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Evaluation of the antirotaviral activity of milk extracellular vesicles using a human intestinal model

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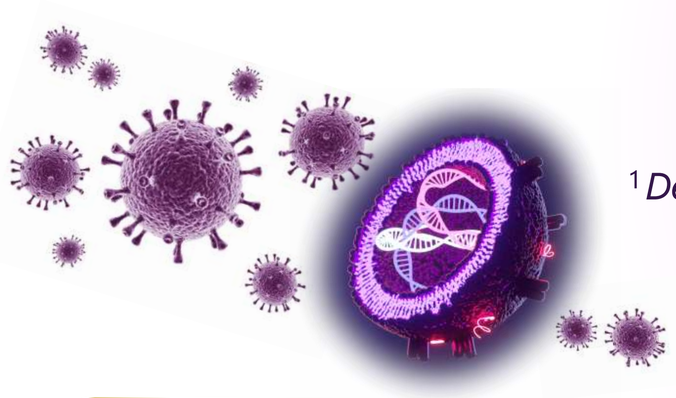


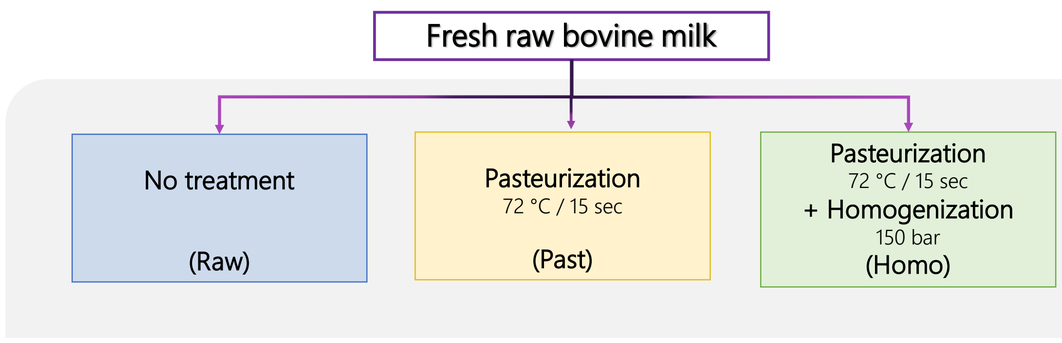
INTRODUCTION

Rotaviruses are the leading etiologic agents of severe gastroenteritis and fatal dehydration in infants and young children. Current live rotaviral vaccines are not completely effective, mainly due to economic and logistical difficulties, and low availability in developing countries. Accordingly, some milk proteins and fractions have been investigated to attenuate rotavirus infectivity, but the role for the hitherto less considered milk derived extracellular vesicles (EVs) in this context is unknown. EVs are nanosized phospholipid membrane-bound entities that encapsulate a soluble content including various proteins and miRNA. They are now central to research in many fields of biology because they constitute an overseen new system of cell-to-cell communication with a potential application within therapeutics. EVs from milk have demonstrated the ability to resist gastrointestinal digestion and are effectively taken up by host cells across species. Furthermore, milk EVs have been found to consist of several components that have exhibited antirotaviral properties, especially glycoproteins like mucins and lactadherin.

