Original Article



# DodecaRNAs (doRNAs) are abundant in cow's milk and differentially enriched in milk ultracentrifugation fractions

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**Background:** Extracellular RNAs (exRNAs) are found in numerous extracellular fluids, including milk. Until recently, microRNAs were the focus of research in this area, leaving aside other exRNAs. Recently, a modified small RNA-sequencing (sRNA-seq) approach led to the discovery of very short, 12 and 13 nucleotides (nt) ribosomal RNA (rRNA) fragments (rRFs), designated as dodecaRNAs (doRNAs), in reference to the number of core nucleotides (12 nt) they contain. Since milk is highly enriched in extracellular vesicles (EVs) and exRNAs, we inquired about the existence of doRNAs and other very short exRNAs in milk and milk EV (mEV)-enriched ultracentrifugation fractions.

**Methods:** We used sRNA-seq to explore exRNAs shorter than 16 nt in cow's milk and milk fractions obtained by ultracentrifugation. Results were validated with high-specificity splint-ligated reverse transcription quantitative polymerase chain reaction (RT-qPCR) using high sensitivity locked nucleic acid (LNA) oligonucleotides.

**Results:** Cow's milk was abundant in doRNAs and c-doRNAs, a doRNA derivative harboring an additional cytosine (C) at its 5' end. Together, these two sequences represented 66.5% of all 8- to 15-nt RNA species. The abundance of doRNAs in milk was 11 to 49 times higher than the most abundant microRNAs. These RNAs were differentially distributed in milk ultracentrifugation fractions; their concentration was highest in the lower speed fractions (12,000 and 35,000 g). We also observed an increased c-doRNA/doRNA ratio with centrifugation speed, suggesting a possible selective release of c-doRNA over doRNA in denser mEVs. RT-qPCR quantification confirmed the presence of doRNAs in milk and supported the differential enrichment of doRNAs in different mEV subsets compared to that of the most enriched milk bta-let-7b, bta-miR-30a-5p and bta-miR-148a, yet not without discrepancies with the sequencing data.

**Conclusions:** These findings suggest that exRNAs might be more diverse in cow's milk than previously thought. As doRNAs were found to be downregulated and to modulate cell proliferation/migration of prostate cancer cells, this could have health implications in adult and infant consumers which warrant further investigations.

**Keywords:** Milk; cow; extracellular vesicle (EV); dodecaRNA (doRNA); extracellular RNA (exRNA)

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## Introduction

Extracellular RNAs (exRNAs) are found in a diversity of biological fluids, but they are especially enriched in milk (1,2). Within this fluid, exRNAs are contained in small membranous extracellular vesicles (EVs), such as exosomes, that protect their labile cargo of exRNAs (3,4) from the degradative conditions that prevail during lactation (e.g., RNase found in milk, IgA hydrolases) (5,6) and digestion [(7) and reviewed in (8,9)].

For the last decade, most studies investigating milk exRNAs in different species focused on microRNAs (4,9-12), small non-coding RNAs (ncRNAs) implicated in a vast array of—if not all—physiological functions and pathologies, such as cancer and inflammatory diseases [reviewed in (13)]. However, our previous discovery of a plethora of milk EV (mEV) subsets (6,14), progress of small RNA sequencing technologies and democratization of their use initiated new lines of research unveiling the relative abundance and diversity of other non-coding exRNAs, such as ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), tRNA fragments (tRFs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) (10,15,16) and coding exRNA (i.e., mRNAs) (17,18).

Recently, the discovery of very short [12–13 nucleotides; (nt)] functional dodecaRNAs (doRNAs) (19) challenged the belief that ncRNAs shorter than 16 nt were merely non-functional degradative byproducts that pollute sequencing results. Most probably derived from the 5.8S rRNA, doRNAs were found to be extremely abundant in the cells, organs and even EVs (i.e., platelet-derived EVs) that were analyzed. Found so far in humans, mice and flies (19), doRNAs displayed a species-specific enrichment and ratio of the two most abundant isomers of doRNAs: the 12-nt doRNA and its 13-nt c-doRNA [a doRNA derivative harboring an additional cytosine (C) at its 5' end] variant (19). Moreover, these very short ncRNAs were notably found to be more abundant than microRNAs in these samples. Interestingly, they were mainly cytoplasmic, interacted with heterogeneous nuclear ribonucleoproteins (hnRNP) A0, A1 and A2B1, and were found to regulate the expression of Annexin II receptor (AXIIR) (19). They were also differentially expressed in prostate cancer cells/tissues (vs healthy controls) and impacted cancer cell migration (19). Along with our previous report on semi-microRNAs (smiRNAs) (20), these results put in question the practice of systematically discarding sequencing data of RNAs shorter than the arbitrary threshold of 16 nt, oftentimes even before library construction (21).

While we previously reported the existence of different RNA biotypes, including canonical rRNA fragments (rRFs) longer than 16 nt in commercial dairy milk (16), whether shorter RNA species derived from rRNA, such as doRNAs, exist in milk remains unknown. Because (I) milk is highly enriched in exRNAs of various types, (II) milk exRNAs resist human digestion (7) and (III) milk exRNAs might exert biological functions (12,22) and impact consumers' health upon ingestion [reviewed in (8,11)], we hypothesized that commercially available dairy cow's milk might contain doRNAs or similar, very short exRNAs, in greater amount than microRNAs.

To fill this gap in knowledge and after an extensive characterization of mEVs (6,7,14,16,23), we used small RNA-sequencing (sRNA-seq) to investigate the existence and abundance of 8- to 15-nt sRNAs, including doRNAs, in commercial dairy cow's milk and validated these results using a new high-specificity reverse transcription quantitative polymerase chain reaction (RT-qPCR) method designed and validated for RNAs shorter than microRNAs (24). We present the following article in accordance with the MDAR reporting checklist (available at https://exrna.amegroups.com/article/view/10.21037/exrna-22-6/rc).

## Methods

### *Cow milk samples*

Commercial skimmed filtered dairy milk (PurFiltre, Lactantia, Toronto, Canada) was purchased in a local store in *Québec* City, Canada. Three packs of milk with different expiration dates were either processed for triplicate analyses (sequencing of unfractionated milk and qPCR validations) or were mixed into one milk solution used for sequencing analyses of milk fractions.

### *Differential ultracentrifugation*

Milk was fractioned following our previously reported protocol (14). Briefly, to solubilize casein and prevent contamination of the mEVs-enriched fractions with this protein, 100 mL of dairy milk was mixed with 1 volume of 2% sodium citrate (in water, Sigma) filtered through 0.22-µm pore microfilters (Corning, Corning, NY, USA) and kept on a rocking table for 20 min at 4 ℃ until milk clarified. The citrated milk samples were then subjected to successive ultracentrifugation at 12,000 (12K), 35,000

(35K), 70,000 (70K) and 100,000 (100K) g for 1 h each at 4 ℃ in a Sorvall WX TL-100 ultracentrifuge, equipped with either a SureSpin 630 or a T-1250 Rotor (Sorvall, through Thermo Fisher Scientific, Waltham, MA, USA). After each step, pellets were carefully suspended in 1 mL of 0.22-µm filtered sterile phosphate buffer saline (PBS), pH 7.4 and kept resuspending overnight at 4 ℃ before RNA isolation.

### *RNA isolation*

For sequencing analyses, total RNA from milk or milk fractions was isolated using TRIzol LS (Thermo Fisher Scientific) following the manufacturer's recommendations prior to resuspension in diethylpyrocarbonate (DEPC)-treated, nuclease-free water (Invitrogen, Carlsbad, CA, USA). Total RNA was purified further using RNeasy mini-kit and subjected to on-column treatment with DNase I, according to the manufacturer's protocol (Qiagen, Hilden, Germany). Total RNAs was kept at –80 ℃ for a few days, after which it was shipped on dry ice to the ArrayStar sequencing platform (Rockville, MD, Canada).

