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Regular Industrial Processing of Bovine Milk Impacts the Integrity and Molecular Composition of Extracellular Vesicles

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ABSTRACT

Background: Bovine milk contains extracellular vesicles (EVs), which act as mediators of intercellular communication by regulating the recipients' cellular processes via their selectively incorporated bioactive molecules. Because some of these EV components are evolutionarily conserved, EVs present in commercial milk might have the potential to regulate cellular processes in human consumers.

Objectives: Because commercial milk is subjected to industrial processing, we investigated its effect on the number and integrity of isolated milk EVs and their bioactive components. For this, we compared EVs isolated from raw bovine milk with EVs isolated from different types of commercial milk, including pasteurized milk, either homogenized or not, and ultra heat treated (UHT) milk.

Methods: EVs were separated from other milk components by differential centrifugation, followed by density gradient ultracentrifugation. EVs from different milk types were compared by single-particle high-resolution fluorescence-based flow cytometry to determine EV numbers, Cryo-electron microscopy to visualize EV integrity and morphology, western blot analysis to investigate EV-associated protein cargo, and RNA analysis to assess total small RNA concentration and milk-EV-specific microRNA expression.

Results: In UHT milk, we could not detect intact EVs. Interestingly, although pasteurization (irrespective of homogenization) did not affect mean ± SD EV numbers ($3.4 \times 10^8 \pm 1.2 \times 10^8$–$2.8 \times 10^8 \pm 0.3 \times 10^7$ compared with $3.1 \times 10^8 \pm 1.2 \times 10^8$ in raw milk), it affected EV integrity and appearance, altered their protein signature, and resulted in a loss of milk-EV-associated RNAs (from 40.2 ± 3.4 ng/μL in raw milk to 17.7 ± 5.4–23.3 ± 10.0 mg/μL in processed milk, $P < 0.05$).

Conclusions: Commercial milk, that has been heated by either pasteurization or UHT, contains fewer or no intact EVs, respectively. Although most EVs seemed resistant to pasteurization based on particle numbers, their integrity was affected and their molecular composition was altered. Thus, the possible transfer of bioactive components via bovine milk EVs to human consumers is likely diminished or altered in heat-treated commercial milk. *J Nutr* 2021;151:1416–1425.

Keywords: bovine milk, cow milk, raw milk, commercial milk, extracellular vesicles, microvesicles, exosomes, microRNA, pasteurization, ultra heat treated

Introduction

Milk is nature's first nutrition for mammals and it is due to its high nutritional value that bovine milk has become part of the human diet (1–3). Because raw milk is not sterile and might contain pathogens, processing of milk by heat treatment is needed to make bovine milk safe for human consumption. There are several heating methods, for instance pasteurization whereby milk is heated for ≥15 s at temperatures ranging from 72°C to 78°C for minimally 15 s, or ultra heat treatment whereby milk is heated for ≥1 s to ≥135°C (4). In addition, bactofugation or membrane filtration can be applied in order to remove bacteria (5). Milk processing also often includes homogenization, a process that reduces the size of milk fat globules (MFGs) to uniformly small particles that distribute evenly in milk, thereby preventing separation of cream during storage. However, all these processing steps can have an impact on the biological activity of milk components because they might affect their structure and function (6–8).

The various milk components each have distinct functions, with the recently discovered extracellular vesicles (EVs), including exosomes and microvesicles, being biologically active

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macromolecular components (9–13). EVs are secreted, lipid bilayer–enclosed, nano-sized particles that are heterogeneous in size and composition and are tailor-made multicomponent vehicles for intercellular communication (14). Various routes of interaction with target cells exist, allowing for the delivery of EV cargo (15), which is composed of specific lipids (16), EV-enriched proteins (17–19), and nucleic acids, like microRNAs (miRNAs) (20, 21). Recently, miRNAs have gained much attention because these small noncoding RNAs regulate posttranscriptional gene expression and thereby influence a wide range of cellular processes (22). Milk-EV-associated miRNAs are protected from enzymes, allowing the functional transfer of miRNAs between cells within an individual, between mother and newborn, and even between species owing to conserved sequences (23, 24). For instance, bovine milk–derived EVs can be taken up in vitro by a variety of human cells, including macrophages (25), intestinal epithelial cells (26–28), and vascular endothelial cells (29).

Currently it is unknown whether industrial processing steps like heat treatment affect milk EVs. Hence, a comparison of the different milk EV studies aforementioned is difficult because in some of these studies raw milk was used as a source of milk EVs, whereas in other studies this was commercial milk. Furthermore, because the total milk EV population is heterogeneous (30, 31), and different milk EV separation procedures are used, this will influence the composition of the final EV-enriched samples that are studied. Therefore, we used a single purification procedure that we previously established (31) to isolate bovine milk EVs and performed a comprehensive analysis to study the consequences of heat treatment (with or without homogenization) on EV numbers, morphology, and EV-associated proteins and miRNAs.

Methods

Milk samples

Raw bovine milk was obtained from tank milk within 24 h after the first milking. The commercial milk sources were all purchased in a local grocery store in Wageningen, Netherlands and included pasteurized milk (organic full cream and bactofuged milk "Demeter"), pasteurized and homogenized milk (organic full cream and bactofuged milk "Campina Boerenland"), and ultra heat treated (UHT) milk (full cream and homogenized milk "Friesche vlag lang lekker"). EVs were isolated from all milk types using the same protocol.