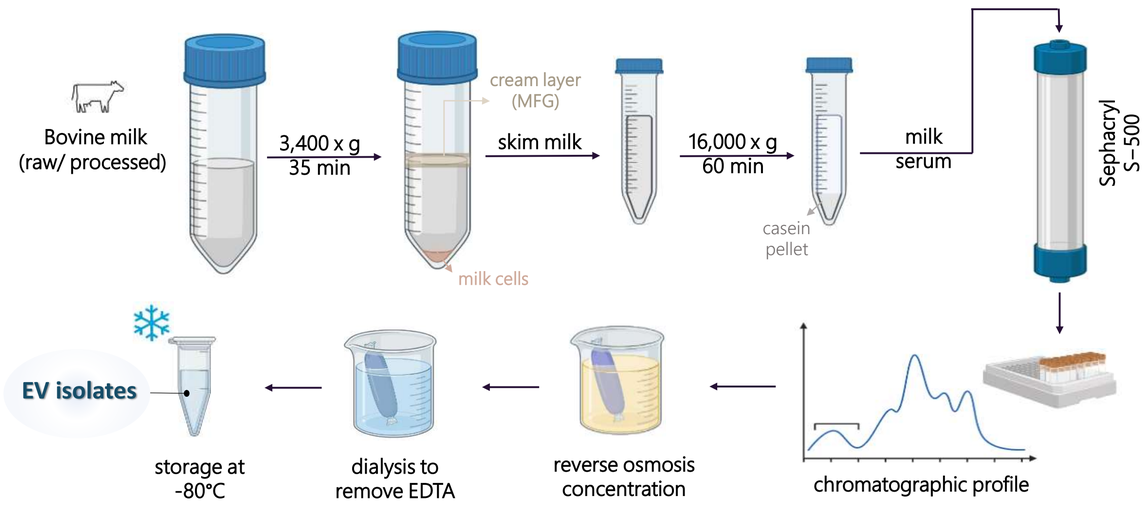
The aim of this study was to evaluate the protective activity of EVs isolated from bovine milk against rotavirus in a model of human intestinal epithelium. EVs were isolated from bovine milk serum, originating from raw or processed milk, using centrifugation followed by size exclusion chromatography. Western blotting was carried out to identify the specific membrane markers on the EVs and nanoparticle size analysis was implemented to further characterize the samples. Additionally, proteoliposomes (PLs) were created to serve as negative control in the *in vitro* assays. PLs were formulated by mixing liposomes containing a similar phospholipid distribution as EVs, with purified milk fat globule membrane (MFGM) protein incorporated into the liposome membrane.

METHODS

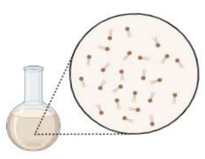
MILK PROCESSING



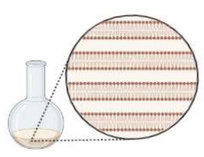
EV ISOLATION



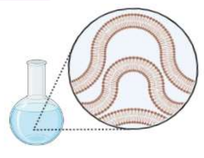
PROTEOLIPOSOME FABRICATION



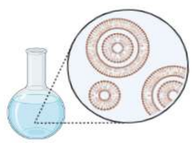
① **Lipids dissolved in chloroform in the desired ratio**



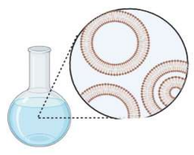
② **Dried lipid film formation:** Organic solvent is evaporated under a stream of nitrogen gas



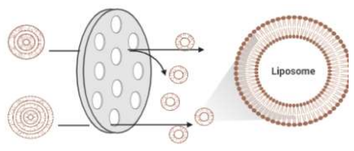
③ **Hydration**: Dried lipid film swells by adding PBS buffer with 0,1% deoxycholate



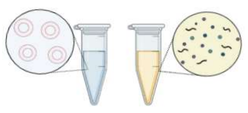
④ **Hydration and Agitation:** Lead to multilamellar vesicle formation



⑤ **Freeze-thaw 10 circles:** Repeatedly exposure to liquid nitrogen and hot water: Lead to unilamellar vesicles and a more homogenized sample



⑥ **Extrusion** through polycarbonate membrane filters (0,2 µm pore size) to obtain unilamellar vesicles



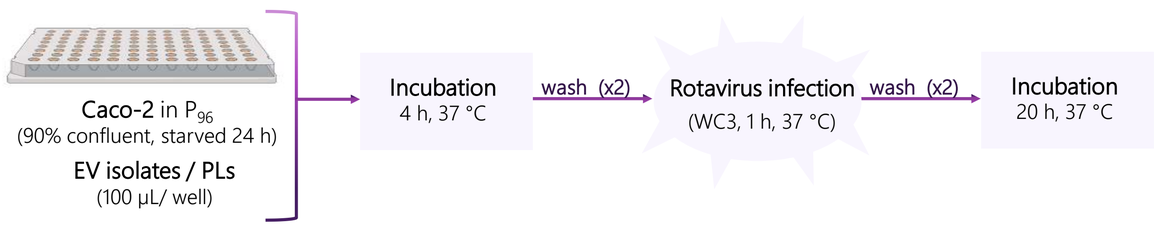
⑦ **Incorporation of MFGM proteins into the liposomes:** ratio 4:1 - 3 h rotation

