ExRNA
AN OPEN ACCESS, PEER-REVIEWED JOURNAL FOR INNOVATIVE RESEARCH IN EXTRACELLULAR RNA

Peer Review File
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Reviewer A

General comment

1. By using a modified small RNA-sequencing (sRNA-seq) approach, the investigators discovered a new family of unusually short 12 and 13 nucleotides (nt) ribosomal RNAs (rRNAs), which they designated as dodecaRNAs (doRNAs), in whole commercial cow milk and milk ultracentrifugation fractions (12,000xg, 35,000xg, 70,000xg and 100,000xg). The highest read count was found in the 12,000xg fraction. Interestingly, the abundance of doRNAs exceeded the numbers of the most prevalent milk and milk EV miRNAs (let-7b, miRNA-148a, miRNA-30a). The detection of doRNAs in whole milk and ultracentrifugation fractions of whole milk implies that they are not artefacts produced by the exposure to high gravitational forces.

2. Preliminary evidence indicates that doRNAs are bioactive and may modulate gene expression. The interaction of doRNAs with hnRNP A0, A1 and A2/B1 implicates their functional role in translation regulation and mRNA stability, among other activities as discussed in a previous publication (Lambert M, Benmoussa A, Diallo I, Ouellet-Boutin K, Dorval V, Majeau N, Joly-Beauparlant C, Droit A, Bergeron A, Têtu B, Fradet Y, Pouliot F, Provost P. Identification of Abundant and Functional dodecaRNAs (doRNAs) Derived from Ribosomal RNA. Int J Mol Sci. 2021 Sep 9;22(18):9757).

3. The transfer of doRNAs by EV signalosomes derived from the lactation genome may have an important impact of milk-mediated gene regulation and epigenetic programming of the infant.

4. The discovery of abundant short ribosomal RNAs in milk and EV density fractions is a novel and exciting finding, which is of high interest for milk research with potential impact on milk´s physiology and human pathologies related to the consumption of pasteurized bovine milk.

Specific comments

Methods and Results
Sound and convincing.

Discussion
Comment 1: The authors may discuss the potential interaction of doRNAs in the rRNA biogenesis and the nucleolar assembly, including diverse rDNA intergenic spacers, snoRNAs, circular RNAs, other long non-coding RNAs or even microRNAs according to the recent review of Kaliatsi EG, Giarimoglou N, Stathopoulos C, Stamatopoulou V. Non-Coding RNA-Driven Regulation of rRNA Biogenesis. Int J Mol Sci. 2020 Dec 20;21(24):9738.

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Response 1: We appreciate the Reviewer's suggestion and have added a small paragraph to the Discussion section accordingly. However, we feel that delving too deeply into this matter would be beyond the scope of our current paper, which focuses on milk exRNAs rather than their effect inside the cells. We believe that a more thorough discussion of this topic would be more appropriate for a future publication focused specifically on doRNAs biogenesis and function in cells.

Added text: Moreover, doRNAs might also impact the maturation or degradation of ribosomal RNA by binding to the 5' end of 28S ribosomal RNA [1]. This could interfere with the proper assembly and integrity of nucleolus, affecting the accurate synthesis and processing of rRNAs and, if leading 28S to degradation or edition, potentially modulating the diversity of ribosomal RNA [2]. In turn, this could impact the function of ribosomes and disrupt mRNA translation. Therefore, doRNAs may have an impact on cell viability by affecting rRNA biogenesis [3].

REFERENCES

1. Lambert, M. *et al.* Identification of Abundant and Functional dodecaRNAs (doRNAs) Derived from Ribosomal RNA. *Int J Mol Sci* **22**, 9757 (2021).
2. Lambert, M., Benmoussa, A. & Provost, P. Small Non-Coding RNAs Derived from Eukaryotic Ribosomal RNA. *Noncoding RNA* **5**, 16 (2019).
3. Kaliatsi, E. G., Giarimoglou, N., Stathopoulos, C. & Stamatopoulou, V. Non-Coding RNA-Driven Regulation of rRNA Biogenesis. *Int J Mol Sci* **21**, 9738 (2020).

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Reviewer B

This is an interesting paper in a novel line of research. The manuscript needs to be strengthened by addressing the following concerns before it should be considered for publication.

Comment 1: The EVs should be characterized beyond fractionation by centrifugation, e.g., marker proteins and zeta potential.

Response 1: We thank Reviewer B for their thorough review and valuable input. We agree with the Reviewer that milk EV characterization is extremely important and believe that it should be given the attention it deserves. We have worked for four years to characterize the milk EVs we dealt with in this in-depth study, and the current manuscript is the culmination of this arduous process.

The work in this paper is based on well-established protocols and previous research by the first author of this paper, which included the characterization of the EV subsets studied here at the morphologic, proteomic and transcriptomic levels [1–5].