For qPCR analyses, total RNA was isolated from 250 µL of whole milk or resuspended mEVs after mixing with 750 µL TRIzol LS (Thermo Fisher Scientific) spiked with UniSp2 exogenous synthetic RNA oligonucleotide (Qiagen, MD, USA, Cat No. 339306, product number: YP00203950), used as an internal control, following the manufacturer's recommendations. Isolated RNA was further treated with DNase I (M0303S, New England Biolabs, MA, USA), as per manufacturer's protocol, to remove contaminating DNA. RNA was stored at –80 ℃ for a few days before reverse transcription.

### *Complementary DNA (cDNA) library preparation*

Library preparations were performed following modified standard operating procedures (SOPs) at ArrayStar platform to allow for a larger RNA size window (8–35 nt). The purity, quality, and concentration of total RNA samples were determined with NanoDrop ND-1000 (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The miRNA sequencing library was prepared from total RNA through: (I) 3'-adapter ligation; (II) 5'-adapter ligation; (III) cDNA synthesis; (IV) PCR amplification, and (V) size selection of approximately 120 to 155 bp of PCR-amplified fragments (corresponding to approximately 8 to 35 nt of small RNA). The complete libraries were analyzed and quantitated by Agilent 2100 Bioanalyzer.

### *sRNA-seq*

The cDNA library samples were then diluted to a final concentration of 8 pM and denatured as single-stranded DNA. Cluster generation was performed on the Illumina cBot using TruSeq Rapid SR cluster kit (#GD-402–4001, Illumina, San Diego, CA, USA) and sequenced for 51 cycles on Illumina HiSeq 2000, using TruSeq Rapid SBS Kits (#FC-402–4002, Illumina), following the manufacturer's instructions.

### *Bioinformatics analysis of sequencing data*

Adapter sequences were then trimmed from the reads that passed the quality control filter (clean reads) leaving clean sRNA trimmed reads. All analyses displayed here were provided through the ArrayStar standard analysis pipeline and refined using R (R Foundation for Statistical Computing, Vienna, Austria). Only the reads that were identical, both in length and sequence, were considered as a unique read. Small RNA biotypes were determined by mapping trimmed reads against bovine noncoding RNA database [Bos_taurus.UMD3.1.ncrna, http://bovinegenome.org/; Elsik *et al.*, 2016 (25)] using Blastn tool (National Library of Medicine, National Center for Biotechnology. Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi). For miRNA, trimmed reads were aligned to miRBase pre-microRNA database (miRBase release 22.1, http://www.mirbase.org/). Sequences known to be contaminant confounders from RNA isolation procedures were discarded [Heintz-Buschart *et al.*, 2018 (26)]. sRNA read counts were expressed as reads per million (RPM) sRNAs within the specified size windows.

### *Adapter-ligated RT-qPCR*

The splint bridging sequence and adapter used to quantify sRNAs were annealed together, as described in our previously published protocol (24). The annealed adapter/splint was added to 200 ng of total RNAs (10 µL) and 18 µL of ligation master mix containing 10 units T4 RNA ligase (New England Biolabs, M0437M, Whitby, Ontario, Canada), 10% dimethyl sulfoxide (DMSO), 25% polyethylene glycol (PEG) 8000 (New England Biolabs, Whitby, Ontario, Canada), 1 mM adenosine triphosphate (ATP) (New England Biolabs, M0437M, Whitby, Ontario, Canada) and 20 units SUPERase•In RNase inhibitor (Thermo Fisher Scientific, AM2694, Waltham, MA, USA)

in T4 RNA ligase reaction buffer [50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.5]. The ligation (in 31 µL) was allowed to proceed for 1 h at room temperature (RT), after which 17 µL of stop ligation buffer [1 M Tris-HCl, 0.1 M ethylene diamine tetraacetic acid (EDTA), pH 8.0] was added. This step lengthened doRNA and c-doRNA to 22 and 23 nt RNAs, respectively. Two µL of ligated total RNA, along with UniSp6 RT spike-in, were used for RT using the miRCURY locked nucleic acid (LNA)-modified microRNA PCR Assay (QIAGEN Inc., Toronto, ON, Canada) and the oligo-d(T) primer with 5' universal tag included in the miRCURY LNA RT Kit (QIAGEN Inc., Toronto, ON, Canada; Cat. No. #339340). After cDNA 1/10 dilution, qPCR was performed using miRCURY LNA SYBR® Green PCR Kits (QIAGEN Inc., Toronto, ON, Canada) in 0.1 mL MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystem™, Cat. No. 4346907) StepOne™ Real-Time PCR System (Cat. No. 4376357) and specific Custom LNA Oligonucleotides for the doRNA (No. 339317, ad3-d-621278, Cat. No. YCP0054421, QIAGEN Inc., Toronto, ON, Canada) and c-doRNA (No. 339317, ad3-C-d-621381_1, Cat. No. YCP0054420, QIAGEN Inc., Toronto, ON, Canada) or the LNA PCR primer assays (QIAGEN Inc., Toronto, ON, Canada) for miR-30a-5p (No. 339306, miR-30a-5p, Cat. No. YP02104140), miR-148a-3p (miR-148a-3p, Cat. No. YP00205867) and Let-7b-5p (Let-7b-5p, Cat. No. YP00204750). We used the following thermal PCR cycle program: denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/elongation at 57 °C for 1 min.

### *Standard curve for absolute doRNAs and miRNAs quantification*

DoRNA, c-doRNA, miR-30a-5p, miR-148a-3p and Let-7b-5p copy numbers were determined using a standard curve established using the corresponding synthetic RNA oligonucleotides (IDT, Coralville, IA, USA) serially diluted 1/10th to obtain between $5.7\times10^9$ and $5.7\times10^3$ copies, covering a range of concentration of 6 logs (Figure S1). Diluted synthetic RNA was then subjected to adapter-ligated RT-qPCR. For each standard curve, the cycle quantitation (Cq) values with the corresponding copy numbers were plotted, and the linear curve equation and correlation coefficient (R2) were calculated (Figure S2). doRNA and miRNA quantifications were normalized using internal spike-in controls to ensure comparable isolation efficiency (isolation and splint-RT-qPCR quality controls available in Figure S3).

### *Statistical analysis*

All statistical analyses were performed using Prism 9.2.0 (GraphPad Software Inc., La Jolla, CA, USA). Unfractionated milk and validation experiments were performed in biological triplicates (n=3). Statistical significance was determined by one-way ANOVA with Holm-Sidak's *post-hoc* correction for multiple comparisons or Student's *t*-test after validation of statistical assumptions and prerequisite for each test and with type error α set to 0.05 (i.e., 5%, P value below 0.05 considered significant).

### *Illustrations*

Figures were generated using R (R Foundation for Statistical Computing), Inkscape software (http://inkscape.org/), InteractiVenn (27) and Prism 7 (GraphPad Software Inc.).

## Results

### *doRNAs are highly abundant in commercial cow's milk*

There was a higher abundance of sRNAs in the 8–15 nt compared to the 15–30 nt size window of our unfractionated commercial cow's milk sRNA sequencing data, although this 1.4-fold difference did not reach statistical significance (*Figure 1A*).

Analysis of size distribution of sRNAs in the 8–15 window revealed a marked, and significant, 6- to 33-fold enrichment of 12-nt sRNAs and 3- to 17-fold enrichment of 13-nt sRNA, in comparison to the other fractions ($P<0.05$ to $P<0.0001$, *Figure 1B*). The sRNA detection threshold in this experiment averaged 10.06 RPM (*Figure 1C*), with 34±21 sRNAs representing 80% of the entire 49,612 detected sequences (*Figure 1C*). Most of the sRNAs in these samples mapped to rRNAs (77.9%) and mRNAs (17.1%, *Figure 1D*). Among the 20 most expressed sRNAs, two sequences (GACUCUUAGCGG and CGACUCUUAGCGG, 12 and 13 nt in length, respectively) represented 66.52% of all 8–15 nt sequences (*Figure 1E*). These two rRFs were designated as doRNAs, in reference to the number of core nucleotides (12 nt) they contain.