⑧ **Dialysis:** 7 days

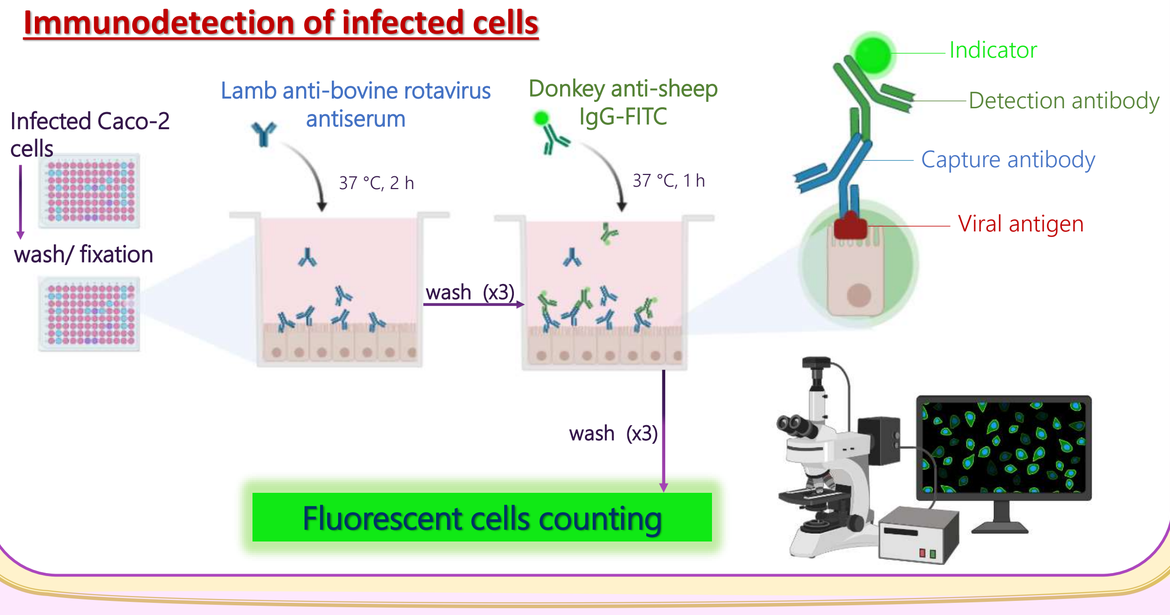
⑨ **Proteoliposome** (PL)