Isolation of milk EVs

Isolation of milk EVs was performed as previously described (31, 32) (**Supplemental Figure 1**). In short, milk was centrifuged twice at 3000 × *g* at 4°C for 10 minutes in a Beckman Coulter Allegra X-12R, followed by 5000 × *g* and 10,000 × *g* centrifugation at 4°C for 30 minutes in an Avanti J-26 XP with a JA-12 rotor (Beckman Coulter). The 10,000 × *g* supernatant of each milk type was loaded on top of a sucrose density gradient, which was made by layering successive sucrose (Mallinckrodt Baker) solutions of decreasing density (2.0 M–0.4 M) on top of 2.5 M sucrose. The gradient was centrifuged at 192,000 × *g* for 15–18 h at 4°C in a Beckman Coulter OptimaL-90K with a SW40 rotor (k-factor 144.5). Density fractions were collected and 3 fractions were pooled together as follows: high-density fractions 1–3 (mean density: 1.28–1.26 g/mL), EV-enriched fractions 4–6 (mean density: 1.24–1.20 g/mL), EV-enriched fractions 7–9 (mean density: 1.18–1.13 g/mL), and low-density fractions 10–12 (mean density: 1.12–1.08 g/mL). The pooled density gradient fractions were transferred to a polyallomer SW28 tube (Beckman Coulter) containing 32 mL PBS (Gibco, Invitrogen) and centrifuged at 100,000 × *g* for 65 min at 4°C in a Beckman Coulter OptimaL-90K with a SW28 rotor, after which the pellets were used for further experimentation. All relevant data of the experiments have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV200099) (33).

Fluorescent labeling of milk EVs for high-resolution flow cytometric analysis of milk EVs

Pelleted samples of the pooled density fractions were resuspended in 40 μL PBS, of which 10 μL of resuspended EV samples were fluorescently labeled with PKH67 (Sigma-Aldrich) and separated from unbound PKH67 and protein aggregates by overnight density gradient centrifugation at 192,000 × *g* for 15–18 h at 4°C using an SW40 rotor, according to the previously described protocols (34, 35). From the top of the tube, 12 fractions of 1 mL were collected in Eppendorf tubes (Eppendorf) and stored at 4°C until further analysis.

High-resolution flow cytometric analysis of milk EVs

High-resolution flow cytometric analysis of PKH67-stained samples was performed by fluorescent threshold triggering on a BD Influx cell sorter (BD Biosciences) that was dedicated to and optimized for detection of submicron-sized particles. Detailed descriptions of both the hardware adaptations and methods used have been previously published (34, 36, 37). To exclude detection of EV swarms, serial dilutions of samples were performed. All 12 fractions from the second density gradient were then individually measured at optimal dilution. Data analysis was performed with FlowJo version 10.5.0. (FlowJo) To determine the total number of PKH67-labeled particles present in the pooled density gradient fractions 4–6 and 7–9 derived from the first gradient, the sum total of PKH67-positive events in fractions 3–12 of the second density gradient was calculated and corrected with the dilution factor. Fractions 1–2 were omitted, because these contained unbound PKH67 dye aggregates, not EVs.

Cryo-electron microscopy

Pelleted samples of the pooled density gradient fractions were resuspended in 40 μL PBS, of which 10 μL was kept on ice for 1–2 h until vitrification using a Vitrobot™ Mark IV system (FEI), after which 3 μL was directly placed onto a glow-discharged 2/2 copper grid (Quantifoil). Excess sample was removed with 595 filter paper (Schleicher & Schuell, Whatman) in the Vitrobot chamber for 1 s at 100% relative humidity, with subsequent plunging into liquid ethane (3.5 purity). Residual ethane was removed with filter paper and grids were stored in cryo-boxes under liquid N_2 for later imaging. For Cryo-electron microscopy (Cryo-EM), grids were transferred to a Gatan 626 cryo-holder (Gatan Inc.), which was inserted into a Tecnai™ 20 transmission electron microscope (FEI) with an LaB_6 filament operated at 200 kV. Images were acquired with a 4000 × 4000 Eagle charge coupled device camera (FEI) at a 19,000× magnification, with 5–10 μm under focus.

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Abbreviations used: Cq, quantification cycle; Cryo-EM, Cryo-electron microscopy; EV, extracellular vesicle; HRP, horseradish peroxidase; MFG, milk fat globule; miRNA, microRNA; rw-FSC, reduced wide-angle forward scatter; TSG101, Tumor susceptibility gene 101; UHT, ultra heat treated.

SDS-PAGE and western blot

Pelleted samples of the pooled density gradient fractions were resuspended in sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol), heated for 5 min at 100°C, and stored at −20°C. Samples were diluted 1:1 with PBS, or were reduced 1:1 with PBS + 0.3 M β-mercapthoethanol (BioRad) + 0.02 M DL-dithiothreitol (Sigma-Aldrich) before loading on a 4%–20% Criterion gradient gel (BioRad). The separated proteins were transferred to 0.45-μm polyvinylidene difluoride membranes (Merck-Millipore) and blocked in PBS containing 0.2% fish skin gelatin (Sigma-Aldrich) and 0.1% Tween-20. Proteins of interest were detected by immunoblotting using mouse-anti-human CD9 (clone HI9a; BioLegend; diluted 1:1000), mouse-anti-bovine CD63 (clone CC25; BioRad; diluted 1:2000), mouse-anti-human-flotillin-1 (clone 18; BD; diluted 1:500, in reducing buffer), mouse-anti-human-Tumor susceptibility gene 101 (TSG101) (clone C-2; Santa Cruz Biotechnology; diluted 1:100, in reducing buffer), rabbit-anti-human MFG-E8 (polyclonal; Sigma-Aldrich; diluted 1:1000), rabbit-anti-bovine casein (polyclonal; GeneTex; diluted 1:500), and rabbit-anti-lactoglobulin-β-horseradish peroxidase (HRP) (polyclonal; Abcam; diluted 1:1000). The secondary antibodies used were goat-anti-mouse-HRP (Jackson ImmunoResearch; diluted 1:10,000) or goat-anti-rabbit-HRP (Agilent Technologies; diluted 1:5000). HRP-conjugated antibodies were detected using the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Images were acquired using a ChemiDoc MP and Image Lab 5.1 (BioRad). Band intensities were quantified using Image Lab software.