DoRNAs and c-doRNAs were respectively 25 to 49 and 11 to 30 times more enriched in these milk sequencing data

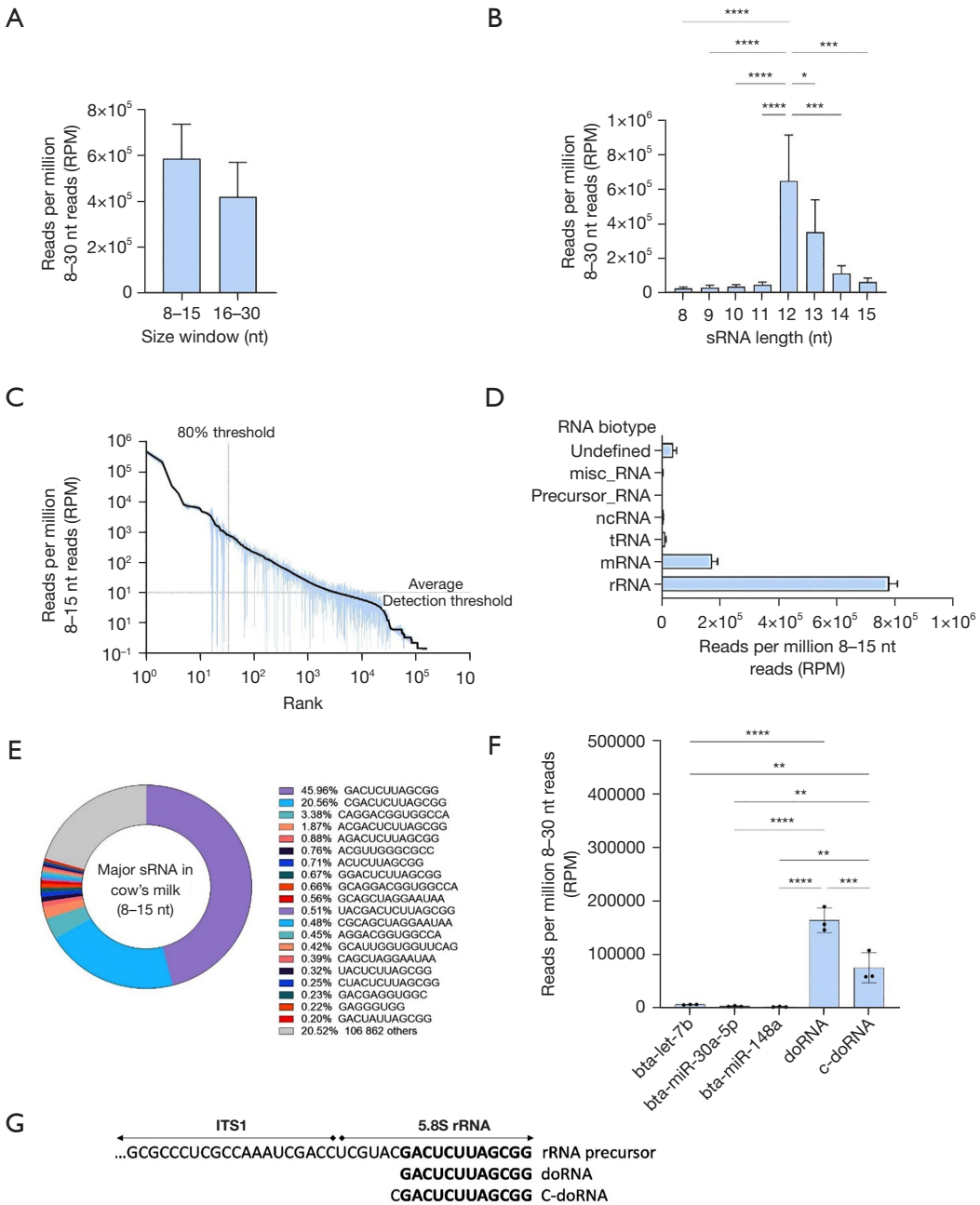


**Figure 1** Sequencing and identification of very-short RNAs in commercial cow's milk. Total RNA from 100 mL of commercial cow milk was subjected to small RNA sequencing in the 8–30 nt size window (n=3 independent experiments). (A) Abundance of sRNAs in the 8–15 nt windows in comparison to the canonical 15–30 nt RNA sequencing window, expressed as RPM reads in the 8–30 nt size windows. (B) Size distribution of very-short RNAs, expressed as RPM in the 8–15 windows. (C) Number of different sRNAs detected in the 8–15 nt size window, expressed as RPM and defining average detection threshold. (D) Small RNA biotype abundance (in RPM). (E) The 20 most abundant sRNAs in commercial cow milk (percent of total). (F) Abundance of doRNA and c-doRNA in comparison to milk's most abundant microRNAs (bta-miR-30a, bta-miR-148a and bta-miR-let7b) expressed in RPM in the 8–30 nt size window. (G) doRNA and c-doRNA sequences compared to the 5.8S rRNA from which they likely derive [adapted from (19)]. Data are displayed as average ± SD and analyzed as described in the Methods section. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA; nt, nucleotide; RPM, reads per millions; sRNA, short RNA; ncRNA, non-coding RNA; mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA; SD, standard deviation.

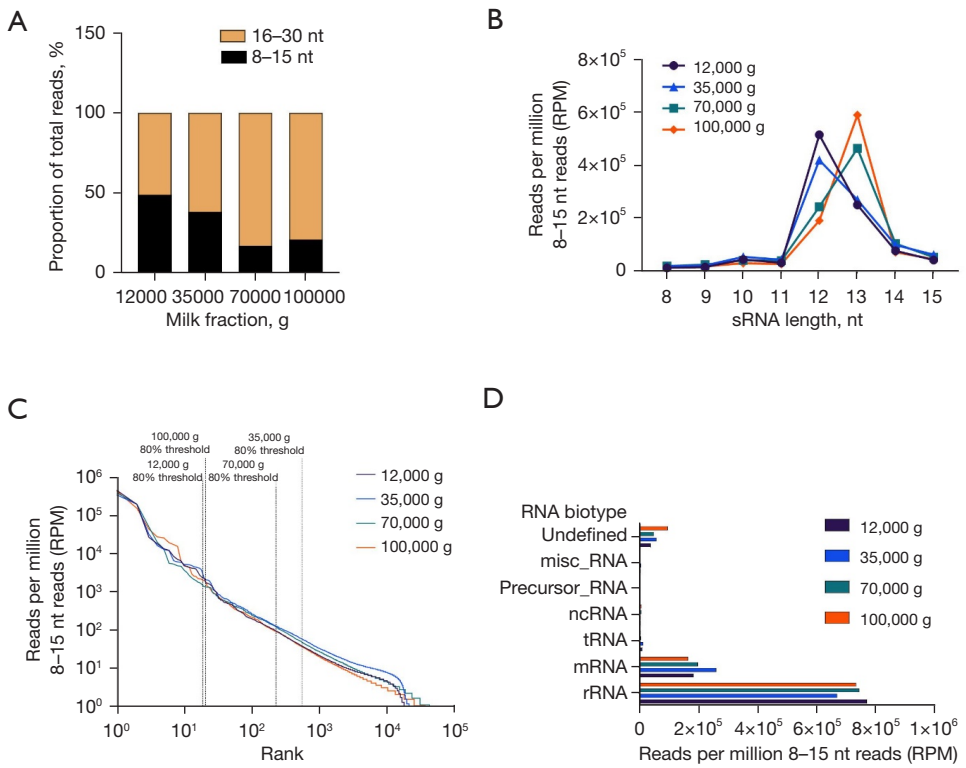


**Figure 2** Sequencing and identification of very-short RNAs in ultracentrifugation fractions of commercial cow's milk. Three milk samples were pooled and subjected to differential ultracentrifugation at 12,000, 35,000, 70,000 and 100,000 g, respectively. Total RNA isolated from each pellet was subjected to sRNA sequencing in the 8–30 nt size window. (A) Abundance of sRNAs in the 8–15 nt windows in comparison to the canonical 16–30 nt sRNA sequencing window, expressed as a percentage of total reads in the 8–30 nt size windows. (B) Length distribution of very-short RNAs in the 8–15 nt windows for each fraction, expressed as RPM. (C) Number of different sRNAs detected in the 8–15 nt size window, expressed as RPM. (D) Small RNA biotype abundance in each fraction (in RPM). doRNA, dodecaRNA; nt, nucleotide; RPM, reads per millions; sRNA, short RNA; ncRNA, non-coding RNA; mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA.

than bta-let-7b, bta-miR-30a-5p and bta-miR-148a; three of the most enriched milk microRNAs (28) from the same sequencing datasets (P<0.01 to P<0.0001, *Figure 1F*). These two sequences are the doRNA and c-doRNA we previously reported in other species (19) and derive most likely from the 5.8S rRNA (*Figure 1F,1G*).