As you will notice, these works and the present one followed the guidelines set by the ISEV for characterizing EVs in four milk fractions (12,000 g, 35,000 g, 70,000 g and 100,000 g). The methods used include transmission electron microscopy along with dynamic light scattering to study the physical properties of EVs, density measurements using ultracentrifugation coupled with Iodixanol density gradient, high-sensitivity flow cytometry, and nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS), which allowed for in-depth characterization of milk EV proteomics. To further prove that these fractions contained cytoplasm-enclosing phospholipid bilayered membrane vesicles, fluorescent lipophilic carbocyanine DiOC18 (DiR) was used as a marker. Western blot analysis of commonly used EV markers revealed that milk EV fractions were associated with common EV marker proteins, including tumor susceptibility gene 101 (TSG101), Heat Shock Protein 70 (HSP70), ALG-2 interacting protein X (ALIX), Cluster of differentiation 63 (CD63) and xanthine oxidase (XDH). The latter is found in proteins extracted from exosomes and was shown to be specifically enriched in larger EV subsets sedimenting at a lower speed compared to EVs of the 100,000 g fraction [1,3,4].

Additionally, the sequencing data we present here were from the analysis of the same samples on which we performed and reported microRNA profiling in milk and ultracentrifugation pellets [3]. Therefore, all the previous in-depth characterization process of milk EVs that we performed is directly relevant to the results we report in this manuscript.

Finally, please find below our usual quality control (QC) analyses in the form of Western blots of common EV-associated markers, in addition to SDS-PAGE and Coomassie blue staining of the proteins extracted from the pellets used in this study.

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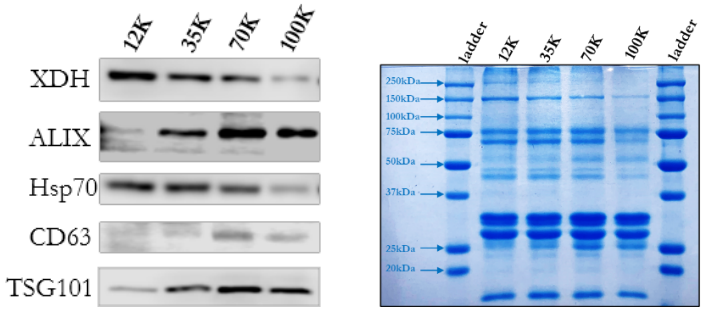


Changes in the text: We made slight modifications to the text to further clarify the depth of characterization that was previously performed.

Added text: Introduction: "To fill this gap in knowledge and after an extensive characterization of milk EVs…"

Comment 2: Biological functions of doRNAs have yet to be established. The reference to a role in cancer seems premature.

Response 2: We agree that much work remains to be done before assigning biological functions to doRNAs. In this paper, we shed light on the 'potential' role of these short RNAs in cancer, as our lab has previously studied these doRNAs in the context of prostate cancer [6]. Using human normal and cancerous prostate tissue samples, it was shown that doRNA, but not C-doRNA, was lower in cancerous tissues compared to normal tissues. Moreover, wound closure assays, using normal and cancer prostate cell lines, showed that overexpression of C-doRNA resulted in a decrease in migration/proliferation in both cancerous and normal prostate cell lines. What we are trying to highlight here (based on the difference of doRNA levels in cancerous vs normal prostate tissues) is that further investigations and more research work from independent groups is warranted. This will help elucidate the biogenesis of these short RNA sequences and highlight their role as potential biomarkers or therapeutic targets.

Changes in the text: Discussion: We revised our manuscript to tone down any claim and make clearer that we deal with possibilities, not certainties. We replaced "bear the risk for promoting prostate cancer" by "might affect the etiology of prostate cancer" and highlighted the speculative character of this hypothesis through this sentence: "This link, although speculative, calls for further investigations to clarify this issue." We also revised the abstract to clarify the possible health implication: ", this could have health implications in adult and infant consumers which warrant further investigations."

Comment 3: To this reviewer's best knowledge, the bioavailability of doRNAs remains to be demonstrated. What is the merit of assessing their abundance in milk if they may not be absorbed? In a manuscript revision it may not be enough to refer to previous publications on the bioavailability of milk EVs, because the authors make the point that they looked at distinct subsets of EVs.

Response 3: It is true that the bioavailability of milk EVs and/or their associated cargos upon oral ingestion remains to be established, despite the intensive study of the subject and very recent evidence of their bioavailability in humans [7]. The controversy is even greater when it comes to the ability of milk EVs to deliver their RNA cargos (like microRNAs or short RNA sequences) at levels sufficient to trigger a change in gene expression. Recently, several papers reported the transfer of milk EVs to mouse tissues upon oral ingestion [8–11]. It is true that, in these papers, the authors focused on what is commonly referred to as "milk exosomes" or, more specifically, those milk EVs sedimenting at high speed (100,000 g to 120,000 g) rather than on all milk EVs including exosomes and those sedimenting at lower speed (12,000 g, 35,000 g or even 70,000 g), as those reported in our study. Whether these studies [8–11] apply to these subsets milk EV subsets remains to be investigated.