Small RNA isolation

Pelleted samples of the pooled density fractions were resuspended in 700 μL QIAzol Lysis Reagent (Qiagen) and RNA was isolated according to the miRNeasy micro kit protocol (Qiagen). The RNA was eluted from the spin column membrane using 12 μL RNase-free water and was stored at −80°C until analysis. The concentration of RNA was determined using the Agilent RNA 6000 Pico kit (Agilent Technologies) and the Agilent 2100 Bioanalyzer instrument (Agilent Technologies).

RT-qPCR and cDNA synthesis

Isolated RNA (5 μL) from each milk type was converted into cDNA using the miScript cDNA synthesis kit (Qiagen) according to the manufacturer's instructions. The PCR was performed (Alpha Thermal Cycler, Bibby Scientific) at 37°C for 60 min, 95°C for 5 min, and kept at 4°C. The cDNA sample was stored at −20°C until use. The cDNA sample was diluted 100 times in RNAse-free MilliQ water, 2 μL of the diluted sample was transferred to a qPCR plate (BioRad) and 6 μL of a mastermix containing 2 × SensiMix SYBR & Fluorescein (Bioline Reagents), and 100 nM forward primer and 100 nM reverse primer were added (**Supplemental Table 1**). Cyclin conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 20 s. RT-qPCR reactions were performed on the Bio-Rad iQ5 Multicolor Real-Time PCR detection System (BioRad). Quantification cycle (Cq) values were determined using Bio-Rad CFX software. Thresholds were set in the linear phase of the amplification curve. For all our qPCR reactions melt curve analyses were performed and no RT controls were included. In all cases the difference between the no RT control and the highest Cq value of the specific sample was minimally 10 cycles. For the 4 miRNAs lowest in abundance we also determined the limit of detection based on dilution curves and melt curve analysis: miR-223 (no RT control: 41.9 with detection limit 40.2), miR-21-5p (no RT control: 41.2 with detection limit 39.3), miR-148a-3p (no RT control: 33.3 with detection limit 31.5), and miR-181d (no RT control: 44 with detection limit 36).

Statistical methods

Data normality was assessed by the Shapiro–Wilk test and homogeneity of variances was confirmed with the use of the Brown–Forsythe test. Normally distributed data were analyzed by 2-tailed paired t test when comparing 2 groups, or 1-factor ANOVA with Tukey's multiple comparisons test was performed when comparing >2 groups. Data are reported as means ± SDs and were considered significant if $P < 0.05$. Analysis was performed using GraphPad Prism software version 8.3.0.

Results

Ultra heat treatment of milk resulted in a loss of EVs, whereas EV numbers after pasteurization were not affected

To analyze the effect of regular industrial milk processing on the quantity of EVs, we labeled purified milk EVs for high-resolution flow cytometric analysis (34). Most PKH67-labeled EVs were detected in fractions 7–9, with comparable numbers of events present in raw milk ($3.1 \times 10^8 \pm 1.2 \times 10^8$), or milk that was pasteurized, either with ($2.8 \times 10^8 \pm 0.3 \times 10^7$) or without homogenization ($3.4 \times 10^8 \pm 1.2 \times 10^8$). Strikingly, UHT milk contained very low numbers of fluorescent events in both fractions 4–6 and 7–9 (**Figure 1**A). Similarly, for fractions 4–6, substantially lower numbers of PKH67-labeled EVs were detected in raw and pasteurized milk than for fractions 7–9 (Figure 1A). Analysis of the light scatter properties of EVs showed typical EV-scattering (34, 35) and similar patterns for EVs isolated from raw milk and each of the pasteurized milk types, whereas the fluorescent events from the UHT milk lost the typical low reduced wide-angle forward scatter (rw-FSC) population and contained high rw-FSC particles with dim PKH67-fluorescence (Figure 1B). Collectively, these results demonstrate that industrial processing of milk by pasteurization did not significantly affect the number of EVs in bovine milk, whereas UHT resulted in a substantial loss of EVs and changed the light scatter characteristics of the remaining fluorescent events.

Both ultra heat treatment and pasteurization of milk affected the morphology of milk EVs

We next investigated the impact of industrial processing on the morphology and integrity of milk EVs by performing Cryo-EM on the EV-enriched fractions 4–6 and fractions 7–9 of the different milk types. In concordance with the flow cytometry results, fractions 4–6 contained fewer vesicular structures than fractions 7–9 and in UHT samples hardly any spherical membrane-enclosed vesicular structures were detected (**Figure 2**). EVs isolated from raw milk were spherical with a clear lipid bilayer and differed in size and electron density, showing the heterogeneous character of EVs. In contrast, the morphology of EVs subjected to pasteurization, both in the absence and in the presence of homogenization, was remarkably different compared with EVs derived from raw milk. EVs isolated from pasteurized milk samples were not perfectly spherical and the membrane integrity was affected. Thus, although the number of EVs detected by single EV-based flow cytometry was not significantly altered after pasteurization (Figure 1), based on Cryo-EM we unveiled that the integrity of EVs was affected after pasteurization. This raised the question whether pasteurization, in the presence or absence of homogenization, also affected EV-associated cargo.

