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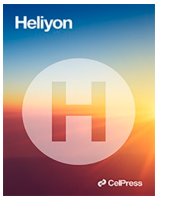

Research article

Goat milk extracellular vesicles: Separation comparison of natural carriers for theragnostic application

Jessie Santoro, Silvia Nuzzo, Monica Franzese, Marco Salvatore, Anna Maria Grimaldi *

IRCCS SYNLAB SDN, Via Emanuele Gianturco 113, 80143, Napoli, Italy

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ABSTRACT

Goat milk is a complex biological fluid, which in addition to having a high nutritional value, it is an interesting source of extracellular vesicles (EVs). Despite the countless potential applications that they offer in many biological fields, is not easy to compare the different proposed systems, and this is a major limitation for the real translatability of these natural nanoplatforms for theragnostic purposes. Thus, it is useful to further investigate reproducible methods to separate goat milk EVs. The choice of methods but also the preprocessing of milk has an immense impact on the separation, quality, and yield of EVs. Here, we tested four protocols to separate EVs from unpasteurised goat milk: two based on differential ultracentrifugation (DUC) and two on size-exclusion chromatography (SEC). Moreover, we assessed two different approaches of pre-treatment (acidification and precipitation) to facilitate milk protein discharge. To the best of our knowledge, a similar comparison of all performed protocols on raw goat milk has never been published before. Therefore, enriched EV samples were successfully obtained from goat milk using both DUC and SEC. Taken together, our results may be helpful to obtain natural carriers for future theragnostic applications in personalised medicine.

1. Introduction

Extracellular vesicles (EVs) are a heterogeneous population of small lipid-bilayer structures, with a diameter of 50 nm–2 μm, that derive either from the endosomal compartment or as a result of shedding from the plasma membrane. They are secreted by multiple cell types, under physiological as well as pathological conditions [1]. Since EVs were discovered over four decades ago [2], they continue to gain increased attention from the scientific community. EVs are particularly fascinating for clinical applications because they play key roles in intercellular communication, signaling, and regulation. They are nanocarriers of proteins, lipids, coding, and noncoding RNA signatures of pathological or physiological states, and may influence or regulate recipient cells [3]. Because of their unique source, structure, and physiological functions, EVs are considered an innovative and ideal natural endogenous tool for the development of nanomedicine [4]. EVs can be detected and purified from nearly all biological sources, including milk. Milk, generally considered safe and capable of providing important nutritional benefits, is one of the most promising sources for the mass production of EVs. Indeed, the availability, cost, and absence of toxicity make milk a scalable natural source of EVs, suitable for multiple uses [5]. In support of this idea, in recent years, several experimental studies have proposed milk-isolated EVs as cargo for the delivery of small

* Corresponding author.
E-mail addresses: jessie.santoro@synlab.it (J. Santoro), annamaria.grimaldi@synlab.it (A.M. Grimaldi).

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molecules [6], nucleic acids [7], and or contrast agents [8–10], strengthening its use as nanoplatform that combines diagnostic and therapeutic approaches to provide the potential for personalised treatment and real-time monitoring of the effectiveness of treatments.

Due to their nanostructural composition with cell membranes, milk EVs represent advantageous delivery platforms, offering low toxicity, high stability, overcoming natural barriers, and improving tumor/other organ-homing ability. However, up to date, the use of milk EVs in medical sciences is low and the first bottleneck for this gap can be related to EVs separation procedures. Indeed, it may be not completely achievable with simple protocols (i.e. commercial kits or polymer based methods) [11,12]. For instance, exoEasy Maxi Kit, ExoQuick-TC (SBI), Total Exosome Isolation Reagent and MagCapture Exosome Isolation Kit have also been used to separate EVs from milk. Although these kits are based on rapid isolation and are easy to operate, the quality and quantity of EVs remain poor. In addition, co-separation of protein aggregates and non-EV small fragments may occur during the separation process [13]. Therefore, methodological development aimed to ensure consistent and reproducible EVs separation protocols is itself a valuable area of research. In fact, numerous studies have explored different methods to separate milk EVs from other species [14,15]. Despite that, to date, there is not an appropriate method that can be applied to all types of milk, including goat. In this regard, the International Society for Extracellular Vesicles (ISEV) has developed appropriate protocols, recommendations, and requirements for obtaining highly purified EVs and continues to update and optimise experimental procedures [16].