We previously reported that the milk EV subsets we study here had the ability to transfer their microRNA content to human cultured cells and regulate reporter gene expression in a specific manner [3]. Moreover, our previous reports highlighted the ability of these four EV subsets to resist simulated human digestion and protect their RNA cargo [2]. While this bioaccessibility and potential bioavailability require further confirmation, we believe the potential to be important enough to report it to the scientific community, as it highlights a new realm of exRNAs.

Moreover, regardless of whether these EVs are being absorbed or not, assessing the abundance of these short RNA sequences or other RNA species in milk might be of great interest when studying the potential of these EVs to be used as RNA therapeutic vehicles through routes of administration other than the oral route [12] or simply as biomarkers of mammary gland disorders or infections for instance, or even for the transfer of this approach to other biological fluids (e.g. plasma, cerebrospinal fluids, etc.) in which the transfer of EVs' RNA content is well established.

Finally, we believe that all scientific discoveries are important to share with the community, as their apparent usefulness or functionality may rapidly evolve over time.

Changes in the text: We modified the Discussion section to highlight the current limited data on the potential for these doRNAs to be bioavailable. We added this sentence: "Additionally, as for other exRNAs in milk, their transfer to recipient cells remains to be fully demonstrated, despite reports supporting this hypothesis (1-3).".

Comment 4: Line 73: The authors overstate their own previous findings (reference 19). Reporter gene knockdown was achieved by transfecting cells with doRNA, which is a far cry from the oral delivery of doRNAs through milk EVs.

Response 4: We agree that, although it is commonly used to study the gene regulatory effects of certain RNA species, transfection has its own limitations when it comes to determining the role of the studied RNAs. Even more so when performed to support the transfer of RNA from bioavailable milk EVs or RNA delivery through oral consumption of milk EVs. For milk EV-contained doRNAs to have a function in consumers following oral consumption, many challenges have to be overcome, from resisting to digestion to efficient absorption, homing to tissues and being transferred to target cells in sufficient amount to regulate gene expression.

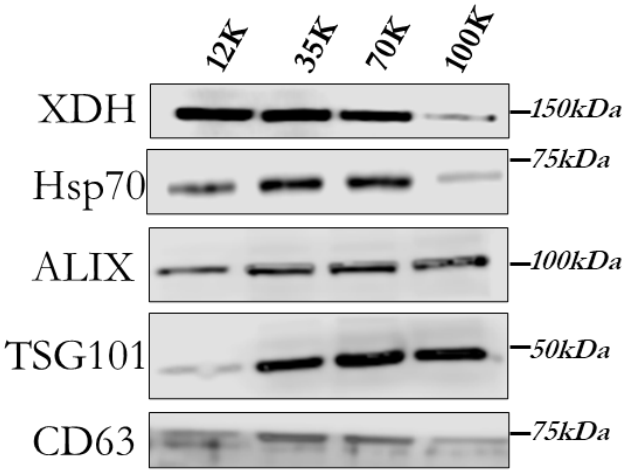
However, the transfer of RNA (specifically microRNAs) from milk EVs to cells *in vivo* (upon oral or intravenous administration; our own unpublished data) or *in vitro* have been reported in different studies [7,9]. In our previous work [3], we have reported an increase in miR-223 levels in cultured human cells upon incubation with either of the four milk EV subsets. It is still, however, very critical to study the ability of these EVs to transfer their RNA cargo to tissues upon oral delivery. We are currently working on replicating this microRNA work to doRNAs and are exploring the bioavailability of milk exRNAs in mice using genetically engineered cow's milk samples. While we have promising preliminary data, we do believe it is best to deal with these issues systematically in a dedicated work to be shared when the results are full and confirmed to be sound.

In any case, we modified our manuscript to tone down any claim of bioavailability and functionality (see previous changes that encompass this comment).

Comment 5: Report any EV authentication experiments performed, and data obtained.

Response 5: As mentioned above, the four milk EV fractions investigated in this study have been thoroughly characterized in four previous publications of the first author. Accordingly, all the studies performed in our lab on these milk EV fractions are mere replications of the same protocol established before and every time validated by Western blotting and DLS quality controls [5]. Moreover, the same protocol was also used to isolate the four EV subsets from raw unprocessed milk and other milk brands. Interestingly, upon characterization by Western blot (EV markers) and transmission electron microscopy, we have found that the four pellet fractions (12,000 g, 35,000 g, 70,000 g and 100,000 g) have the same EV markers enrichment as those found in commercially available skim milk (please see attached below).

Changes in the text: We clarified that the characterization part of this work was extensively performed over the course of 4 years and reported in 4 previous highly detailed manuscripts.