Milk processing altered the protein profile of milk EVs

Because milk EVs are abundant in proteins (32, 38, 39), we performed western blot analysis to assess the presence of proteins commonly enriched in EVs, as well as milk proteins that are not associated with EVs (31, 32). As expected, EV-associated markers were abundantly present in the EV-enriched

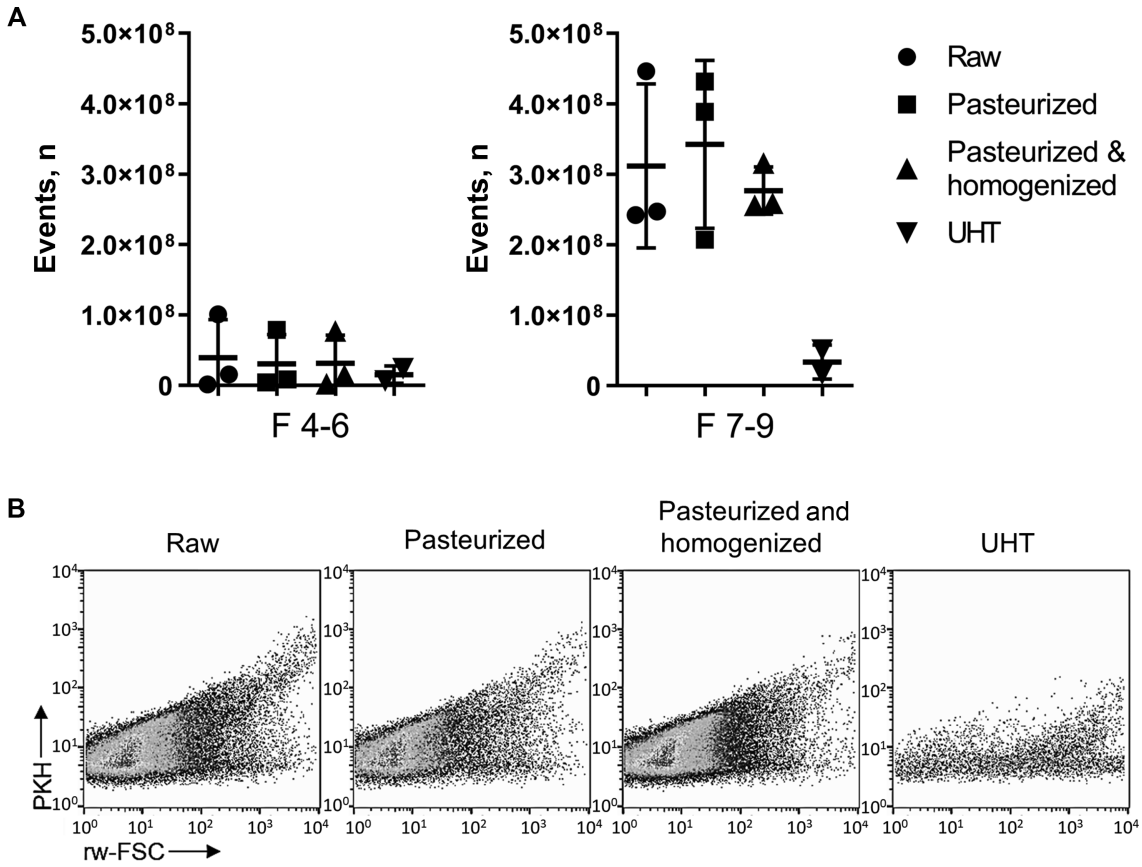

FIGURE 1 Quantitative EV analysis of raw and differently processed bovine milk using single-particle EV-based high-resolution flow cytometry. (A) Quantitative high-resolution flow cytometric analysis of EVs showing the total number of PKH67-labeled events of the pooled EV fractions 4–6 and 7–9 derived from raw or differently processed bovine milk. Data are mean ± SD, $n = 2$–3 individual samples that are derived from $n = 2$–3 independent experiments. (B) Typical scatterplots of each milk type showing PKH67 fluorescence and rw-FSC of PKH67-positive EVs in the most EV-enriched density fraction of the second sucrose gradient (density: 1.15 g/mL) for pooled EV fractions 7–9. EV, extracellular vesicle; rw-FSC, reduced wide-angle forward scatter; UHT, ultra heat treated.

fractions isolated from raw milk, with fractions 7–9 containing the highest signals of CD9, CD63, flotillin-1, and TSG101 (**Figure 3**A-left panel, B). MFG-E8 was most abundant in fractions 7–9 and fractions 10–12 and its detection was not affected by pasteurization. Also, the detection of CD9 was nearly influenced in pasteurized milk. In contrast, the signals of CD63 and flotillin-1 were clearly reduced, whereas the signal for TSG101 was lost completely in EV-enriched fractions of pasteurized samples (Figure 3A-left panel, B). These data suggest that some EV subsets might be more susceptible to pasteurization whereas others are more rigid and temperature resistant. As expected, in UHT samples all signals of EV-associated proteins were strongly reduced or completely absent, even after an extended exposure time (Figure 3A-right panel). This is in line with the reduced EV numbers and the impact of UHT on EV integrity in this type of milk. Furthermore, signals of abundant non-EV-associated milk proteins, like casein and β-lactoglobulin, were very low in the EV fractions 4–6 and 7–9 isolated from raw milk. However, these signals were increased after industrial processing and especially casein was highly abundant in UHT samples (Figure 3C). Collectively, these data show that any type of industrial processing involving heat treatment affects the protein cargo of milk EVs or can lead to the loss of EV subsets, whereas no additional detrimental effects of homogenization were observed.