These results support the existence and high enrichment of the very short doRNAs in cow's milk, less diverse but more abundant than the most abundant milk microRNAs.

### *Milk ultracentrifugation fractions have specific 8–15 nt sRNA enrichment profiles*

In our previous reports, we fully characterized the mEVs contained in the 12K, 35K, 70K and 100K g fractions obtained upon sequential ultracentrifugation of commercial cow's milk (6,16,23). We replicated the above-described analysis on these isolated fractions, as described in the Methods sections (*Figure 2*).

In all fractions, and contrary to unfractionated milk, most sequences (51.3% to 83%) were in the 15–30 nt size window (*Figure 2A*). There was a fraction-specific ratio between 8–15 and 15–30 nt sRNAs, with an increasing proportion of 16–30 nt sRNAs in the later fractions (70K and 100K g, *Figure 2A*). Similarly, 12K and 35K g milk fractions had higher proportion of 12-nt sRNAs, while subsequent fractions were more enriched in 13-nt sRNAs (*Figure 2B*).

Detection thresholds and total sRNA count in these samples were comparable (*Figure 2C*). In 12K and 100K g fractions, 19 and 20 sequences represented 80% of 8–15 nt reads, respectively (*Figure 2C*). In 35K and 70K g fractions,

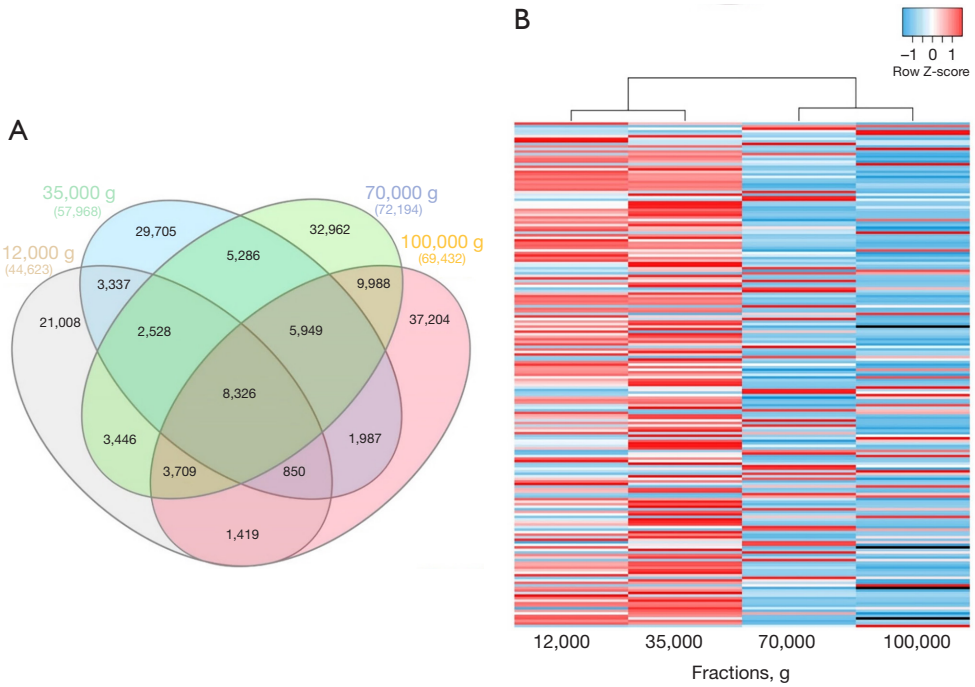


**Figure 3** Comparison of sRNA profile between milk ultracentrifugation fractions. (A) Venn diagram displaying the number of 8–15 nt sRNAs shared or not between cow's milk ultracentrifugation fractions. Interactable version providing all shared sequences is available as supplementary file. (B) Heatmap and clustering of milk ultracentrifugation fractions based on the 200 most enriched 8–15 nt sRNAs in milk (Spearman's rank, expressed as row Z-scores). nt, nucleotide; sRNA, short RNA.

this proportion accounted for 562 and 230 sequences, respectively, suggesting a higher diversity of sequences in these two intermediate fractions (*Figure 2C*). As for unfractionated milk, biotype analysis revealed that most of the 8–15 nt sRNAs, as in all fractions, mapped to rRNAs (73–77%) and mRNAs (16–25%) (*Figure 2D*).

Further comparison of the four milk fractions revealed that each contained between 21,008 to 37,204 specific sequences and shared over 8,326 common reads (*Figure 3A*). Enrichment distribution of the 200 most enriched 8–15 nt sRNAs in milk (~80% of all sequences) revealed that those were most enriched in the 12K and 35K g fractions (*Figure 3B*). Clustering by these sRNAs confirmed 12K and 35K g fractions were more closely related to each other (*Figure 3B*). Similar observation was drawn for 70K and 100K g fractions (*Figure 3B*).

These results suggest 8–15 nt sRNAs, a high proportion of which is around 12–13 nt in size, distribute differentially between milk ultracentrifugation fractions, distinguishing and clustering these in two subsets (12K–35K and 70K–100K g groups).

### *The majority of 8–15 nt sRNAs in milk fractions are doRNAs that are more abundant than most abundant milk microRNAs*

Like unfractionated milk, doRNA (GACUCUUAGCG, 12 nt) and c-doRNA (CGACUCUUAGCGCC, 13 nt) accounted for 56% to 67% of all 8–15 sRNAs (*Figure 4A-4D*).

When looking specifically at these two doRNAs, 12K and 35K g fractions were more closely related to each other, with their most abundant sequence being the 12-nt doRNA (*Figure 4A,4B*). In subsequent fractions (70K and 100K g), the most abundant 8–15 sRNA was the 13-nt c-doRNA (*Figure 4C,4D*). These findings translated in a c-doRNA/doRNA ratio of 0.42, 0.56, 1.99 and 2.85 in 12K, 35K, 70K and 100K g fractions, respectively, which tended to increase with ultracentrifugation speeds (*Figure 4A-4D*). A closer inspection of the distribution profiles of c-doRNA and doRNA (*Figure 4E*) revealed that the c-doRNA/doRNA ratio is increasing mainly because doRNA sequences sediment at lower speeds, leaving less of these sequences for the subsequent fractions, while c-doRNA remains constant

