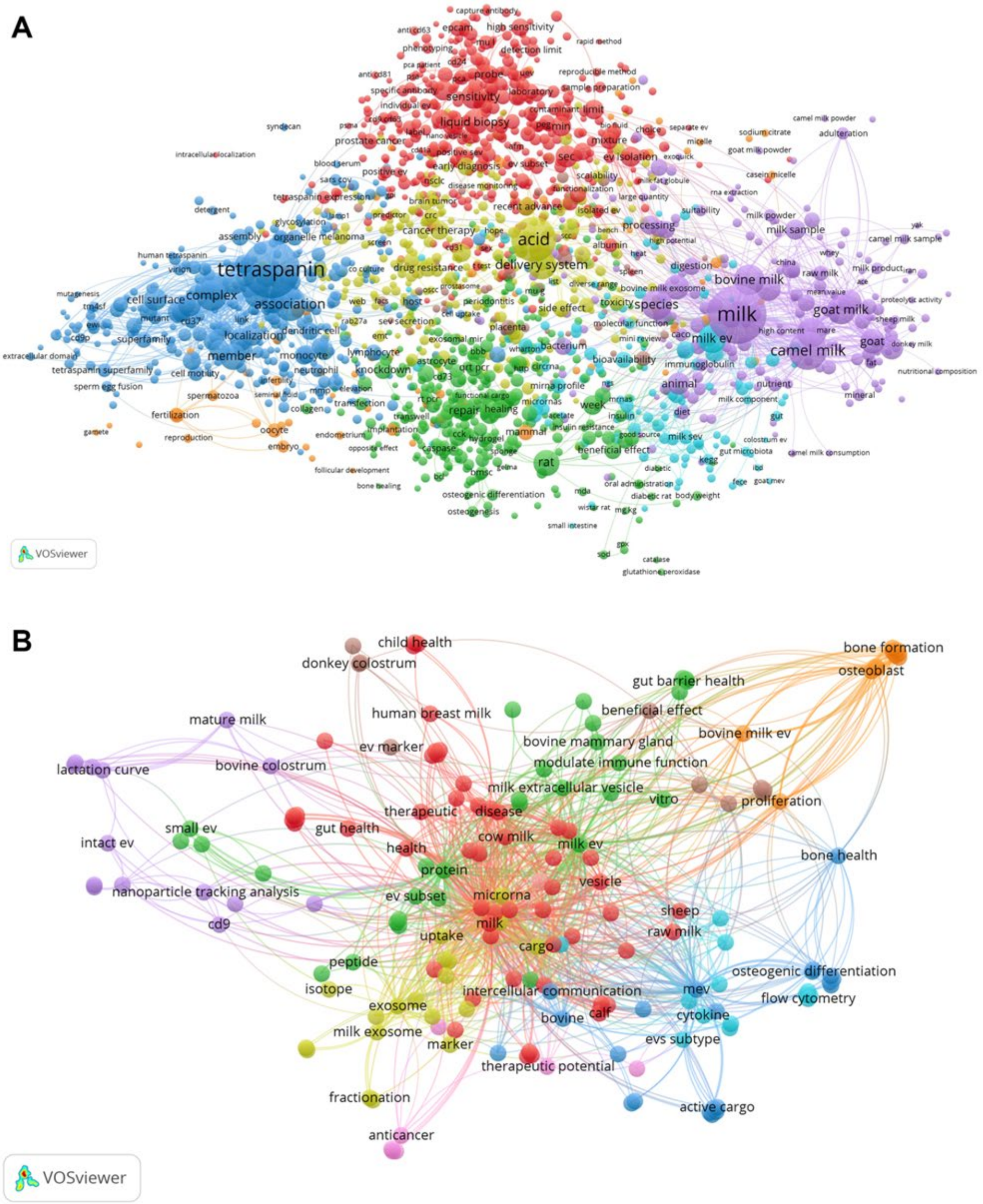


Figure 5. The VOSviewer analysis presented in both maps (A and B) illustrates the current research trends in milk EVs, highlighting various scientific investigations ranging from their fundamental characterization and biological mechanisms to their significant therapeutic potential in areas such as cancer treatment, bone formation and gut health. A) Red cluster focuses on the use of milk EVs in cancer treatment and related chemical/food processing topics. Green cluster reveals how EVs interact at a cellular level, their localization and their potential as delivery systems. Blue cluster highlights specific biological components like tetraspanins, cell surface interactions and cellular fusion processes. Purple cluster focuses on the origin of the milk (camel, bovine, goat) and immune-related components like immunoglobulins. B) Orange cluster refers to osteogenic differentiation, bone formation processes and the role of osteoblasts induced by milk EVs. Green cluster focuses on gut health induced by different sources of milk EVs. Red cluster highlights the ability of milk EVs to modulate the immune system and their use in treating various diseases. Purple/Pink cluster concerns the active cargo within milk exosomes with anticancer potential and the scientific methods used for analysis. Light blue cluster focuses on the characterization and analysis of EVs found in various types of raw milk. From the above presented VOSviewer analyses it is possible to easily note that blue and purple clusters of Figure 5A are at both ends of the graph and, although few interconnections are visible, this distribution summarizes what expressed several times in the main text, namely the difficulty of obtaining the reagents needed (mainly anti-tetraspanin antibodies) for

the precise characterization of EVs in the milk of many animal species. In Figure 5B this info is underlined, revealing CD9 (enclosed in the analyses of the Purple/Pink cluster) as the more recurrent antigen detected in colostrum and milk EVs.

13. Conclusion

Admyre [128] first reported in 2007 that human breast milk is rich in exosomes, but the origin of these exosomes remains uncertain. They may originate from breast epithelial cells [211,212], macrophages, lymphocytes, or even cells from other parts of the body, which reach breast milk through the blood circulation. Similar to other body fluids, mEVs are composed of a variety of RNA, lipids, proteins and so on. Several factors influence the content and biological activity of mEVs [213], such as gestational age, lactation period, maternal diet or nutrition, maternal disease, lifestyle and stress. With regard to the future application of mEVs, their proper isolation and storage is necessary to maintain the biological activity. Supplementing infant formula with physiological levels of mEVs may prevent certain neonatal diseases, such as necrotizing enterocolitis. Furthermore, the potential of mEVs as therapeutic agents and pharmaceutical carriers warrants further investigation.

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