Goat milk has high nutritional value; it is a good source of various macro- and micro-nutrients, with a good proportion of protein, fat, carbohydrates, and other nutritional components [17]. In 2012, the European Food Safety Authority concluded that goat milk is suitable as a source of protein and bioactive peptides for infant nutrition [18].

Goat milk has several health benefits and lower production costs [19]. It is perceived to be easier to digest than cow milk, because of the small size of fat globules [20]. Moreover, is considered an alternative food with less allergenicity than cow milk and proteins of higher biological value due to its lower concentration of α-casein than β-casein [21].

Besides being a beneficial nutritional and healthy source, it is a scalable source of EVs with great potential for biomedicine, as natural carriers for diagnostic and therapeutic applications. Goat milk EVs can be used for successful delivery of agents in different organs and can be extended for therapy and non-invasive imaging techniques. One field of application that we look at with particular interest is the use of EVs as theragnostic nanocarriers to ascertain cellular localisation/distribution and tissue accumulation/bio-distribution and pharmacokinetics of molecules or contrast agents. Goat milk EVs were isolated to develop natural probes for optical [22] and single-photon emission computerised tomography imaging [23]. Moreover, the uptake of goat milk EVs was proven to detect inflammatory processes *in vivo*, which supports their potential use as diagnostic markers [22]. Yenuganti et al. highlighted potent antiviral activity against the dengue virus of goat milk exosomes [24]. Recently, Franzoni et al. demonstrated that goat milk EVs were able to polarise porcine macrophages towards an M1-like phenotype, which can be useful to enhance defense against intracellular pathogens and malignancies [25]. The examples given here concern detection and response to inflammatory processes, but the use of

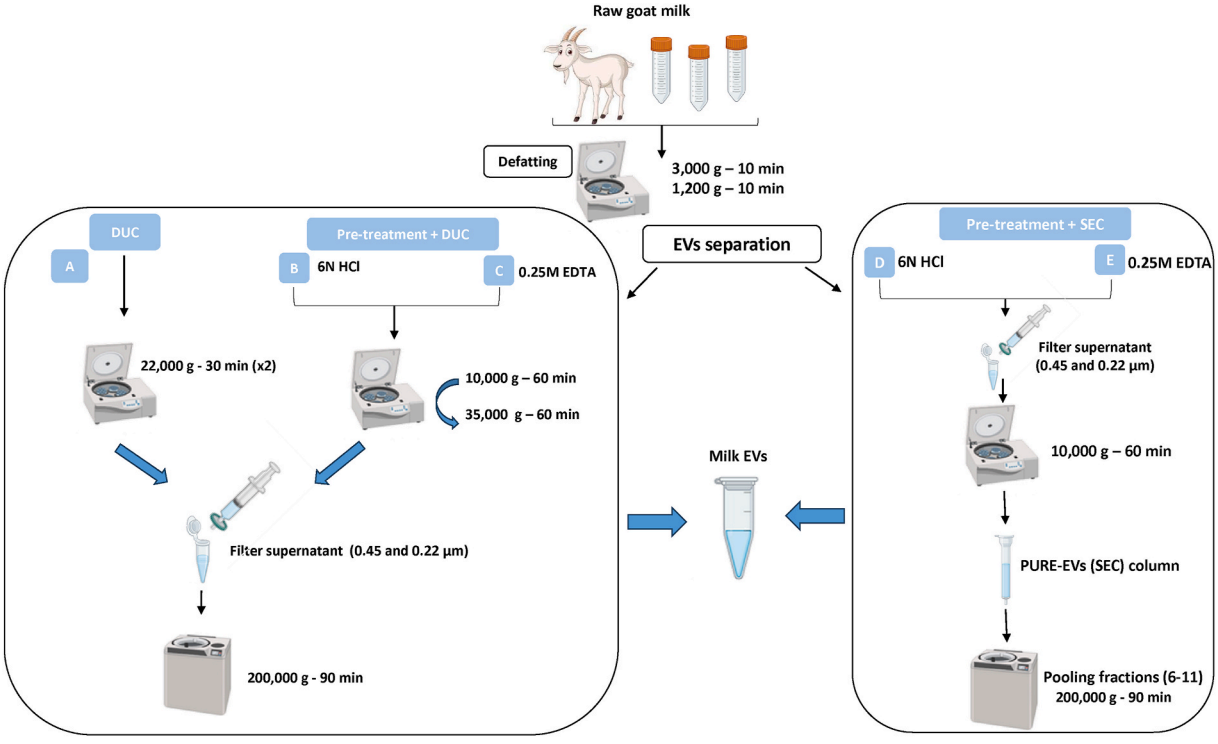

Fig. 1. Flowchart of protocols tested for EVs separation. Differential ultracentrifugation (DUC) and size exclusion chromatography (SEC) were performed as methods for EVs separation. Within each method we performed two types of pre-treatments, acidification (HCl) and chelation (EDTA).