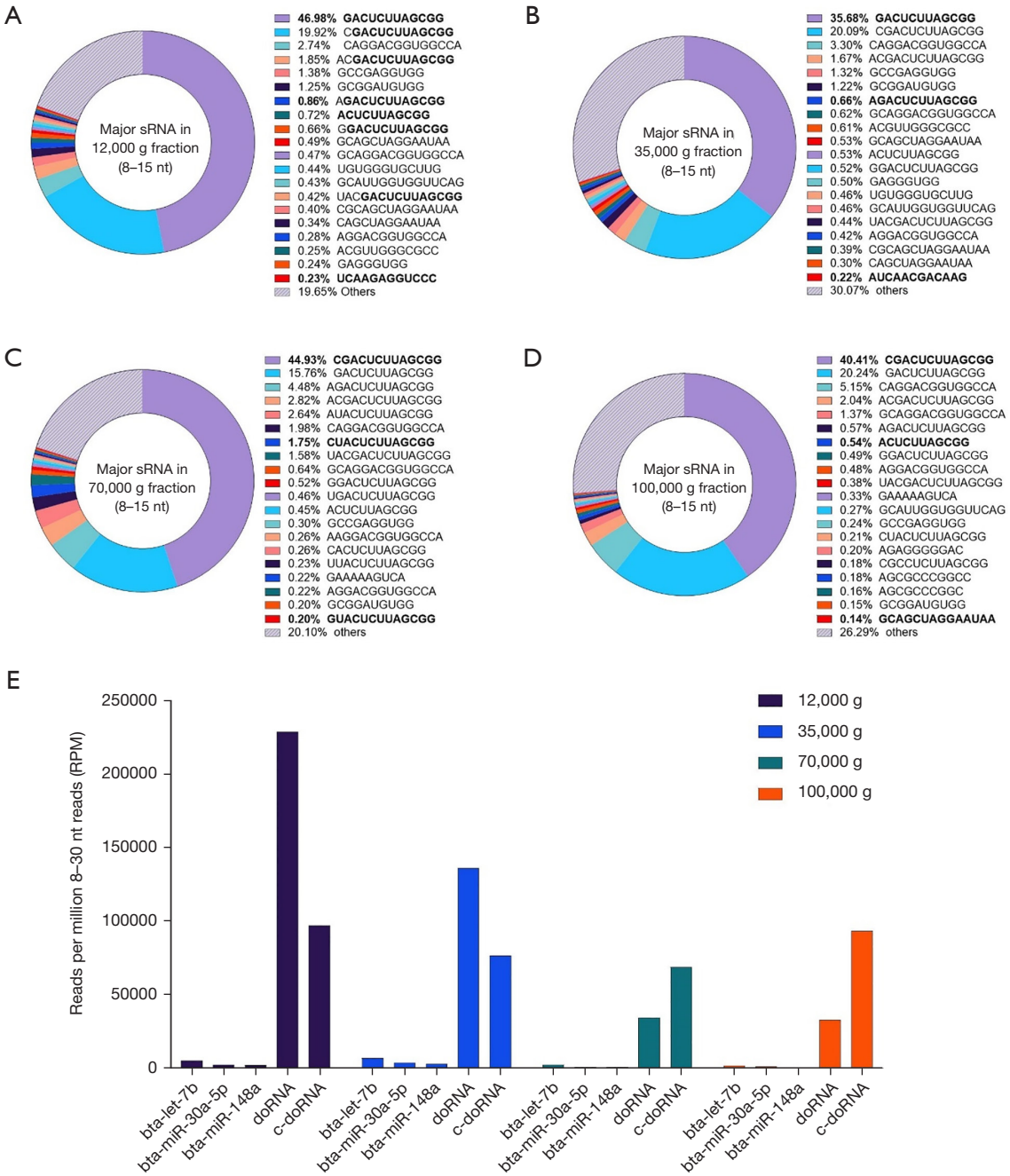


**Figure 4** Identification of the most abundant 8–15 nt sRNAs in milk fractions and comparison with most abundant microRNAs. Twenty most abundant 8–15 nt sRNAs in 12,000 g (A), 35,000 g (B), 70,000 g (C) and 100,000 g (D) milk ultracentrifugation fractions. (E) Enrichment of doRNA, c-doRNA, and microRNAs bta-let-7b, bta-miR-30a and bta-miR-148a in milk ultracentrifugation fractions, expressed as RPM 8–30 nt reads. c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA; nt, nucleotide; RPM, reads per millions; sRNA, short RNA.

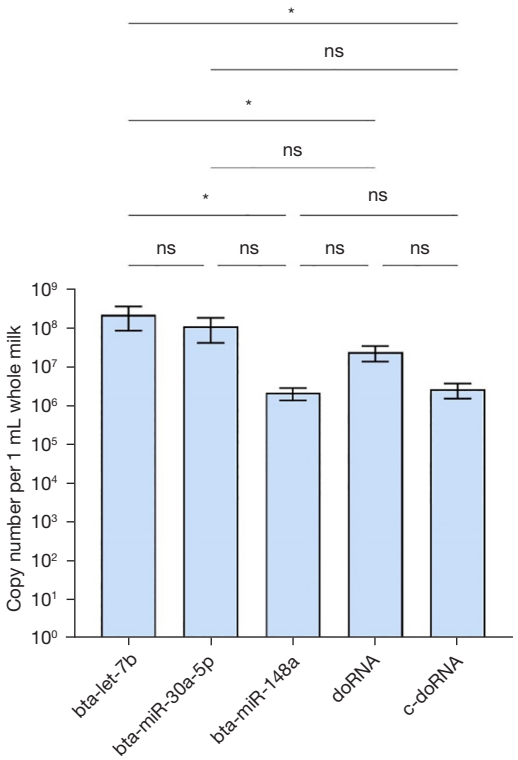


**Figure 5** Validation by LNA-based RT-qPCR of doRNA and c-doRNA existence in milk and their proportion relative to most abundant milk microRNAs. Total RNA isolated from milk was subjected to splint-ligation LNA-based qPCR for the detection of doRNAs and most abundant milk microRNAs in unfractionated milk. Copy number of each RNA was calculated using a standard curve produced by a serial dilution of the synthetic form of each RNA. Data are displayed as means ± SD (n=3) and statistically compared as described in the Methods section. *, P<0.05. ns, not significant; c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA; LNA, locked nucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SD, standard deviation.

across all the fractions (*Figure 4E*). Therefore, these observations suggest a specific enrichment of doRNA in the lower speed fractions and potential association with the large exRNA-enriched mEVs it contains (6).

When compared to milk's most enriched microRNAs (i.e., bta-let-7b, bta-miR-30a-5p and bta-miR-148a), doRNA and c-doRNA were systematically more abundant (*Figure 4E*). Twelve-nt doRNA was 16 to 194 times more enriched in these fractions than these three microRNAs, while 13-nt c-doRNA reached 12- to 553-fold higher abundance compared to bta-let-7b, bta-miR-30a-5p and bta-miR-148a (*Figure 4E*).

These sequencing results suggest that 12- and 13-nt doRNAs (I) are possibly more abundant than the most abundant milk microRNAs, (II) are specifically distributed across milk ultracentrifugation fractions, and (III) might be associated to the specific milk mEV subsets that sediment at the indicated speeds.

### *qPCR validation and comparison to the most enriched milk microRNAs*

As sequencing data often trade large spectrum of detection for precision in quantification, we aimed to confirm these results using a complimentary and previously validated splint-ligation LNA-based RT-qPCR method designed to detect sRNA shorter than 15 nt (24).

We first looked at the enrichment of doRNA and c-doRNA in unfractionated pasteurized milk, and compared those to the most enriched commercial milk microRNAs (16), namely bta-let-7a, bta-miR-30a-5p and bta-miR-148a (*Figure 5*). Both doRNA and c-doRNA were detectable in unfractionated commercial milk at a concentration of $10^6$ to $10^7$ copies per 1 mL milk (*Figure 5*). While doRNA and c-doRNA were slightly, yet not significantly, more enriched than bta-miR-148a, their levels were not higher than bta-miR-30a-5p and significantly lower than bta-let-7b (*Figure 5*).

We observed similar results in milk fractions, with bta-let-7b systematically being the most enriched of the five small RNAs surveyed in all fractions (*Figure 6*). In the first two fractions (12K and 35K g), doRNA ranked second, followed by bta-miR-30a-5p, c-doRNA and, finally, bta-miR-148a (*Figure 6A,6B*). In the latter fractions, bta-miR-30a-5p ranked second, while doRNA was relocated to the third position and c-doRNA to the last, translating a difference in enrichment pattern between the fractions (*Figure 6C,6D*). When looking at distribution patterns across the fractions (*Figure* 7), distribution profiles of milk microRNAs matched our previous reports (6,16), with the bulk of bta-let-7b and bta-miR-148a significantly concentrating within the 12K and 35K g, while bta-miR-30a was distributed more evenly across the fractions (*Figure* 7*A*-7*C*). Similarly, doRNA and c-doRNA were more enriched within the first two milk fractions (*Figure* 7*D*,7*E*), confirming a differential enrichment of doRNA between milk fractions and associated mEVs.