Selective loss of small RNAs and milk-EV-associated miRNAs after milk processing

Besides bioactive proteins, milk EVs contain RNA molecules like miRNAs (10, 23, 24, 40). Therefore, we investigated the effect of industrial processing on the RNA cargo of milk EVs. We first assessed the total small RNA concentration in the pooled density gradient fractions of raw and processed milk. In all UHT milk–derived samples, as well as in the fractions 1–3 and fractions 10–12 of the other milk types, very low amounts of small RNAs were detected (**Figure 4**A). In contrast, small RNAs were readily detectable in the EV-enriched fractions 4–6 and 7–9 of raw, pasteurized, or pasteurized and homogenized milk (Figure 4A). In EV-enriched fractions 4–6 no significant effects on total small RNA concentration were observed to have been caused by pasteurization, either in the absence or in the presence of homogenization (Figure 4A). The highest concentration of small RNAs was detected in the EV-enriched fractions 7–9 from raw milk (40.2 ± 3.4 ng/μL), corresponding to the aforementioned flow cytometry, Cryo-EM analysis, and western blot data. Notably, in EV-enriched fractions 7–9 of pasteurized, and pasteurized and homogenized milk, the total concentration of small RNAs was substantially lower than in raw milk (23.2 ± 10.0 ng/μL and 17.7 ± 5.5 ng/μL compared with 40.2 ± 3.4 ng/μL, respectively, $P < 0.05$) (Figure 4A). These findings again indicate that industrial processing may affect different EV subsets.

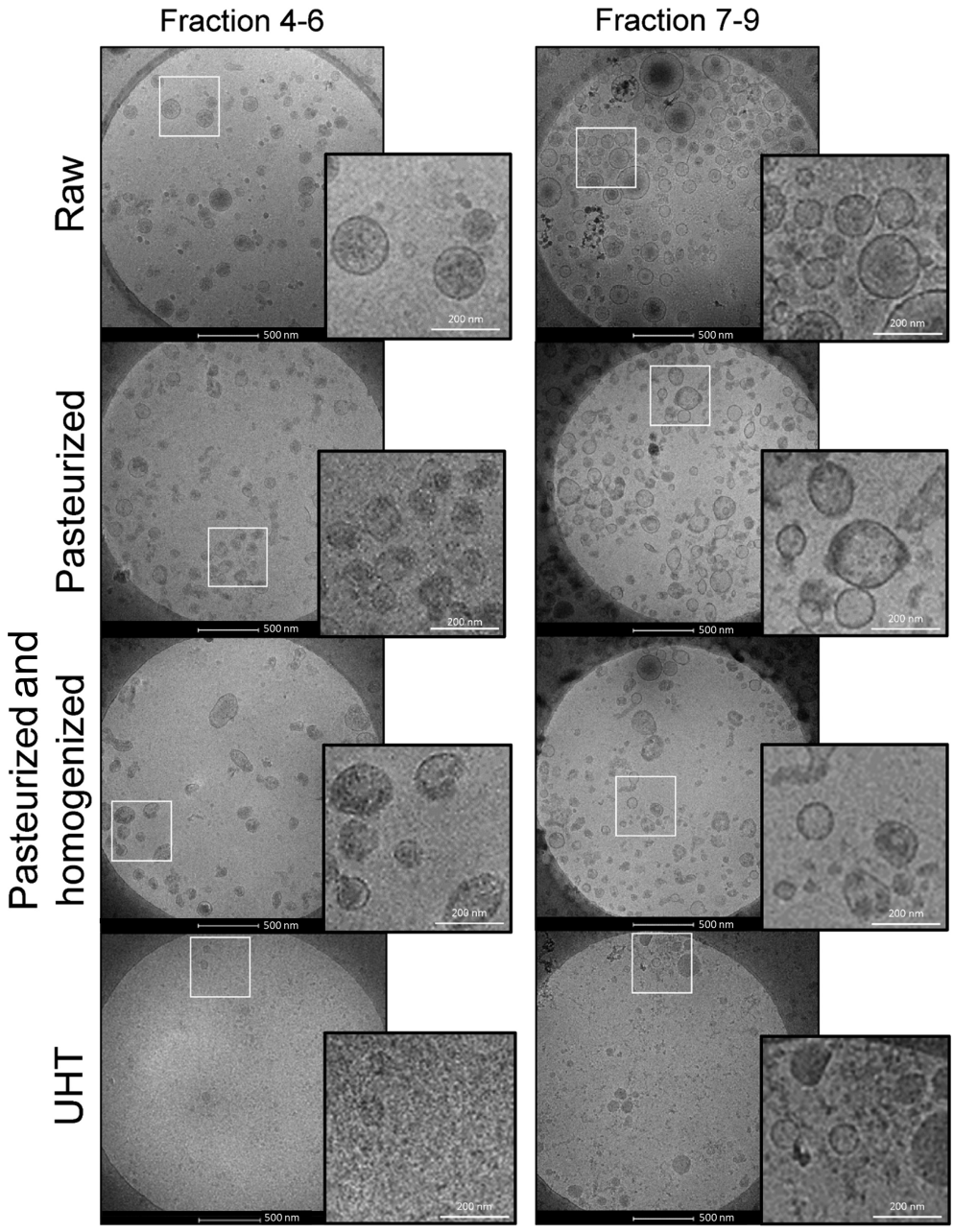

FIGURE 2 Cryo-EM analysis for the morphology of EVs isolated from raw and processed bovine milk. Overview and close-up Cryo-EM images of milk EVs from pooled density gradient fractions 4–6 or fractions 7–9 of raw milk or industrially processed milk. Data are single samples representative of 2 independent experiments. Cryo-EM, Cryo-electron microscopy; EVs, extracellular vesicles; UHT, ultra heat treated.

Next, we examined the effect of milk processing on milk-EV-associated miRNAs. Because we did not observe major differences in the total RNA concentration between pasteurized or pasteurized and homogenized milk in either EV-enriched fractions 4–6 or fractions 7–9 (Figure 4A), we compared the more frequently consumed pasteurized and homogenized milk to raw milk for further miRNA profiling. We determined the amounts of 8 miRNAs that are abundantly present either in EVs from bovine milk (10, 25, 30, 41) or in milk from other species (23, 42–47), including 2 evolutionarily conserved miRNAs (miR-148a-3p and let-7a-5p) (23). In both EV-enriched fractions 4–6 (Figure 4B) and fractions 7–9 (Figure 4C), 7 out of the 8 EV-associated miRNAs tested were reduced in EVs isolated from pasteurized and homogenized milk. In contrast, the concentration of miR-223 increased in both EV-enriched fractions 4–6 and 7–9 after pasteurization and homogenization (Figure 4B, C).