EVs as powerful tools, can be extended in multiple fields of precision medicine, for instance, in cancer, intestinal disease and regenerative medicine.

Despite, goat milk EVs are receiving much attention due to their potential diagnostic and therapeutic applications, they have not been widely investigated, especially in comparison with bovine milk EVs. One drawback of the use of goat milk EVs for biomedical purposes is related to its chemical composition and specific peculiarities. The high fat content of goat milk, as well as the significant casein content, affect the efficiency of EVs isolation. Therefore, we performed a comparative methodological study for EVs separation from goat milk.

Differential ultracentrifugation (DUC) [24,25] and size exclusion chromatography (SEC) [22,23] are commonly used to separate EVs from milk, which are preferred to commercial kits using a complex matrix, such as milk [13]. DUC consists of sequential centrifugation with progressively higher centrifugal force and time. This method is very effective for isolating EVs from high sample volumes, but it ensures partially pure preparation because other particles might be pelleted with EVs. In comparison, SEC is a finer separation methodology based on the difference in molecular size accomplished only by gravity or low-speed centrifugation, has high recovery rates and is suitable for small sample volumes. However, it is a much longer, and more complex methodology compared to DUC. Moreover, when high centrifugal force is used, EVs separation can be compromised by the co-precipitation of milk proteins, which form gelatin at the bottom of the tubes [26]. This gelatin is very sticky and difficult to resuspend and can trap subpopulations of EVs in its matrix. To avoid its formation, it may be useful to remove caseins. Therefore, milk can be mixed with acids [27] or cold EDTA to precipitate caseins before EVs separation [27–29].

Thus, not only the choice of methods for separation but also the preprocessing of milk has a significant impact on the quality and yield of EVs. Here, we compared four protocols to separate EVs from unpasteurised goat milk: two based on DUC and two on SEC. Moreover, we assessed two different approaches of pre-treatment (acidification and precipitation) to facilitate milk protein discharge.

2. Results and discussion

2.1. Pre-treatment of goat milk prior to EVs separation

Goat milk was treated either with 6 N HCl and/or 0.25 M EDTA to precipitate caseins and protein aggregates (Fig. 2). To this regard, removal of casein micelles is highly recommended, because they might have a similar size as EVs and they can misconduct further analysis. Indeed, it was previously reported that acid treatment efficiently removed casein in bovine milk which was subsequently used to separate EVs [30].

On the other hand, Mecocci et al. [31] used only 0.25 M EDTA but not HCl to remove casein micelles and aggregates. Their approach to separate milk EVs was used for high throughput analysis (transcriptome and metabolome) from goat, donkey and bovine milk EVs [32,33]. In this study, instead, we performed both pre-treatments on goat milk to validate different approaches for EVs separation from raw goat milk. In fact, we demonstrated that milk whey was clearly separated from the caseins fraction (pellet), represented in Fig. 2A; whereas for further EVs separation, cleared whey was used, as shown in Fig. 2B.

2.2. Particles concentration and size in goat milk EVs by Nanoparticle tracking analysis (NTA)

Next, we analysed the particles concentration and size of milk EVs separated by DUC and SEC, also within each pre-treatment