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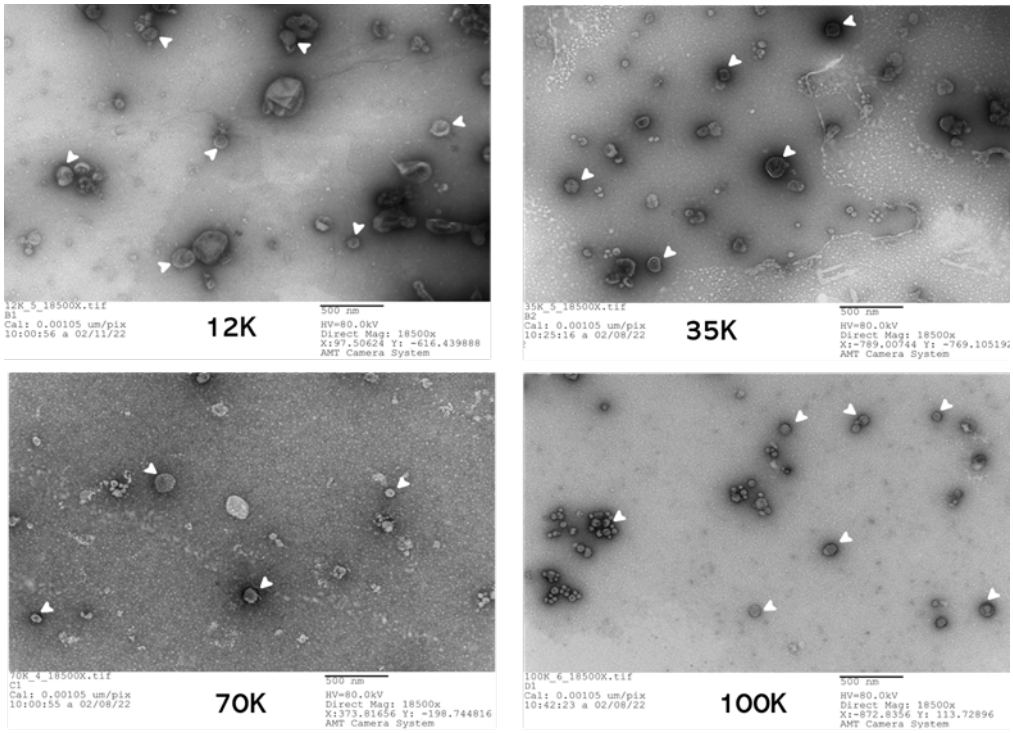


Comment 6: ANOVA: Test for heterogeneity of variances prior to conducting statistical analysis

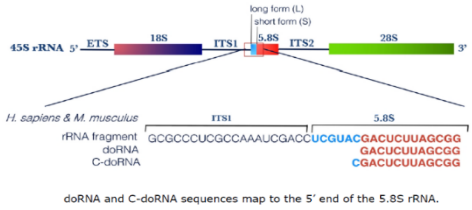
Response 6: Every statistical analysis was performed following prerequisite validations as stated in the methods sections. Both homoscedasticity and normality, among others, were validated before confirming the test selection. For the data of RT-qPCR, the Brown-Forsythe test, for all the comparisons made, showed no significant difference between the variance of the groups, except for graphs in Figures 6b and 6d, in which case the non-parametric equivalent (Kruskal-Wallis ANOVA) was used.

Minor Comments

Comment 1: doRNAs are a rather new concept and need to be clearly defined. This is of particular importance for C-doRNAs, which are jumped at the reader in the abstract without providing any explanation.

Response 1: doRNA and C-doRNA are two short RNA sequences which map to the rRNA 5.8S. They were referred to as dodecaRNAs (doRNAs) based on the number of core nucleotides (12 nt) they contain. doRNA sequence is composed of 12 nucleotides, while its variant C-doRNA contains an additional Cytosine (C) at the 5'end [6,15]. We modified the abstract to bring a smoother introduction to this new concept.

Changes in the text:



doRNA and C-doRNA sequences map to the 5' end of the 5.8S rRNA.

"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)… Cow's milk was abundant in doRNAs and C-doRNAs, a doRNA derivative harboring an additional cytosine (C) at its 5' end."

Comment 2: Why use the qualifier "unusual"? Just call them short RNA or state the length.

Response 2: The word unusually refers to the fact that RNA sequences of such short length are usually discarded and not considered in the studies. However, the word "unusually" was omitted throughout the text to make the whole more readable.

Changes in the text: The word "unusually" was omitted from the text and replaced only by "very short" or its specific length, where applicable.

Comment 3: Define LNA-RT-qPCR when first used in the abstract.

Response 3: Definition added to the Abstract.

Changes in the text: "Results were validated with high-specificity splint-ligated reverse transcription quantitative polymerase chain reaction (RT-qPCR) using high sensitivity locked nucleic acid (LNA) oligonucleotides."

Comment 4. Having single sentences make a paragraph is not great writing (Introduction, Results).

Response 4: We reformatted our work to make it more readable. We aim for each paragraph to convey a message and, sometimes, it is constituted of only one sentence. Further formatting is expected at the proof phase of the manuscript production.

Comment 5: Line 102: This may be journal style, but I found the use of a URL to be unusual. Likewise, for "Illustrations" (line 196) – it seems unusual to state the software used to prepare figures.