Discussion

EVs are biologically active macromolecular components in milk that are thought to play a role in the development of the gastrointestinal tract and the immune system of the newborn (9–11, 13). Because EVs are abundantly present in bovine milk it is likely that human consumers will be exposed to these EVs, because it is generally believed that bovine milk EVs can withstand digestion (27, 41, 47), contain

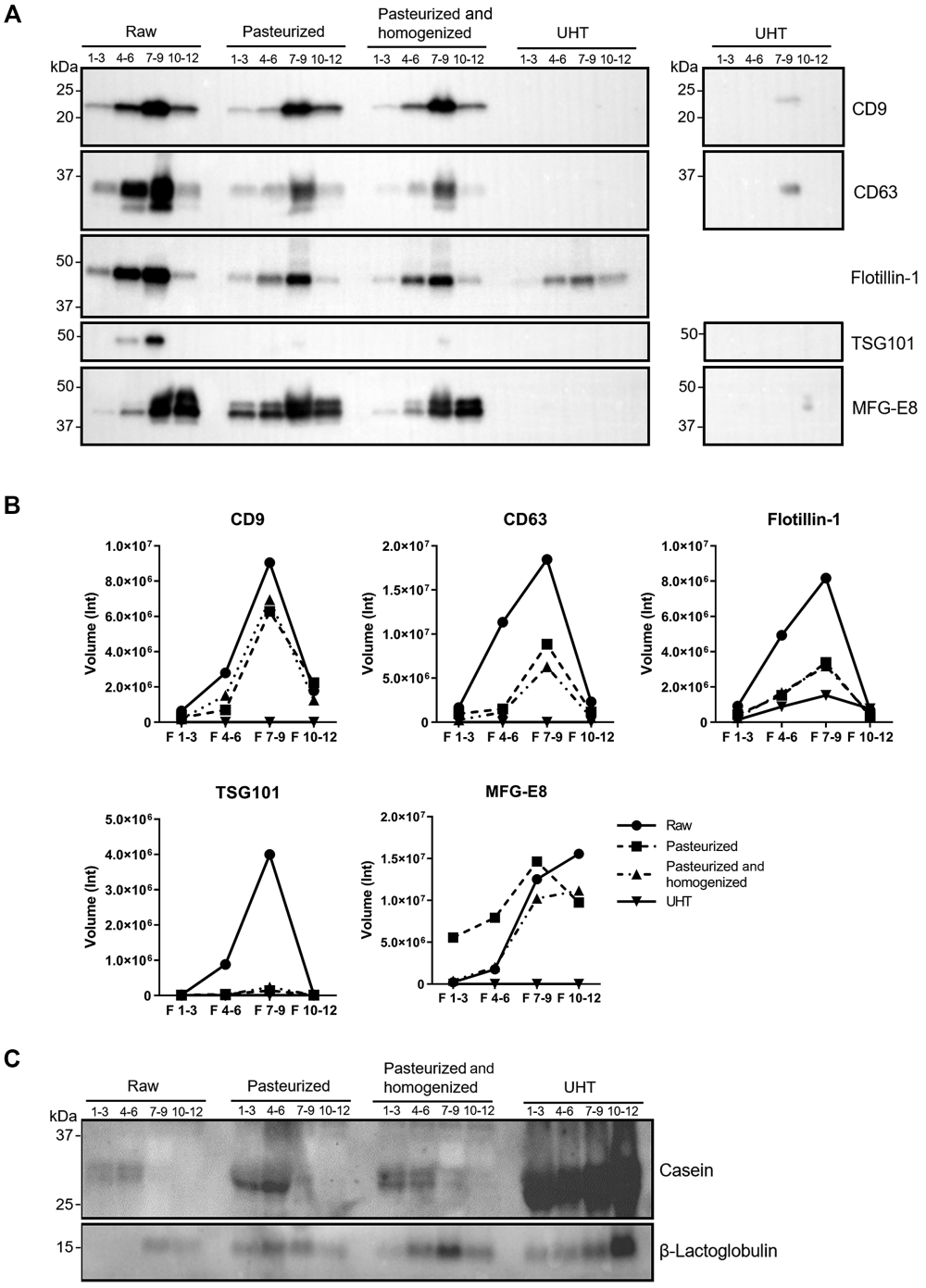

FIGURE 3 Western blot analysis for EV markers and abundant non-EV-associated milk proteins. (A) Western blot analysis for EV-associated markers of the various pooled density gradient fractions derived from raw milk or industrially processed milk. Molecular weight and exposure time: left panel CD9 (~24 kDa, 12 s), CD63 (~26 kDa, exposure time 22 s), flotillin-1 (~48 kDa, exposure time 42 s), TSG101 (~45 kDa, exposure time 260 s), and MFG-E8 (~43 kDa, exposure time 34 s). The right panel shows the same membranes as in the left panel, but after prolonged exposure times for CD9 (~24 kDa, 300 s), CD63 (~26 kDa, exposure time 600 s), TSG101 (~45 kDa, exposure time 1500 s), and MFG-E8 (~43 kDa, exposure time 300 s). (B) Quantitative analysis of the signal intensities of the bands shown in (A). (C) Western blot analysis of the abundant non-EV-associated milk proteins casein (~23–36 kDa, exposure time 30 s) and β-lactoglobulin (~17 kDa, exposure time 4 s). All data presented are single samples that are representative of 3 independent experiments. EVs, extracellular vesicles; MFG, milk fat globule; TSG, tumor susceptibility gene; UHT, ultra heat treated.