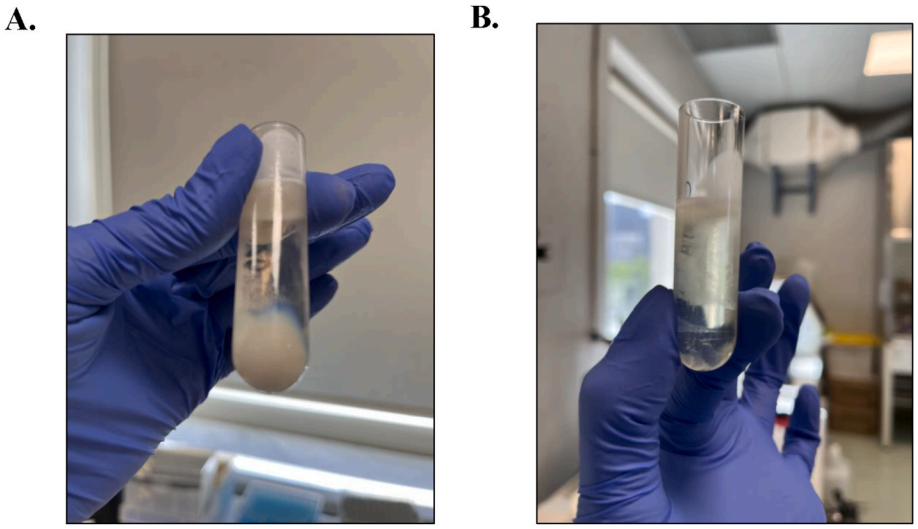

Fig. 2. Goat milk pre-treatment with EDTA and HCl. A) Pellet of caseins and other contaminants obtained after 10,000×*g* centrifugation from treated goat milk. B) Clear whey used for EVs separation.

method, EDTA and HCl (Fig. 3). Specifically, NTA results showed no significant effect on EVs concentration (particles/mL) when pre-treated with HCl and processed by DUC. This similarity of results could be explained by the degree of purity of EVs obtained from the two protocols. DUC alone does not guarantee the removal of casein micelles, which are similar to EVs in size. Therefore, the removal of casein from whey is a critical step to obtaining high amounts of high-purity EVs.

Differently from HCl pre-treatment, EDTA pre-treatment before DUC significantly increased the concentration of separated EVs ($p < 0.001$). Also, performing HCl pre-treatment before SEC, significantly ($p < 0.01$) affected the abundance of EVs separated. On the other hand, no significant differences were observed in particle concentration of EDTA pre-treatment before SEC. These differences can be explained because the NTA instrument is incapable of distinguishing EVs from any other particles with similar Brownian motion and thus the particle concentrations calculated by NTA may include a mixture of EVs and other structures [34]. Despite of that, the NTA is highly recommended by the ISEV community for EVs quantification [16]. The number of particles in goat milk without pre-treatment was $2.8 \times 10^{11} \pm 2.6 \times 10^{10}$ which was significantly lower compared to goat EDTA DUC and HCl SEC samples which had $1.0 \times 10^{12} \pm 7.2 \times 10^{10}$ and $7.1 \times 10^{11} \pm 3.8 \times 10^{10}$, respectively (Fig. 3A). Instead, particle concentration of goat EVs obtained by HCl DUC and EDTA SEC protocols ($4.2 \times 10^{11} \pm 2.1 \times 10^{10}$ and $3.1 \times 10^{11} \pm 9.4 \times 10^{9}$) was comparable to what was achieved by the DUC alone. Overall, the EDTA DUC protocol provided higher particle concentration (Table 1), followed by HCl SEC and HCl DUC methods. Interestingly, the particle concentration in our EDTA pre-treatment followed by DUC samples (10^{12}) was much higher compared to the data (10^{11}) observed by Mecocci et al. [31], where they applied the same methodology. Therefore, these results confirmed that milk pre-treatments followed by DUC or SEC increased the particle concentration and generated highly enriched EV samples.

Regarding particle size, across the different separation approaches, the smaller one was recorded when goat milk was pre-treated with HCl before DUC (Fig. 3B and Table 1). Otherwise, a larger particle size was obtained by combining EDTA pre-treatment with DUC, which was significantly ($p < 0.001$) higher compared to the DUC only. Thus, in the context of the DUC method, the choice of pre-treatment affected the size yield of separated EVs. Differently, within the SEC, the type of pre-processing did not appear to make a dimensional variation in the EV samples.

In Table 2, we reported in detail the mean and mode of particle size in all the samples with the associated D10, D50, and D90 extrapolated from the NS300. Moreover, NTA results indicated that all assessed methods provided a single peak corresponding to the modal size smaller than 200 nm (Fig. 4A–E). Accordingly, Franzoni et al. [25] reported goat milk EVs of similar size within 200 nm as reported here. Regardless of the methodology used in this study, overall NTA analysis showed a narrow range associated with a homogenous population of particles in all the EVs samples.