The trend of increasing c-doRNA/doRNA ratio with increasing ultracentrifugation speeds observed in our

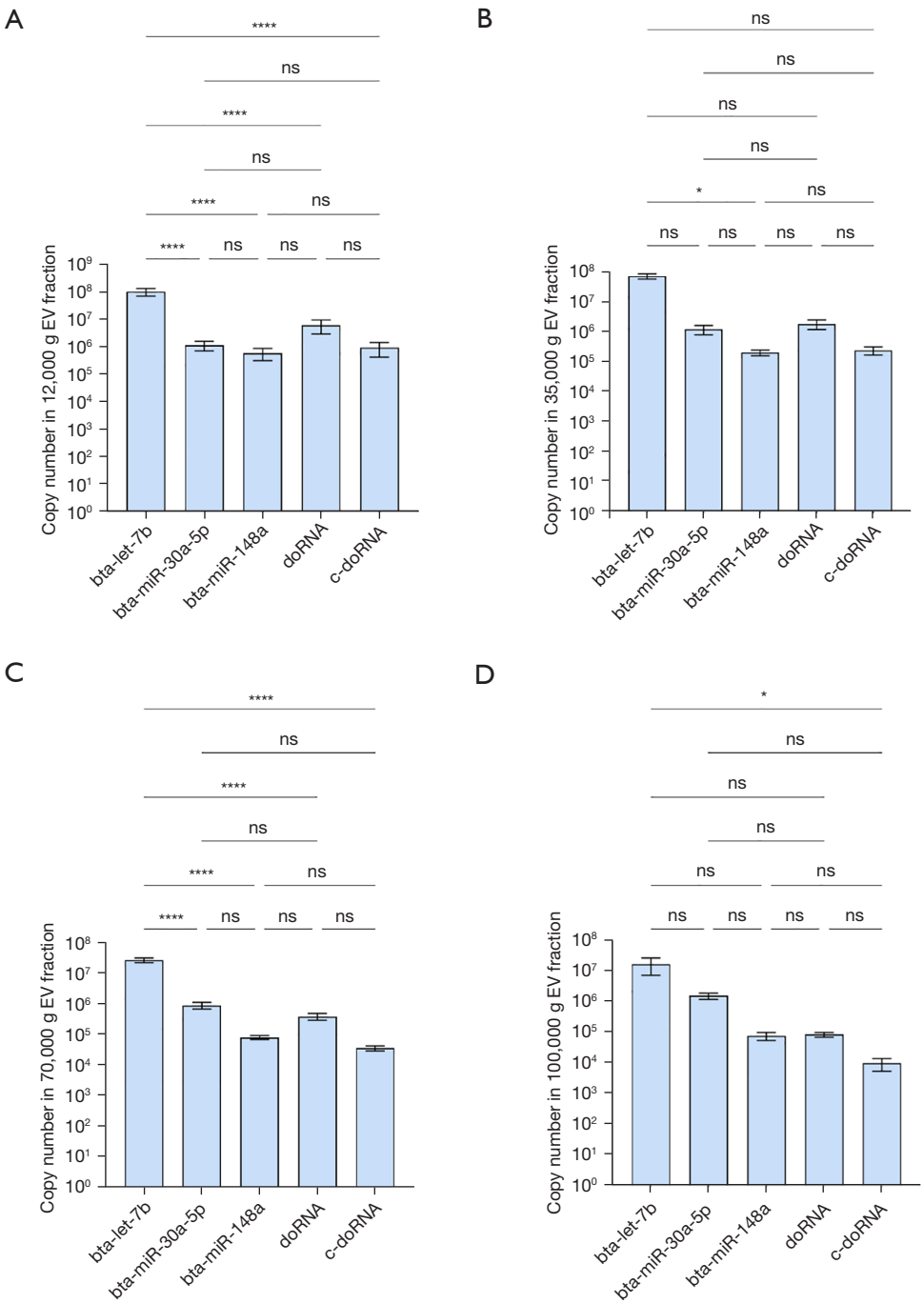


**Figure 6** Validation by LNA-based RT-qPCR of doRNA and c-doRNA existence in milk fractions and their proportion relative to most abundant milk microRNAs. Total RNA isolated from milk fractions was subjected to splint-ligation LNA-based RT-qPCR for the detection of doRNAs and most abundant milk microRNAs in 12,000 g (A), 35,000 g (B), 70,000 g (C) and 100,000 g (D) milk fractions. Copy number of each RNA was calculated using a standard curve produced by a serial dilution of the synthetic form of each RNA. Data are displayed as means ± SD (n=3) and statistically compared, as described in the Methods section, either by parametric or nonparametric tests depending on prerequisite validation. *, P<0.05; ****, P<0.0001. ns, not significant; c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA; EV, extracellular vesicle; LNA, locked nucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SD, standard deviation.

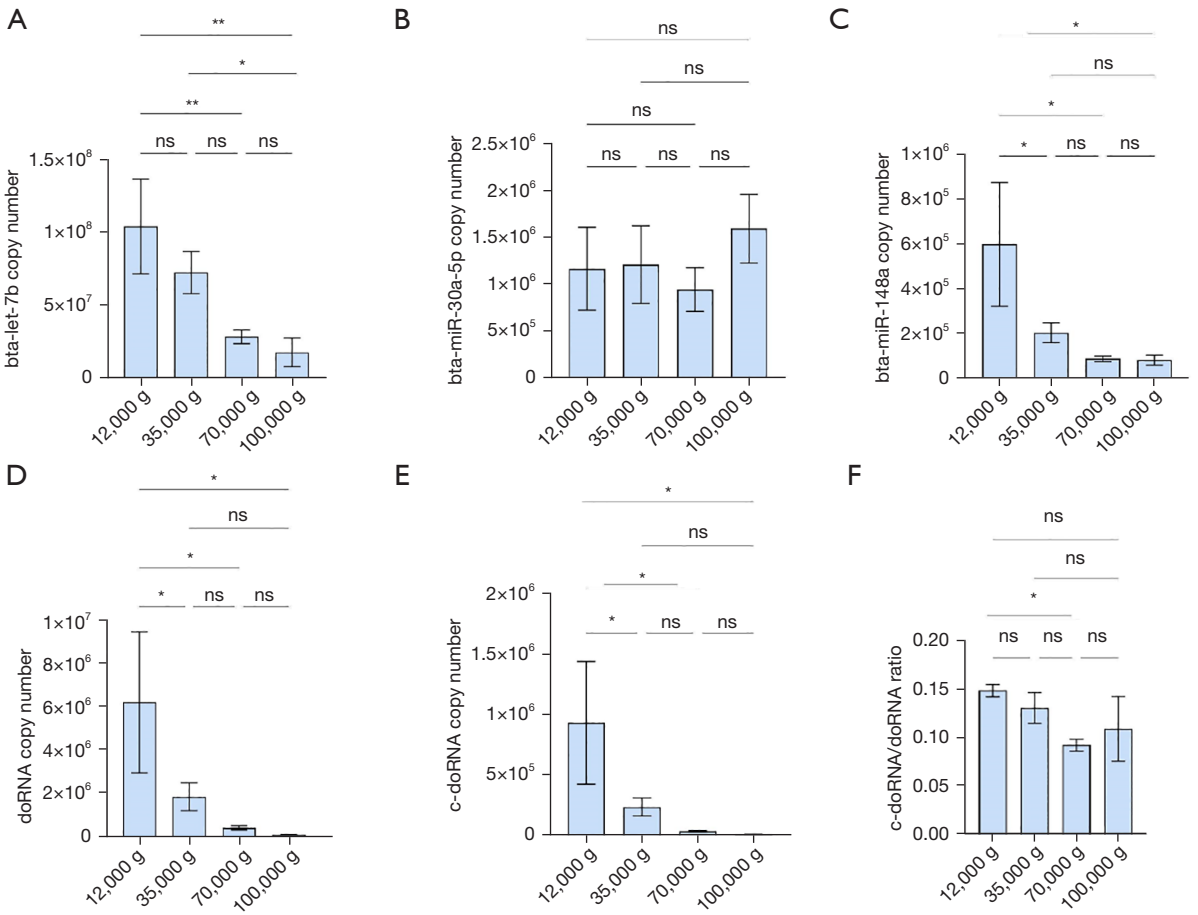


**Figure 7** Distribution of doRNA and c-doRNA in milk fractions compared to most abundant milk microRNAs. Total RNA isolated from milk fractions was subjected to splint-ligation LNA-based RT-qPCR for the detection of bta-let-7b (A), bta-miR-30a-5p (B), bta-miR-148a (C), doRNA (D), and c-doRNA (E) across milk fractions, and calculation of the doRNA/c-doRNA ratio in those fractions (F). Copy number of each RNA was calculated using a standard curve produced by a serial dilution of the synthetic form of each RNA. Data are displayed as means ± SD (n=3) and statistically compared as described in the Methods section. *, P<0.05; **, P<0.01. ns, not significant; c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA; LNA, locked nucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SD, standard deviation.

sRNA-seq data was not confirmed by RT-qPCR data (*Figure 7F*). There was, however, a significant difference in the c-doRNA/doRNA ratio between the 12K and 70K g fractions, supporting the potential specific loading of certain doRNAs within certain EV subsets (*Figure 7F*).