evolutionarily conserved cargo (23, 24), and can be taken up by human cells (25–29). After ingestion and cellular delivery, the milk EVs' evolutionarily conserved cargo might be involved in the regulation of host cellular processes (48). The possible physiological role of dietary-derived EVs in interorganism and cross-species intercellular communication is fascinating, but the concept remains controversial mainly because of bioactivity and bioavailability issues (24, 48). In that respect it is important to investigate the abundance and integrity of milk EVs in commercial milk before concluding

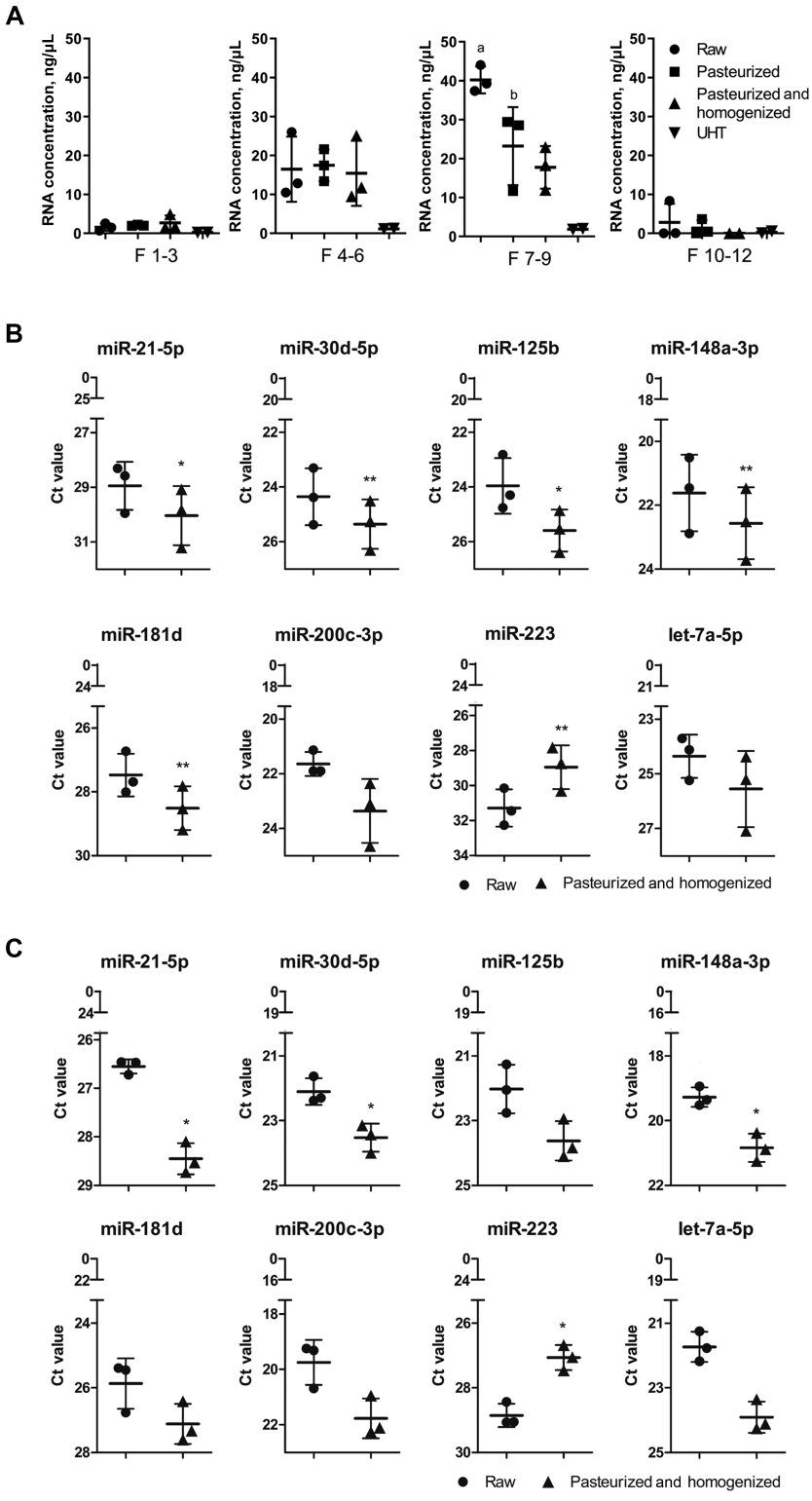

FIGURE 4 Quantification of total small RNAs and EV-associated miRNAs in raw and processed bovine milk. Small RNAs were isolated from pooled density gradient fractions 1–3, fractions 4–6, fractions 7–9, and fractions 10–12 of raw milk or industrially processed milk. (A) Total concentrations of small RNA. Significance was calculated by 1-factor ANOVA and labeled means without a common letter differ: $^{a}P < 0.05$ compared with pasteurized and homogenized and UHT milk, $^{b}P < 0.05$ compared with UHT milk. (B) miRNA expression shown as Cq values in pooled EV-enriched fractions 4–6 of raw or pasteurized and homogenized milk. (C) miRNA expression shown as Cq values in pooled EV-enriched fractions 7–9 of raw or pasteurized and homogenized milk. Data are mean ± SD, $n = 2$–3 individual samples that are derived from $n = 2$–3 independent experiments. (B, C) Significance was calculated using a paired t test. Different from raw, $*P < 0.05$, $**P < 0.01$. Cq, quantification cycle; EV, extracellular vesicle; miRNA, microRNA; UHT, ultra heat treated.

that the efficient delivery of intact cargo could even take place.