2.3. Total protein concentration in EV samples

Protein concentration was estimated by Bradford assay. All assessed separation protocols enabled us to collect at least highly enriched goat's milk EVs protein in suspension at concentration a of μg/μL, as quantified by Bradford assay. Although DUC is widely used to separate EVs, it does not ensure that only EVs are pelleted. Within the different methodologies used, the total amount of protein from EVs separated by SEC was lower in goat milk treated with EDTA or HCl compared to the other samples (Table 3). Here, we also evaluated the particles/μg of protein ratio (Table 3), which may aid in comparing EVs purity across several methods [35]. Similarly, the particles/protein ratio was higher in EVs separated by SEC from goat milk pre-treated with either EDTA or HCl, suggesting that enriched EV samples were obtained. Furthermore, Sun et al. [36] found that by DUC pelleted casein micelles of 160–200 nm at a higher speed (approximately 100,000×*g*). Therefore, the trend observed by NTA was similar to the amount of protein analysed in the EV samples pre-treated with HCl and EDTA.

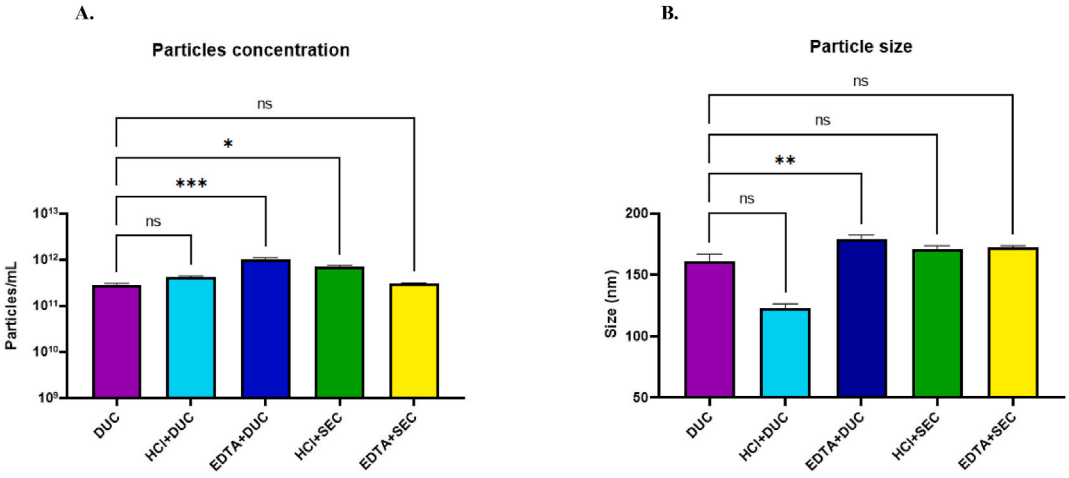

Fig. 3. Characterisation of goat milk EVs using NTA. Analysis of particles concentration (A) and size (B) of goat milk EVs separated according to all performed protocols. The star symbols indicated statistical significance (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

Table 1
NTA size analysis of milk EVs separated from goat milk EVs according to all performed protocols using NS300.

Protocol	Concentration	SEM	Mean	Mode	SEM	D10	D50	D90
DUC	2.8×10^{11}	2.6×10^{10}	161.1	132.1	2.5	103.7	150.3	230.6
HCl + DUC	4.2×10^{11}	2.1×10^{10}	122.6	93.5	1.7	80.4	108.1	182.4
EDTA + DUC	1.0×10^{12}	7.2×10^{10}	179.2	140.7	1.3	119.2	165.7	251.4
HCl + SEC	7.1×10^{11}	3.8×10^{10}	170.8	151.9	1.8	126.6	160.3	225.0
EDTA + SEC	3.1×10^{11}	9.4×10^{9}	179.4	173.8	0.8	127.2	171.4	242.5

Table 2
Pairwise comparison of particles concentration and size from goat milk using different EVs separation methodologies.

NANOPARTICLE TRACKING ANALYSIS		
Comparison between EVs separation protocol	Particles concentration (*p*-value)	Particles size (*p*-value)
DUC *vs* HCl + DUC	ns	ns
DUC *vs* EDTA + DUC	<0.001	<0.01
DUC *vs* HCl + SEC	<0.01	ns
DUC *vs* EDTA + SEC	ns	ns
HCl + DUC *vs* EDTA + DUC	ns	<0.0001
HCl + DUC *vs* HCl + SEC	ns	ns
HCl + DUC *vs* EDTA + SEC	ns	<0.01
EDTA + DUC *vs* HCl + SEC	ns	ns
EDTA + DUC *vs* EDTA + SEC	<0.001	ns
HCl + SEC *vs* EDTA + SEC	ns	ns