## Discussion

Canonical sRNA sequencing pipelines rely on an arbitrary minimal length threshold of 16 nt for library preparation and subsequent bioinformatics analysis (21). This threshold was initially set to ensure higher depth of analysis for longer sRNA, improve the signal-to-noise ratio and normalize the downstream computational analyses. It was chosen on the premise that RNA species shorter than 15 nt may not be specific, might not be mapped with confidence to the genome and would not have biological significance, being most likely degradation products, artifacts or noise "polluting" the sequencing data. While better depth of analysis is indeed of importance in sRNA sequencing, such reasoning follows the same flawed paradigm of "junk short RNA" that hindered the discovery of microRNAs, piwi-interacting RNAs (piwiRNAs), tRFs and other small ribosomal RNAs (srRNAs) in the past (29,30).

The serendipitous discovery of 12-nt smiRNAs, as potentially competing with the function of the microRNA from which they derived, was among the first evidence questioning the relevance of that 16-nt threshold (20). Subsequently, we reported the discovery of other subsets of very-short RNAs, such as doRNAs, in animals (human cells and mouse cells and tissues), plants, yeasts and bacteria, further challenging this paradigm (19). Our experiments and controls confirmed that doRNAs were not mere processing artifacts, but rather biologically relevant and active short rRNAs implicated in cell proliferation and mRNA translation (19). By unveiling the existence of doRNAs in cow's milk and fractions, along with a large array of RNAs shorter than the canonical sRNA-seq threshold of 15 nt, the evidence that we gathered here further supports this paradigm shift and extends the reach of doRNAs to the realm of exRNAs.

In addition, the focus on microRNAs in milk trended in the last decade because of their known important functions and because it was embedded within the larger "nutritional microRNAs" trend in research, leaving aside numerous biologically relevant exRNAs [reviewed in (1,11,28)]. However, mounting evidence suggests the existence of more than microRNAs in biological fluids, with several papers reporting the existence of other exRNAs, such as mRNAs, tRNAs, circRNAs and rRNAs in milk and mEVs (4,10,16,31-35). The results of the present study are in accordance with these reports and further emphasize on the importance of looking at the bigger picture rather than focusing on a single RNA species (36-38).

Interestingly, in this work, milk fractions and the mEVs they contain, were shown to be highly enriched in the 12- and 13-nt doRNAs, which constituted the vast majority of all 8–15 nt exRNAs, while this was not the case for the platelet EVs that we studied and reported in our previous work (19). Along with this observation, the distribution pattern of doRNAs and specific c-doRNA over doRNA ratios across milk fractions suggest that these 12- and 13-nt exRNAs might be specifically secreted within certain mEV subsets, as reported previously for tRNAs, microRNAs and iso-miRNAs (16,39-41). However, because of their length, further research is needed to clarify if these very small RNAs are bound by the same rules that guide other small RNAs with specific motives towards EVs or if it is their potential association with certain RNA-binding proteins that make doRNAs differentially enriched in milk fractions (19,42,43).

It was previously suggested that ultracentrifugation might be a degradative process (44,45). We cannot exclude the possibility that the observed impoverishment of doRNA within the last two fractions might be due to the loss of mEVs protecting these very-short RNAs from degradation by milk ribonucleases (46,47). Therefore, investigation of doRNAs in the different mEV subsets isolated through less disruptive methods (e.g., tangential filtration or size-exclusion chromatography) is warranted before drawing definitive conclusions about the specific loading of milk doRNAs within the larger mEVs found in the 12K and 35K g ultracentrifugation fractions (6). Moreover, as there is a possibility for exRNAs to be associated with more than simply EVs, including exosomes, there is a possibility that doRNAs in milk are associated with non-vesicular particles and ribonucleoproteins (48-50). Accordingly, further exploration of doRNA resistance to digestion and the exact mechanism of their secretion and transport in milk remains to be fully elucidated.

As exRNAs in milk have been previously found to resist digestion and potentially impact the health status of the "consumer", be it cells, mice or humans (18,22,51,52), one might speculate that these new exRNAs in milk also contribute to the bioactivity of mEVs [reviewed in (8,10,28,53)]. In this line, we previously reported that doRNAs might be involved in prostate cancer progression, in which they are underexpressed in patient's cells (19). These doRNAs also seemed to limit cell proliferation and regulation of the expression of AXIIR, which is an important receptor involved in the etiology of this disease (19). Overexpression of the doRNA-binding protein hnRNP A2/B1 in prostate cancer (54), its importance in the progression of this malignancy and for the resistance to apoptosis (55) would make of this protein a "hub"—and association with doRNAs (19)—potentially linking doRNAs and prostate cancer. While the link between milk consumption and prostate cancers remains controversial, with inconsistent evidence in the general population of men (56-58), milk and its bioactive components, including exRNAs, might affect the etiology of prostate cancer, more likely in those with digestive tracts more permeable to mEVs, as seen for bacterial lipopolysaccharide (LPS)-bearing EVs (59,60). This link, although speculative, calls for further investigations.

DoRNAs might also impact the maturation or degradation of rRNAs by binding to the 5' end of 28S rRNA (1). This could interfere with the proper assembly and integrity of the nucleolus, affecting the accurate synthesis and processing of rRNAs and, if leading 28S

 

rRNA to degradation or edition, potentially modulating the diversity of rRNAs (2). In turn, this could impact the function of ribosomes and disrupt mRNA translation. Therefore, doRNAs may have an impact on cell viability by modulating rRNA biogenesis (3).

To our knowledge, our study is the first to explore such very short exRNAs in milk, any other biological fluid and mEV-enriched ultracentrifugation fractions. Our sequencing results were supported by complementary high sensitivity, high specificity, LNA-based RT-qPCR, further confirming the existence of these extracellular doRNAs along with other very short exRNAs in commercial dairy cow's milk.

While original, our work is, however, limited in several aspects. First, these experiments were conducted on a specific milk brand that is pasteurized and ultrafiltered. In accordance with previous reports [reviewed in (8,37,61,62)], milk processing for consumption highly impacts the exRNA content (63), with marked differences between ultra-high temperature (UHT) processing and pasteurized fluids (64). Even though we detected such very short exRNAs in other milks during our investigations and protocol developments (data not yet published), the results we report here might not be inferable to all cow milks around the globe, UHT milks, pascalized fluids (65) or milks from other species. Additionally, as for other exRNAs in milk, their transfer to recipient cells remains to be fully demonstrated, despite reports supporting this hypothesis (8,28,51).

In addition, because of their novelty, so far, the discovery and exploration of doRNAs in various tissues and conditions remains monocentric and requires replication from independent research groups worldwide, which we call for.

Slight modifications to any sRNA sequencing pipeline, aimed to allow for the detection of sequences shorter than 16 nt, might easily allow replication of these results and exploration of other very short exRNAs in other bodily fluids. Additional methods and information on how to explore these very small RNAs were also reported in our latest work and could serve as a guideline for exploring such small RNAs by sequencing across species, including bacteria, yeast and plants (43). However, one should exercise caution when analyzing sequencing data because of the discrepancies we report in this study between the sequencing data and quantitative analyses. Indeed, because classic RT-qPCR would not differentiate between these doRNA and 5.8S rRNA (or longer rRFs), we set, validated, and shared the protocol of a splint-ligation LNA-based RT-qPCR method which allows the highly specific, absolute quantification of doRNAs in any sample and under any condition of health and disease (24). Using this method, we confirmed that doRNAs were highly enriched in milk (at levels comparable to the most enriched milk microRNAs), but their concentration did not seem to be above that of microRNAs, as suggested by the initial sequencing analysis. Moreover, the important differences in doRNA/c-doRNA ratio observed in sequencing data were less pronounced when assessed by LNA RT-qPCR.

