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1 # Feed restriction during the suckling period of ewe Assaf lambs (F0)
2 # modifies milk quality and milk exosomal miRNAome of the filial
3 # generation (F1)

4 Alba Martín[a], Carlos García-Estrada[b], Estela Melcón-Fernández[b], Raffaele Calogero[c],
5 Maddalena Arigoni[c], Fabrizio Ceciliani[d], Cristina Lecchi[d], Mahsa Dehnavi[a], F. Javier
6 Giráldez[a], Sonia Andrés[a*]

7 [a]Instituto de Ganadería de Montaña (CSIC-Universidad de León). Finca Marzanas s/n, E-
8 24346 Grulleros, León, Spain

9 [b]Departamento de Ciencias Biomédicas, Facultad de Veterinaria, Universidad de
10 León, Campus de Vegazana s/n, 24071 León, Spain

11 [c]Department of Biotechnology and Health Sciences, Molecular Biotechnology Center,
12 Università di Torino, 10126, Turin, Italy

13 [d]Università degli Studi di Milano, Department of Veterinary Medicine and Animal
14 Sciences, Lodi, Italy

15

16 [*]Corresponding autor: Sonia Andrés. Instituto de Ganadería de Montaña, CSIC-
17 Universidad de León, Finca Marzanas s/n, 24346, Grulleros, León (Spain). E-mail
18 address: sonia.andres@eae.csic.es

CFU: colony forming units; CPM: counts per million reads; FC: fold change; IBC: impulse bacteria count; IEC: intestinal epithelial cell; IGF1R: insulin-like growth factor-I receptor; lncRNA: long non-coding RNA; MEC: mammary epithelial cells; miRNAs: micro RNAs; ncRNAs: non- coding RNAs; NGS: Next Generation Sequencing; SCC: somatic cell count; UTR: untranslated region.

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19 **ABSTRACT**

20 Feed restriction during the early life of ewe lambs (F0) triggers the transfer of epigenetic
21 marks to the next generation, thus impairing the reproductive performance of F1.
22 However, the effects of this factor on milk production and composition, including its
23 abundance in regulatory miRNA (many of which are contained in exosomes, small
24 vesicles of endocytic origin that play a role in the modulation of immune response of the
25 offspring) has not been tested so far. Therefore, in this study, the replacement ewe lambs
26 (F0) obtained in a previous project (a group of ewes fed milk replacer ad libitum –ADL–
27 vs. a group of ewes restricted –RES– to 62.5% the intake level of milk replacer during
28 the suckling period) were raised under similar post-weaning conditions and mated to
29 obtain the progeny (F1). The F1 female lambs (F1-ADL female lambs born from F0-ADL
30 and F1-RES female lambs born from F0-RES) were also mated to obtain F2. Milk
31 production was controlled during the peak lactation period of F1, and milk samples were
32 obtained for each gland separately to measure chemical composition, somatic cell counts
33 (SCC), and bacteriology. Moreover, exosomes were also isolated from the milk of each
34 gland separately to obtain the miRNAome following a Next Generation Sequencing
35 approach. No significant differences were found in either milk production, chemical
36 composition of milk (fat, protein, lactase), or bacteriology (colony forming units, CFU).
37 However, SCC was reduced significantly in milk samples of F1-RES dairy sheep,
38 whereas the abundance of five miRNAs was also modified. Thus, oar-miR-150, oar-miR-
39 221, oar-miR-23a, oar-miR-27a, oar-miR-376c were all down modulated in F1-RES
40 when compared to F1-ADL. Most of these miRNAs have been found to play a role in
41 biological functions such as development, apoptosis, muscle differentiation,
42 reproduction, or milk production. However, the exosomes extracted from the milk of
43 these sheep (F1-RES) did not affect the production of IL-9 and IL-2 cytokines after in

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44 vitro culture with CaCo-2 cells. This study reveals that nutritional programming events
45 such as feed restriction may drive the abundance of not only SCC but also some milk's
46 bioactive components such as miRNAs, although it is not clear if these changes may
47 modulate the immune response at the intestinal level of the offspring.