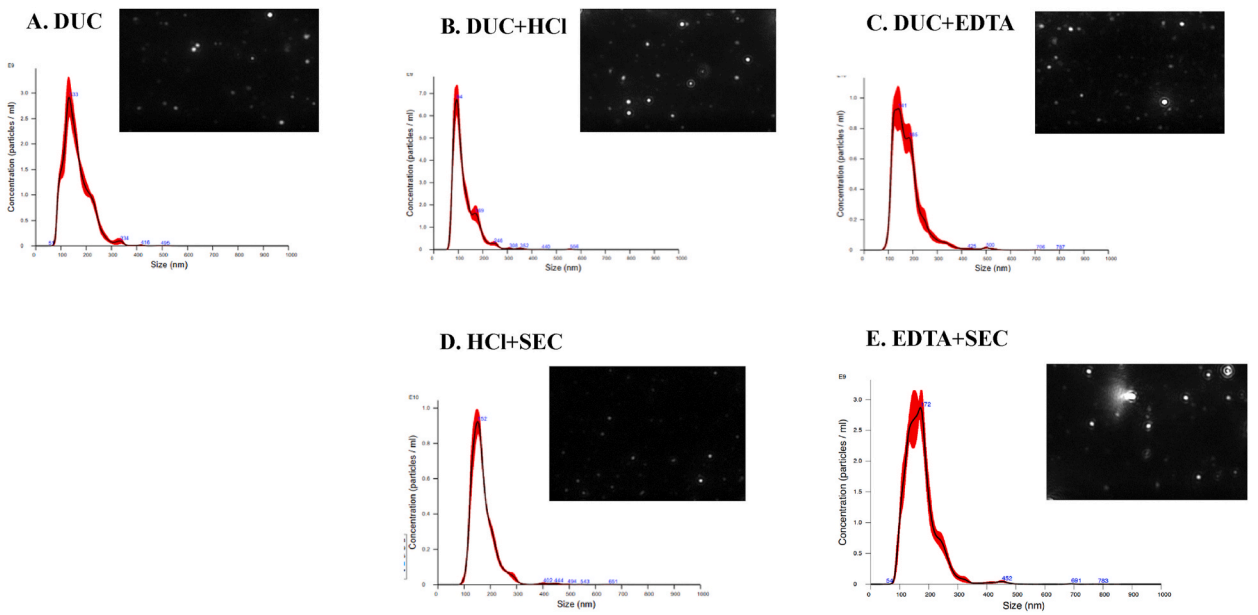

Fig. 4. Representative graphs and particles in motion of analysed goat milk EVs samples by NS300.

Table 3
Protein yield of EVs separated from goat milk in the different protocols tested.

EVs separation protocol	Protein yield by Bradford assay (μg/μl)	Particles/μg of protein by NTA
DUC	5.9	4.7×10^{13}
HCl + DUC	3.6	1.2×10^{14}
EDTA + DUC	4.1	2.5×10^{14}
HCl + SEC	0.8	8.1×10^{14}
EDTA + SEC	0.01	2.04×10^{16}

2.4. EVs markers in goat milk EVs samples analysed by immunoblotting

The presence of EVs in all the samples was assessed by immunoblotting analysis, using EVs markers accepted by the MISEV2023 guidelines [16]. Specifically, TSG101, an internal marker, CD81, a surface marker of the tetraspanin family, and a non-EVs associated marker, calnexin were probed for all the samples. Our results showed that the positive EVs marker, TSG101, and CD81 were both clearly detected in all the samples, with a higher signal in EVs separated by SEC from goat milk previously treated with HCl, as shown in Fig. 5 and Suppl. Fig. 1. Furthermore, the CD81 band appeared weaker for EVs separated by DUC only, confirming the importance of casein removal steps.

In contrast, calnexin was not detected in any of the samples. Therefore, using different approaches, we efficiently separated enriched EVs samples from raw goat milk, which was also confirmed by the presence of EV-associated proteins.

3. Conclusion

To date, current methods for separation EVs from goat's milk do not ensure such a high quality preparation suitable for clinical-grade therapeutic.

Both methods used here were found useful, with some peculiarities that could potentially be contextualised. In particular, within the methodology applied, DUC is preferred for high sample volumes and is better suited to industrial scale production. Conversely, SEC is efficiently used to obtain higher purity with smaller volumes, which might be more appropriate for the application of goat milk EVs in personalised medicine.