ROTAVIRUS ASSAYS

Cell Preincubation with EVs

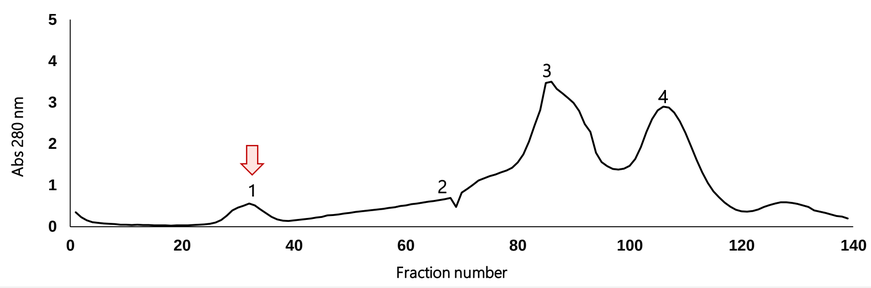


Immunodetection of infected cells

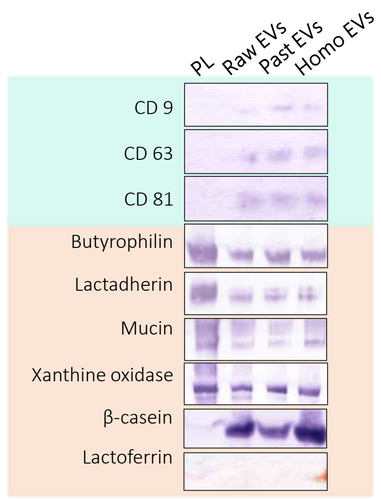


RESULTS

EV ISOLATION AND CHARACTERIZATION

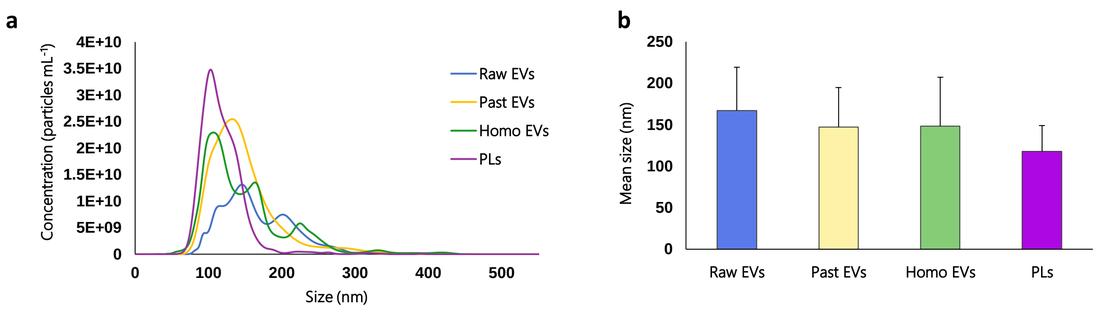


Elution profile of raw milk serum subjected to size exclusion chromatography. Red arrow indicates the peak that correspond to EVs fraction. The insert on the right of the chromatograms is the Coomassie blue stained SDS-PAGE with fractions of the chromatogram as indicated.



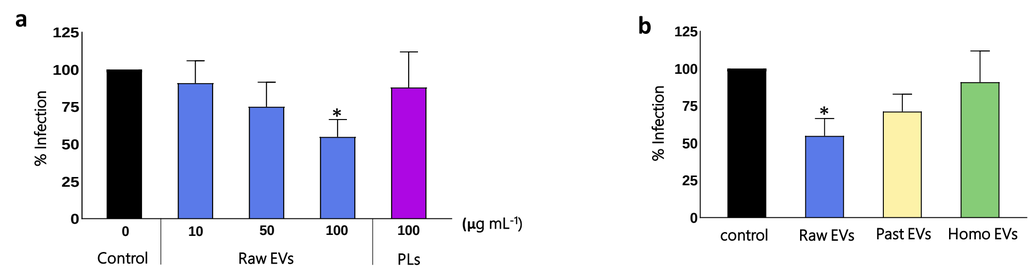
Western blot analysis of proteins shared between milk EVs and PLs (light orange background) and protein markers specific for milk EVs (blue background).

- Milk EVs but not PLs were positive for the three EV markers CD9, CD81 and CD63.
- All samples were positive for the membrane glycoproteins lactadherin and mucin appearing the bands with more intensity in the PL lane. Similar band profile was observed in the case of butyrophilin and xanthine oxidase.
- EV isolates, but not PLs were positive for β-casein.
- None of the samples was found to contain the iron binding protein lactoferrin.



Particle descriptions of the three EV isolates and the proteoliposomes. a) The particle size distributions are presented as curves for raw milk EVs (blue), pasteurized milk EVs (yellow), pasteurized + homogenized milk EVs (green) and artificial proteoliposomes (purple). b) Mean particle size + SD of the four samples.

ANTIROTAVIRAL ACTIVITY



Antirotaviral activity of milk EVs and PLs tested on Caco-2 cells presented as percentage of downregulation of infection. a) The cells were pretreated with 10, 50, or 100 µg/ mL EVs and 100 µg/ mL PLs for 4 h before rotavirus infection for 1 h. b) Effect of industrial processing of milk on the antirotaviral activity of the isolated EVs. The cells were pretreated with 100 µg/ mL EVs for 4 h followed by rotavirus infection for 1 h. Data are presented as mean +SD (n ≥ 6), asterisks indicate statistically significant differences (*p < 0.05) in relation to non-treated sample (control).

CONCLUSIONS

- Centrifugation followed by size exclusion chromatography is a gentle isolation approach to obtain casein reduced native milk serum EVs.
- EV isolates' protein profile resembles to that of the milk fat globule membrane proteome.
- MFGM protein incorporated in PLs was free from EV material.
- Preincubation with EVs isolated from raw bovine milk serum presents inhibitory activity against Caco-2 cells infection by rotavirus, in a dose response way.
- Pretreatment of the cells with PLs failed to result in downregulation of rotavirus infection. Therefore, the neutralization mechanism of EVs might be related to the protective effect of their content rather than the blockage of viral proteins.
- Industrial processing of milk, such as pasteurization and homogenization, lead to reduction of the antirotaviral activity of the corresponding EV isolates.

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