Response 5: We added the URL as a direct, easy-to-use link, which provides additional information on how the milk in the study has been processed. To keep the whole neutral, we omitted the said URL in this revision. Concerning the stated software, we strongly support transparency in data preparation and reporting and believe the scientific community should strive to ensure reproducibility in both data collection/processing and reporting. Accordingly, we think it is important to share which tools were used to prepare these figures.

Changes in text: The URL was omitted in "Commercial skimmed filtered dairy milk (PurFiltre, Lactantia, Toronto, Canada) was purchased in a local store in Quebec City, Canada.".

Comment 6: Line 107: Would there be any benefit in depositing the protocol and EV authentication protocols in EV-TRACK? Lots of politics there, it seems and just a point to consider!

Response 6: We thank the Reviewer for its suggestion. We try our best to stay out of any scientific political concerns. In our view, EV-TRACK is an interesting tool meant at helping with reproducibility, which is a goal we support. Accordingly, a detailed version of our protocol for the isolation of milk EVs was made available at the online Bio-protocol platform and our characterization work of these EVs published in the JEV, along with the MISEV checklist. We will explore how we can centralize all this work on EV-TRACK. As we used more than a dozen of different techniques to characterize the EVs we speak of here across 6 manuscripts, this might take time, but we will strive to have it done in the next months to further live by our principles of transparency and reproducibility in science.

Comment 7: Lines 115 and 195: Lower case p in phosphate, and v in value.

Response 7: Corrected in the text.

Changes in the text: … sterile phosphate buffer saline (PBS)… (i.e., 5%, p value below 0.05 considered significant).

REFERENCES

1. Benmoussa, A. *et al.* A subset of extracellular vesicles carries the bulk of microRNAs in commercial dairy cow's milk. *J Extracell Vesicles* **6**, 1401897 (2017).
2. Benmoussa, A. *et al.* Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions. *The Journal of Nutrition* **146**, 2206–2215 (2016).
3. Benmoussa, A. *et al.* Complexity of the microRNA transcriptome of cow milk and milk-derived extracellular vesicles isolated via differential ultracentrifugation. *Journal of Dairy Science* **103**, 16–29 (2020).
4. Benmoussa, A., Gotti, C., Bourassa, S., Gilbert, C. & Provost, P. Identification of protein markers for extracellular vesicle (EV) subsets in cow's milk. *J Proteomics* **192**, 78–88 (2019).
5. Benmoussa, A., Michel, S., Gilbert, C. & Provost, P. Isolating Multiple Extracellular Vesicles Subsets, Including Exosomes and Membrane Vesicles, from Bovine Milk Using Sodium Citrate and Differential Ultracentrifugation. *Bio Protoc* **10**, e3636 (2020).
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12. Somiya, M., Yoshioka, Y. & Ochiya, T. Biocompatibility of highly purified bovine milk-derived extracellular vesicles. *J Extracell Vesicles* **7**, 1440132 (2018).
15. Lambert, M., Benmoussa, A. & Provost, P. A New Specific and Sensitive RT-qPCR Method Based on Splinted 5′ Ligation for the Quantitative Detection of RNA Species Shorter than microRNAs. *Noncoding RNA* **7**, 59 (2021).

1. Benmoussa A, Provost P. Milk MicroRNAs in Health and Disease. Compr Rev Food Sci Food Saf. 2019;18(3):703-22.
2. Melnik BC, Stremmel W, Weiskirchen R, John SM, Schmitz G. Exosome-Derived MicroRNAs of Human Milk and Their Effects on Infant Health and Development. Biomolecules. 2021;11(6).
3. Mutai E, Ramer-Tait AE, Zempleni J. MicroRNAs in bovine milk exosomes are bioavailable in humans but do not elicit a robust pro-inflammatory cytokine response. ExRNA. 2020;2(1):2.

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Reviewer C

Authors describe for the first time the presence of short exRNAs in cow's milk. While the findings are novel and relevant for the field, the discrepancies between RNAseq results and qRT-PCR validation suggest a possible problem in the analysis. This does not invalid the interesting results. Indeed, this reviewer acknowledge the limitations of the study in a paragraph of the discussion section.

Comment 1: Authors suggest that different speed fractions may contain different small RNAs types. Authors must show data of nanoparticle tracking analysis (NTA) of each fraction to confirm this. NTA can be easily performed and must be included. If possible, electron microcopy data could be included.

Response 1: First and foremost, we would like to express our sincere gratitude to Reviewer C for its thorough revision of this manuscript and its valuable input.

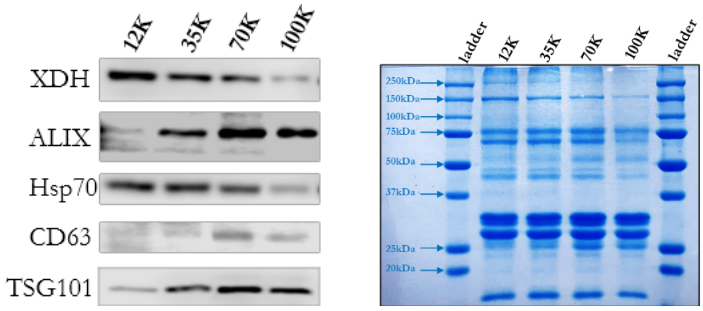
We agree with the Reviewer that characterization is extremely important and believe that it should be given the attention it deserves. We have worked for four years to characterize the EVs in depth leading to 4 characterization manuscripts, and the current manuscript is the culmination of this process.