Taken together, our results demonstrate that EVs can be efficiently separated from a complex matrix such as goat milk within different protocols. Although few studies have performed milk pre-treatment [30,31,37], it is highly recommended to obtain an EVs enriched sample, especially for goat milk. In this study, the use of EDTA pre-treatment combined with DUC resulted in higher concentration of separated EVs. However, the EVs separated in this way appeared to be the largest and the most heterogeneous. Conversely, the smallest and least heterogeneous sample of EVs was obtained by combining HCl pre-treatment with DUC. Furthermore, we showed that the highest yield (particles/ug protein) was obtained by combining EDTA pre-treatment with SEC and that all EVs positive markers were readily detected in all the samples. Although, we are aware that our EVs characterization is not entirely exhaustive, our results can be used to compare and suggest a suitable methodological choice when performing EVs separation from goat milk. In addition to the methods discussed here, another advisable technique for large scalability is tangential flow filtration (TFF) -based method, which may be a valid alternative for large amounts of purified milk EVs [38]. Therefore, the choice of one preferred EVs separation technique to another should be dictated not only by different milk types, but also by the starting volume, downstream analyses, and especially the ultimate purpose of using EVs.

4. Material and methods

4.1. Sample collection and processing

Raw goat milk was kindly gifted by Nelmiocampo farm (Avellino, Campania) and immediately processed upon collection. All samples were aliquoted and centrifuged first at 3000×*g* twice to remove milk cells, debris, and milk fat globules. Next, the pellet was discarded, and the supernatant was used for another centrifugation step at 1200×*g*, twice. Finally, cleared supernatants were used immediately for the next steps. The remaining unused sample was stored at −80 °C.

4.2. Casein removal pre-treatment

HCl and EDTA precipitation were performed to remove caseins from defatted milk. Specifically, a 6 N HCl was added to defatted milk to reach the isoelectric point (pH 4.6) [39]. Similarly, a 1:1 volume of 0.25 M EDTA (pH 7.4) was added to defatted milk [31]. Sample thus treated were allowed to stand for 15 min on ice.

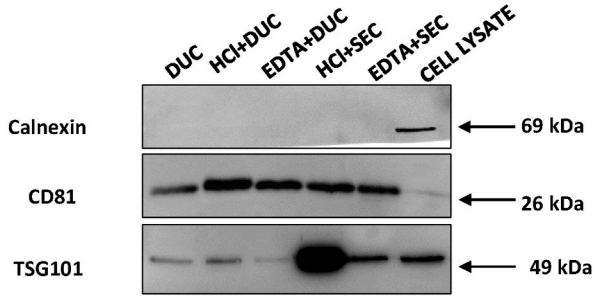

Fig. 5. Immunoblotting analysis of EVs markers in goat milk EVs sample.

4.3. EVs separation

Two methods, that are frequently used for EVs separation, DUC and SEC, were used in this study to separate EVs from unpasteurised goat milk. Moreover, within each method, EDTA and HCl pre-treatment were assessed for promoting casein precipitation and improving EVs separation. DUC without any type of pre-processing was also performed to have a term of comparison (Fig. 1). For every performed protocol, we started from 20 mL of defatted milk.

4.3.1. EVs separation by differential ultracentrifugation (DUC)

Here, DUC - without undergoing any pre-treatment for the removal of casein - was also performed for EVs separation [40]. Specifically, defatted milk was straightforwardly centrifuged at 22,000×*g* for 1h at 4 °C using OPTIMA MAX-XP (Cat# 393315, Beckman Coulter, USA). Next, the pellet was discarded, and the supernatant was filtered using 0.45 μm and 0.22 μm using a syringe filter (GVS North America, Sanford, USA). The resulting samples were processed for a final round of centrifugation at 200,000×*g* for 90 min at 4 °C for pelleting EVs. Finally, the pellet was resuspended in 200 μL of 0.22 μm filtered PBS (Cat# 14190, Gibco) and used for further analysis.

4.3.2. EVs separation by DUC after EDTA and HCl pre-treatment

EVs separation from goat milk after pre-treatment was performed by ultracentrifugation using OPTIMA MAX-XP (Cat# 393315, Beckman Coulter, USA) as previously published, with some modifications [41]. Briefly, goat milk was spun at 10,000×*g* for 1hr to pellet caseins. The resulting supernatant was filtered using 0.45 μm and 0.22 μm using a syringe filter. Afterwards, the filtered supernatant was centrifuged again at 35,000×*g* for 1 h to further collect large particles. Finally, EVs were pelleted at 200,000×*g*, resuspended with 100 μL of 0.22 μm filtered PBS, and used for further analysis.