These differences might have risen from RNA isolation procedure necessarily different for each method. Indeed, while on-column isolation is more suitable for RNA sequencing, TRIzol LS is known to provide with different total RNA yields than columns (66), and both isolation techniques have been reported to have selectivity biases based on GC content (66-68). Also, our splint-ligation RT-qPCR method is reinforced with two internal spike-in controls for extraction and RT-qPCR efficiency estimation, two normalization tools that cannot be used for sequencing purposes. Therefore, while our sequencing analyses led to the discovery of doRNAs as a new kind of exRNAs, we believe our qPCR validation data to be more reliable. Our robust and easily applicable splint-ligation LNA-based RT-qPCR methodology may facilitate replication of our work and allow further exploration of doRNA and very short exRNA biology in different model organisms, biological fluids and contexts.

## Conclusions

In this work, we report the discovery of very short exRNAs, most of which were rRNA-derived doRNAs, in commercially available cow's milk and their specific distribution across EV-enriched ultracentrifugation fractions. The existence of such short exRNAs in milk further challenges arbitrary size paradigms in the molecular biology of sRNAs, which does not go without reminding of the micropeptide paradigm shift in the last decade (69-71). The existence of very short exRNA, such as doRNAs in milk, suggests their potential enrichment in other biological fluids, such as plasma, cell culture mediums, intercellular or amniotic fluid; a possibility to consider when exploring the involvement of exRNAs in physiological processes, pathological conditions or even for basic *in vitro* research (72). Further investigations are warranted to unveil the origin of milk doRNAs, their functions in different conditions, their potential as

 

biomarkers of diseases, especially during lactation disorders and mammary gland infections, their transmission between cells, organs and possibly their bioactivity after transmission between individuals during breastfeeding or species through ingestion of cow's milk.

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*Ethical Statement*: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.



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| Name | Sequence |
|---|---|
| C-doRNA | /5Phos/rCrGrArCrUrCrUrUrArGrCrGrG |
| doRNA | /5Phos/rGrArCrUrCrUrUrArGrCrGrG |
| bta-miR-148a | rUrCrArGrUrGrCrArCrUrArCrArGrArArCrUrUrUrGrU |
| bat-let-7b | rUrGrArGrGrUrArGrUrArGrGrUrUrGrUrGrUrGrGrUrU |
| bta-miR-30a-5p | rUrGrUrArArArCrArUrCrCrUrCrGrArCrUrGrGrArArGrCrU |
| adapter | /5Phos/rGrArCrArArCrCrArUrU |
| splint | CCGCTAAGAGTGTTGTC |

**Figure S1** DNA and RNA oligonucleotides used in this study. c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA.

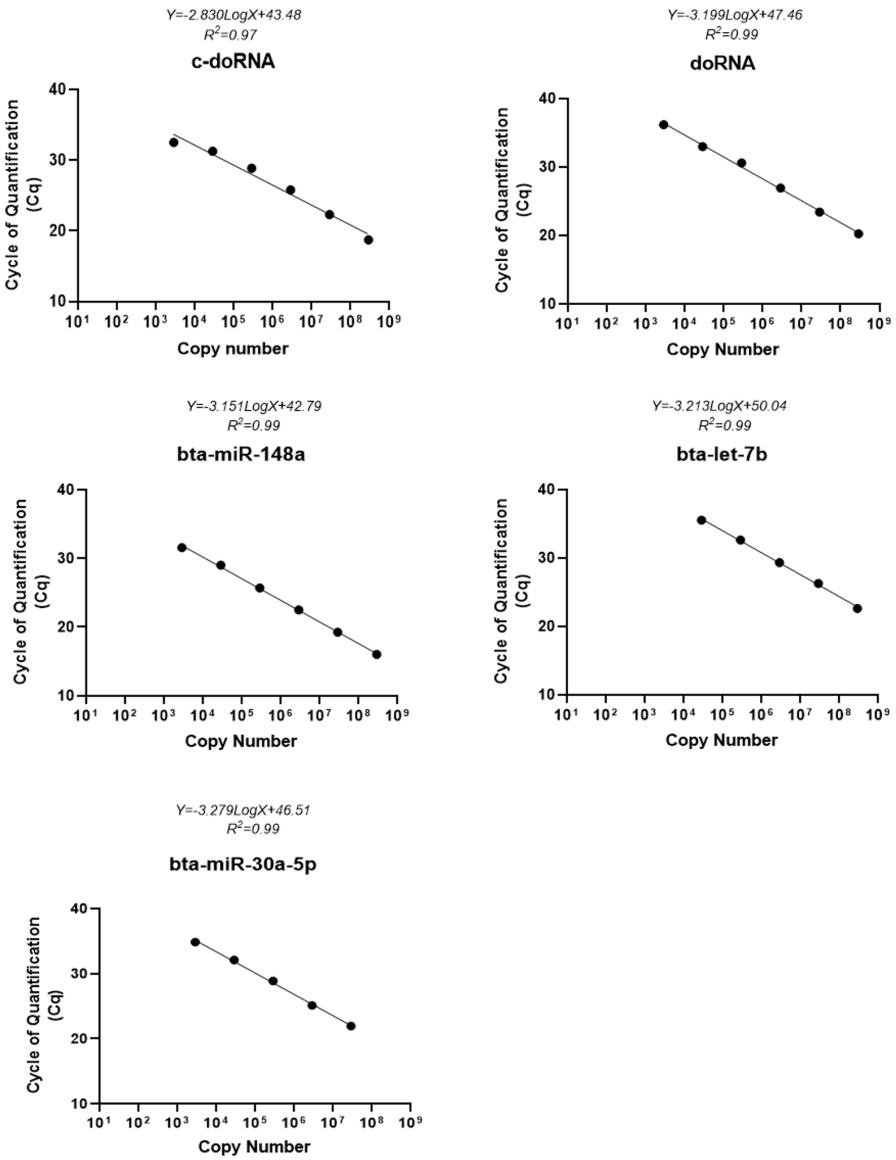


**Figure S2** Standard curves were established using synthetic doRNA, c-doRNA, miR-148a, Let-7b-5p or miR-30a-5p RNA oligonucleotides. c-doRNA, doRNA derivative harboring an additional cytosine at its 5' end; doRNA, dodecaRNA.


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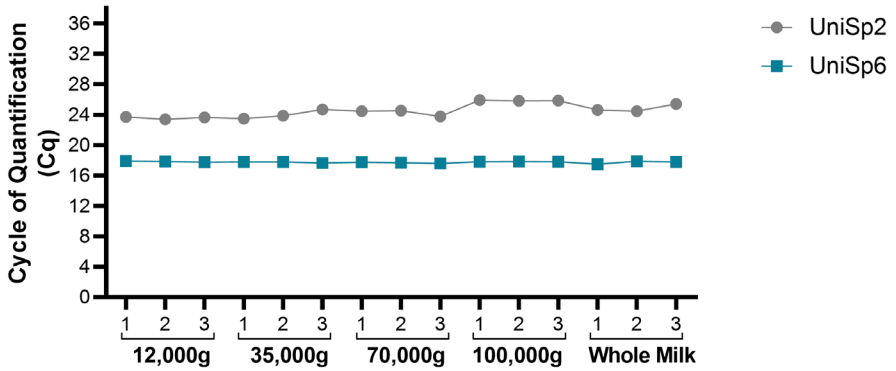


**Figure S3** Raw cycle of quantification data for the two exogenous RNA spike-ins used in the different triplicate samples. UniSp2 is an exogenous RNA oligonucleotide added at the homogenization step with TRIzol to enable RNA isolation quality control. UniSp6, included in the miRCURY LNA RT Kit, is added to the RT reaction to control for cDNA synthesis and PCR efficiency. The numbers 1, 2 and 3 refer to each of the biological triplicate sample (n=3).


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