The work in this paper is based on well-established protocols and previous research by the first author of this paper. The first author's extensive work on milk, and more specifically commercial pasteurized skim milk, included the characterization of new EV subsets at the proteomic and transcriptomic levels[1].

In these works, we did not have access to NTA, but we have used similar techniques and followed guidelines set by the ISEV for characterizing EVs in four milk fractions (12,000 g, 35,000 g, 70,000 g and 100,000 g). Some methods used include transmission electron microscopy along with dynamic light scattering to study the physical properties of EVs, density measurements using ultracentrifugation coupled with Iodixanol density gradient, high-sensitivity flow cytometry with PMT able to detect small particles, and nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS), which allowed for thorough proteomic characterization of EVs. In order to further prove that these fractions contained cytoplasm-enclosing phospholipid bilayered membrane vesicles, fluorescent lipophilic carbocyanine DiOC18 (DiR) was used as a marker. Western blot analysis of commonly used EV markers revealed that milk EV fractions are associated with common proteins, including tumor susceptibility gene 101 (TSG101), Heat Shock Protein 70 (HSP70), ALG-2 interacting protein X (ALIX), Cluster of differentiation 63 (CD63) and xanthine oxidase (XDH). The latter is found in proteins extracted from exosomes and have been shown to be specifically enriched in larger EV subsets sedimenting at lower speed compared to EVs of the 100,000 g fraction [2–4].

Additionally, the sequencing data we present here were from the same samples on which we reported microRNAs profiles in milk pellets [3]. Therefore, all the previous in-depth characterization process we performed is directly applicable to the results we describe here.

For revision purpose, please find below our usual QC controls in the form of Western blot analysis of common EV-associated markers, in addition to an SDS-PAGE and Coomassie blue staining gel of the proteins extracted from the pellets used in this study that we used to ensure the EVs we isolated here are in all ways comparable to those we used in our previous works.



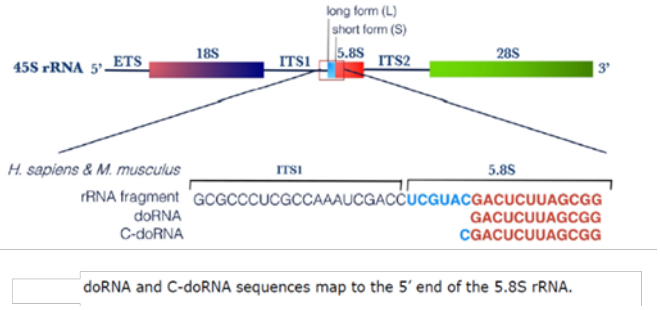
Changes in the text: We modified the text to clearly state that we previously made an extensive characterization of these EVs and that this work is in direct line with the previous ones.

Comment 2: Indicate in the abstract section that there are some discrepancies between the sequencing analysis and the validation analysis that need further research.

Response 2: Thank you for this valuable input. We improved the abstract to highlight these discrepancies.

Comment 3: What is c-doRNA? It is not explained what it stands for

Response 3: doRNA and C-doRNA are two short RNA sequences which map to the rRNA 5.8S. They were referred to as dodecaRNAs (doRNAs) based on the number of core nucleotides (12 nt) they contain. doRNA sequence is composed of 12 nucleotides while its variant C-doRNA is 13-nt in length and it was annotated as C-doRNA because it is composed of the core 12-nt doRNA with an additional Cytosine (C) at the 5'end (please refer to the sequences below)[5,6].



doRNA and C-doRNA sequences map to the 5′ end of the 5.8S rRNA.

Changes in the text: Abstract section, Background subsection: “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.”.

Abstract section, Results subsection: “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.”.

We also modified the abstract to clarify these points from the start.

Comment 4: What is figure 1d? something in missing

Response 4: We apologize for the apparent loss of some characters from the graphs when the manuscript was converted from Word to PDF format for the review process. The complete figure has been added to the manuscript as pictures. We also reported the issue to the Editors.

Changes in the text: Complete figures are now provided.

Comment 5: What is figure 2d? something in missing

Response 5: Please refer to the previous response.

Comment 6: It is suggested to reduce the tone of the last paragraph of the abstract section as this has not been tested experimentally (“with potential health implications….”)

Response 6: We used potential to express potentiality at first, but it seems to be understood as function rather than potential. The sentence was toned down as well as the associated Discussion sections.