4.3.3. EVs separation by size exclusion chromatography (SEC) after EDTA and HCl pre-treatment

The clear supernatant (whey) obtained after casein removal was filtered using 0.45 μm and 0.22 μm syringe filter (GVS North America, Sanford, USA). The collect whey was loaded on top of a PURE-EVs: Size exclusion chromatography columns (Cat# HBM-PEV-10, HansaBioMed Life Sciences, OÜ). The column was equilibrated with 0.22 μm filtered PBS prior to use. Six fractions rich in EVs (fraction 6–11) were pooled to collect EVs by ultracentrifugation at 200,000×*g* OPTIMA MAX-XP (Cat# 393315, Beckman Coulter, USA) and the pellet was resuspended in 100 μL of 0.22 μm filtered PBS and used for further analysis.

4.4. Nanoparticle tracking analysis (NTA)

Particle concentration and size of separated goat milk EVs from each methodology were analysed using Nanoparticle Tracking Analysis (NanoSight NS300, Malvern Instruments Ltd, Malvern, UK). NTA exploits Brownian motion and light scattering to quantify particle size and concentration of EVs. Specifically, EVs samples were diluted (dilution range from 1:100 to 1:1000 with 50–100 particles/frame) and automatically injected into the NTA system under constant flow conditions (flow rate = 50). Five × 60-s videos were recorded and videos of the particles in motion were recorded and analysed using NTA 3.2 software (Malvern Instruments Ltd, Malvern, UK).

4.5. Protein concentration from goat milk EVs

The total protein content in goat milk EVs samples was estimated by Bradford assay. Briefly, 1 μL of EVs samples was added to a cuvette and mixed with 1 mL of Bio-Rad Protein Assay Dye Reagent Concentrate (Cat# 5000006EDU, Bio-Rad Laboratories) diluted 1:5 with distilled water. Absorbances were observed at 595 nm using NanoPhotometer® NP80 (Cat#2309710254, Implen, US).

4.6. Immunoblotting analysis

Immunoblotting analysis was performed to discriminate the presence of markers for EVs characterisation. We used CD81, tetraspanin protein marker, and TSG101, cytosolic associated protein. Calnexin, an endoplasmatic reticulum protein marker, was used as a negative control. Firstly, EVs were lysed using JS lysis buffer (HEPES 1 M; NaCl 5 M; Glicerol 100%; Triton X100; $MgCl_2$ 1 M; EGTA 0.1 M; H_2O). Lysed EVs (30 μg) were resolved on 10% gel by electrophoresis at 120V and proteins were transferred by Trans-Blot Turbo System (Bio-Rad Laboratories, Cat# 690BR024275). Next filters were blocked with 5% milk in TBST containing 0.1% Tween-20 for 1h and incubated overnight at 4 °C with primary antibodies anti-CD81 (1:500; Cat# sc-116029, Santa Cruz Biotechnology, Inc); anti-TSG101 (1:500; Cat# sc-6037, Santa Cruz Biotechnology, Inc) and anti-calnexin (1:1000; Cat# ab10286, Abcam). While secondary antibody used was Goat Anti-Mouse IgG (1:2000; Cat# 10303-05, Biotech) or Goat Anti-Rabbit IgG (1:2000; Cat# 4030-05, Biotech). Imaging was performed using an automated ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Cat# 12003154) and Clarity Max™ Western ECL Substrate (Cat# 1705062, Bio-Rad Laboratories). Caco-2 mammalian cell lysate (CL) was used as a positive control.

4.7. Statistical analysis

All data was displayed as the mean ± standard error of the mean. Statistical analysis and figures were performed using GraphPad

Prism Version 9. For comparison of EVs separated by different approaches we applied non-parametric statistical tests (Krustall-Wallis). *P*-values <0.05 were considered statistically significant.

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230985) [42].

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Data availability

All relevant data of our experiments were deposited to the EV-TRACK knowledgebase (EV-TRACK ID: EV230985).

CRediT authorship contribution statement

Jessie Santoro: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Silvia Nuzzo:** Writing – review & editing, Methodology. **Monica Franzese:** Supervision. **Marco Salvatore:** Supervision, Funding acquisition. **Anna Maria Grimaldi:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27621.

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