To reliably study EVs and to unveil their functional relevance, it is essential to properly separate them from other milk components (31, 32). Furthermore, because milk EVs are very heterogeneous in size, buoyant density, and composition, the selected isolation protocol will greatly determine the final composition of the enriched EV pool (30–32). Apart from the fact that many different protocols are being used to enrich or isolate milk EVs, and thus result in differently composed EV pools, comparison of studies is further hampered owing to the use of different milk sources. In several studies raw milk was used as the source (10, 11, 13, 24, 25, 27, 31, 32, 38, 42–47), whereas other studies have been conducted with milk EVs isolated or enriched from commercially available milk (9, 12, 26, 28–30, 39, 41). Furthermore, the various forms of commercial milk that are on the market are processed in different ways, which may affect milk EVs differently. Thus, what is currently lacking is a comparative study using well-characterized EVs isolated with a standard protocol from unprocessed milk which are compared with EVs isolated from milk processed in different ways. Here, we demonstrate the effects of UHT and pasteurization, in the absence or presence of homogenization, on purified milk EV populations isolated via differential centrifugation and sucrose density gradient isolation.

By implementing fluorescence-based single-particle EV flow cytometric analysis, we detected low numbers of EVs in UHT-derived samples which lacked the typical light-scattering properties of EVs. In pasteurized milk, both homogenized and nonhomogenized, comparable numbers of fluorescent EVs with similar light-scattering properties were detected compared with raw milk–derived EVs. These results indicate that pasteurization did not affect the total number of EVs. However, we cannot formally exclude a possible selective loss of (small) EV subsets, for which the generic fluorescent signal was too low to surpass the detection limit (35).

Although the flow cytometric analysis did not reveal a loss in EV numbers after pasteurization, the Cryo-EM analysis unveiled that pasteurization altered the morphology of EVs and affected their membrane integrity. The impact of pasteurization on milk EVs was further substantiated by western blot analysis of EV-associated markers, because the signals for CD63, flotillin-1, and especially TSG101 were decreased. Thus, it appears that milk contains a heterogeneous milk EV population with heat-resistant EV subsets that are positive for the tetraspanin CD9, and for the membrane-associated protein MFG-E8, which is present in certain EV subsets and in MFGs (30, 49). Milk also contains a subset of heat-sensitive EVs positive for the tetraspanin CD63, and the luminal proteins flotillin-1 and TSG101. Alternatively, it might be possible that the diminished signals for EV-associated proteins were caused by a lack of recognition of epitopes by the detection antibodies. In fact, some milk proteins are heat-sensitive and heating causes denaturation, aggregation, and glycation of these proteins, possibly hampering antibody binding and thereby limiting detection in the western blot analysis (50). To the contrary, the disruption or aggregation of non-EV milk components after UHT might have resulted in the increased signal for casein. Because we did not observe significant differences in EV numbers after pasteurization, possible heat-sensitive EVs either would represent a small subset of all milk EVs present in the sample, or were not detected by flow cytometry.

The impact of pasteurization was also observed in the small RNA analysis, because most miRNAs were reduced in both EV-enriched fractions 4–6 and fractions 7–9, whereas only in EV fractions 7–9 was a reduction of total small RNA found. Importantly, besides miRNAs, other EV-associated small RNA biotypes contribute to the total small RNA concentration (51), which was previously shown in milk EVs prepared from commercial milk, as most of the clean reads appeared as undefined small RNAs, whereas most of the defined small RNAs were miRNAs (40). Our results suggest that the total small RNA make-up of EVs in fractions 4–6 and 7–9 substantially differs, and that heat-sensitive EV subsets are predominantly enriched for certain RNA biotypes or miRNAs. The presence of different milk EV subsets in commercial milk has also been confirmed by others, demonstrating differences in physical properties, small RNA, and protein cargo and functionality (30, 52).

A remarkable finding was the increased abundance of miR-223 in EV fractions of pasteurized milk. Previously, it has been shown that pasteurization has detrimental effects on milk in general (8, 53), or on other membranous macromolecular milk components like MFGs (6). Because MFGs also contain miRNAs, including the miRNAs tested here (54), it cannot be excluded that processing induces novel miR-233-bearing vesicles derived from disrupted MFGs that end up in the EV density fractions (30). Alternatively, the increase of miR-223 might be due to the presence of apoptotic cell–derived EVs in commercial milk, which also contain miRNAs (55, 56). Because cells present in milk rapidly die after milk collection, resulting in the release of novel EVs (31), and because MFGs can disintegrate during prolonged storage (57), the fresh raw milk used in this study will not contain as many apoptotic EVs or disrupted MFGs as the processed milk, also because most native cells and intact MFGs were directly removed after milking. In contrast, commercial milk is typically collected and stored for several days before the first processing steps are performed, which will lead to nonnative EVs in commercial milk.

In summary, we have demonstrated that regular industrial processing of bovine milk affects the integrity and composition of milk EVs. Whereas ultra heat treatment of milk resulted in a substantial loss of milk EVs, lower heat treatment (i.e., pasteurization) of milk hardly affected EV numbers but clearly had detrimental effects on EV integrity and cargo, and might have resulted in processing-induced EV subsets. Homogenization in combination with pasteurization did not induce overt additional detrimental effects. Our findings have also demonstrated the importance of analyzing multiple parameters, because not all EV characteristics were affected equally. Furthermore, the source of milk and type of EV isolation procedures should be carefully considered and reported when performing milk EV research, because they will greatly influence the outcome and conclusions of any downstream analysis. The effective transfer of bioactive components via bovine milk EVs to human consumers is likely diminished or altered in processed milk owing to affected EV characteristics that include a loss of cargo, a possible susceptibility to digestion, or impaired cellular targeting and uptake.

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