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1. Benmoussa, A., Michel, S., Gilbert, C. & Provost, P. Isolating Multiple Extracellular Vesicles Subsets, Including Exosomes and Membrane Vesicles, from Bovine Milk Using Sodium Citrate and Differential Ultracentrifugation. *Bio Protoc* **10**, e3636 (2020).
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3. Benmoussa, A. *et al.* Complexity of the microRNA transcriptome of cow milk and milk-derived extracellular vesicles isolated via differential ultracentrifugation. *Journal of Dairy Science* **103**, 16–29 (2020).
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6. Lambert, M., Benmoussa, A. & Provost, P. A New Specific and Sensitive RT-qPCR Method Based on Splinted 5′ Ligation for the Quantitative Detection of RNA Species Shorter than microRNAs. *Noncoding RNA* **7**, 59 (2021).

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Reviewer D

The paper is rising an interesting problem on the composition of milk in short and very short ribonucleic acids. The arbitrary cut-off value of 16 to discard data from small RNAs sequencing is well presented. By using commercial skim milk, the authors warrant an industrial provider of milk with high standards. In addition, the message of the paper is well-circumscribed regarding the composition. The extraction of total RNA under 250 µL of milk is classic in the field. UniSp2 or 6 are used as spike-in reference. The RT-PCR has been described in previous papers of the authors. The limits of the work are clearly explained.

My opinion is that the paper can be published, but not before properly addressing the major modifications listed below.

Major Modifications

Comment 1: Authors are writing do-RNAs, derived from 5.8S rRNA in DNA code: GACTCTTAGCGG and CGACTCTTAGCGG (line 210).
It would be more helpful for readers to use RNA code: GACUCUUAGCGG or CGACUCUUAGCGG, like shown on Figure-1-g. Or is there any reason to present data like on Figure-1-e – « 45.6 % GACTCTTAGCGG » due to RNA sequencing?

Response 1: First, we wish to thank Reviewer D for their time and their in-depth review. As for this first comment, the main reason is that sequencing provides the data as DNA sequence after reverse transcription of the isolated RNA. Therefore, the dataset made publicly available in support of this manuscript is also as DNA code. While RNA code is better for understanding, DNA code is more applicable to explore the provided dataset without requiring the conversion of the entire sequencing data. We, therefore, replaced DNA code by RNA code in the text to help the reader, while keeping DNA code in both the database and the figure where the sequences are listed to ensure anyone can search the molecules within the provided databases without having to perform conversion.

Comment 2: According to the publication of Garcia-Martin et al. (2022, Nature) and previous works, the GACUCUUAGCGG or CGACUCUUAGCGG do not harbor addressing sequences to exosomes, so the possibility of secretion is not obvious.
I suggest rewriting the hypothesis in line-322 (Along with this observation, the distribution pattern of doRNAs and specific c-doRNA over doRNA ratios across milk fractions suggest that these 12–13 nt exRNAs might be specifically secreted within certain mEVs subsets as reported before for tRNAs, microRNAs and iso-miRNAs (16, 37-39).

Response 2: It is true that the core EXOmotifs (motif sequences believed to govern the sorting of microRNAs into exosomes/EVs) reported in the paper by Garcia-Martin et al. (2022, Nature) are not found in our two sequences (C-doRNA and doRNA). However, though each EXOmotif was common for more than one cell type, there might be other motifs in other cell types (not covered by this paper, like mammary gland cells) that might also affect microRNA sorting into EVs. In fact, this is especially intricate in the case of milk EVs, as their exact cell source is still to be determined (although it is suggested to originate from mammary gland cells of epithelial origin or even from immune cells). Moreover, as these RNAs are very short, it is still unknown if classic addressing

motifs are applicable to their nature. Also, we found in our previous works that C-doRNA and doRNA interact with different proteins. Therefore, it cannot be excluded that it is the ribonucleoprotein complex that is targeted towards specific EVs rather the doRNAs alone [1].

Hence, we believe that the point raised by the Reviewer is of great importance, especially in the context of milk EVs and might help study the origin of microRNAs or other RNA species that are highly enriched in milk EVs. We, therefore, modified our manuscript to highly this issue.
Changes in the text: We added this sentence : "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 makes doRNAs differentially enriched in milk fractions (1-3).".

Comment 3: Did the authors check that the sodium citrate treatment of the milk was sufficient to remove casein but also all protein complexes?
The small RNAs in biofluids can be transported, not only within exosomes, but also at the surface of extracellular vesicles captured by Toll-like receptors (Bosch et al., 2020 Front. Genetics), in macromolecular structures (Turchinovich, A., and Burwinkel, B, 2012 RNA Biol). Your presentation is reducing the problem to small RNA inside exosomes, but this may be understood as an oversimplification.

Response 3: When we first initiated our work on these new milk EV subsets, we hypothesized that what we might have in the ultracentrifugation pellets obtained (12,000 g, 35,000 g and 70,000 g) might be aggregates of protein complexes or aggregates of smaller EVs (like exosomes) carrying the bulk of microRNAs. Hence, we subjected these pellets to proteinase K digestion followed by RNase A treatment, and we observed no significant change in milk miR-125b levels, but a reduction for miR-223. This suggests that our pellets may contain heterogeneous populations of EVs that might carry microRNAs within them or on their surface. Moreover, further purification of milk EVs with Iodixanol density gradient ultracentrifugation showed a peak of microRNAs in IDG fractions enriched in EVs.

Our protocol with sodium citrate is based mainly on the dissociation of casein micelles through chelation of the calcium ions, and hence prevents the micelles from coprecipitating with EVs. Our protocol does not completely deplete EV pellets from casein and other protein complexes, but it allows the isolation of high quantities of EVs sedimenting at low speeds with low coprecipitation of these proteins.

To clarify, we do not think that those EVs are all exosomes, but a complex mix of different EV subsets (see [4] for further details). However, we agree that is still unknown if these doRNAs in milk are like microRNAs, i.e., inside EVs.

Because milk is meant to be ingested and is naturally, highly enriched in RNAses evolved to fight against viral infections, we hypothesize that any naked RNA outside of the EVs in milk in bound to be degraded (a synthetic microRNA added to milk is readily degraded). There is still the possibility of ribonucleoprotein complexes, which might be slightly more resilient and have an impact on the proximal sections of the digestive tract. However, with protein digestion, non-encapsulated RNA is likely to become exposed and be degraded. In the absence of stronger evidence on this issue

specifically related to doRNAs, we worked on our manuscript to bring it in view of the reader.

Changes in the text: We updated our discussion to highly the possibility that these RNAs might be associated to other particles than simply EVs. We added the following two sentences to the Discussion section: “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 (4-6). Accordingly, further exploration of doRNAs resistance to digestion and the exact mechanism of their secretion and transport in milk remains to be fully elucidated.”.

Comment 4: Metabolism of ribonucleic acids.
The cut-off value of SIDT1 capture is not reported by Chen et al (2020, Cell Research). Do you think that milk do-RNA could be captured by SIDT1?

Response 4: The findings reported by Chen et al. (2020, Cell Research) are very interesting. The authors focused on the absorption of naked microRNAs by SIDT1 in the stomach. Though in our work we highlighted EV-associated doRNAs, it is possible that some of these doRNAs exist in protein-bound, unencapsulated form. We have previously reported the existence of many microRNAs in the supernatants obtained after the centrifugation of milk at high speed (100,000 g). If doRNAs are to follow the same patterns, they might possibly exist in a free form released when proteins are digested and hence, they might be candidates for absorption by SIDT1. The authors suggested that the absorption of double stranded RNAs is favored by SIDT1 compared to single stranded RNAs. Our data suggest that doRNA and C-doRNA exist as single stranded molecules sometimes bound by hnRNP proteins. doRNA and C-doRNA may also adopt double-stranded hairpin loop structures that may enhance their stability and absorption, especially under acidic conditions. It is thus of great interest to investigate their potential absorption by SIDT1, especially considering the potential biological functions they may assume. However, the existence of endogenous doRNAs will make this endeavor challenging. One might want to setup a labelling method that does not interfere with SIDT1 absorption and devise a process to allow the study of their transport that includes digestion, while considering they are more labile than double-stranded DNA.

Comment 5: The authors are using a splint bridging sequence and adapter qRT-PCR. Please correct the reference line 158 from Marine et al (2021) to Lambert et al., 2021. Thanks to check every reference for accuracy.

Response 5: We thank the Reviewer for pointing out this issue, which we corrected in the text. We reviewed the references according to the guidelines provided by the Editors and all references should now be in the proper format.

Comment 6: Disregarding very small RNAs in Next Generation sequencing, or when using synthetic miRNA in biological experiments, is frequent. Could you improve your paper by proposing guidelines for handling RNA samples, or do you consider that your work cannot be generalized to all kinds of commercial milk?

Response 6: The issue raised by the Reviewer is very critical. In fact, we have only assessed the

existence and quantities of these two short sequences in one commercial milk type (Lactantia). However, we think that these sequences might also be found in other types of milk. Moreover, these short sequences have been detected in 11 samples derived from six different species [2]. Inclusion of RNA sequences shorter than 16 nucleotides also unveiled the existence of very short bacterial RNAs of 13 nt derived from tRNA in *E. coli* RNA sequencing libraries (Diallo et al., manuscript under revision). Hence, we wish to invite the scientific community to revise the scientific concepts concerning the size window of RNAs that are considered as functional RNAs and not mere degradation products. Looking back at how the scientific community has disregarded microRNAs as being authentic, biologically functional molecules that has delayed their discovery for decades, it is especially important to reconsider the size window of nucleic acids in sequencing analysis. We proposed in the last section of our Discussion ways to study such small RNAs and our very recent manuscript entitled "An expanded landscape of unusually short RNAs in 11 samples from six eukaryotic organisms" provides such guidelines and detailed methods for multiple sample types and species[2].

Changes in text: We added a sentence in the relevant Discussion section to highlight this point and the reference for more details from our latest work on multiple species.

REFERENCES

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