48 **Keywords:** feed restriction; nutritional programming; exosomes; miRNA; milk;
49 cytokines

50 INTRODUCTION

51 Nutritional programming is a relatively new concept that seeks to explain the effects of
52 maternal (fetal programming) and early postnatal nutrition (neonatal programming) on
53 the long-term growth and health of the offspring. According to this concept, postnatal
54 early life is a critical window period for the neonate during which nutrition must be
55 adequate to guarantee the correct establishment of DNA methylation; if this process is
56 disrupted by under-nutrition, the arising epigenetic marks (highly stable) may drive the
57 phenotype of the animal along the whole life, even in the absence of the causative factor
58 (e.g., undernutrition). Accordingly, our previous studies have revealed that several
59 metabolic routes are permanently impaired in early feed-restricted lambs (Santos et al.,
60 2018a, 2018b, 2018c, 2018d), thus showing features related to metabolic syndrome such
61 as resistance to insulin (Frutos et al., 2018a, 2018b), increased fat depots (Santos et al.,
62 2018a) and impaired reproductive traits (Santos et al., 2018c) during the adult life (F0).
63 Moreover, we have also observed inter-generational transmission of epigenetic marks
64 triggered in the germline of these animals (F0); consequently, the reproductive
65 performance of the filial female generation (F1) is impaired by undernutrition of F0 dams
66 (Andrés et al., 2021).

67 Feed restriction may also promote changes in non-coding RNAs (ncRNAs) present in
68 colostrum and milk, many of which are contained in exosomes, small vesicles of

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69 endocytic origin present in milk that play a role in the modulation of the immune response
70 of the offspring, as demonstrated in humans and pigs (He et al., 2021; Lapping-Carr et
71 al., 2020; Sun et al., 2016). However, to our best knowledge, there is no information about
72 the composition of the milk produced by early feed-restricted dairy sheep, thus triggered
73 by nutritional programming factors. Given that milk is rich in signal molecules such as
74 exosomes, which can promote a response in the offspring, the differences caused in these
75 vesicles due to nutritional programming events (e.g., early feed restriction of the ewe
76 lambs) must be clarified. In this respect, research into exosomes in ovine livestock may
77 contribute to improve the health and viability of the lambs, thereby decreasing the
78 incidence of some diseases in the newborn lambs (e.g., diarrhea) and the use of
79 antibiotics, which would be aligned with the challenges (Sustainable Food Production)
80 preconized by the European Common Agricultural Policy and the European Green Deal.

81 As stated beforehand, our previous results have demonstrated that postnatal programming
82 events caused by early feed restriction (first month of life of F0) affect postnatal oogenesis
83 in F0 ewe lambs, thus provoking the transmission of epigenetic marks to the progeny
84 (F1), which impairs the reproductive performance (Andrés et al., 2021). Therefore, we
85 hypothesize that the milk of the F1 generation can also be modified by the
86 intergenerational transmission of these epigenetic marks, having not only implications for
87 milk production and quality but also for the sequence of the miRNA (very conserved
88 between mammalian species; Galio et al., 2013) contained in the exosomes that modulate
89 the immunological response of the offspring. To test these hypotheses, the present study
90 was designed to describe milk production, milk quality, and the miRNAome of the milk
91 obtained from the progeny (F1) born from dairy ewes (F0) feed restricted during the
92 suckling period. Moreover, the Caco-2 cell line is an appropriate model to study the
93 regulation of cytokine secretion by intestinal epithelial cells (IEC) since they produce

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94 several cytokines (Vitkus et al., 1998). Therefore, an *in vitro* trial using this cell line was
95 attempted as a proxy protocol to test the potential effects of these exosomes (miRNA)
96 isolated from the milk of F1 (Kar et al., 2021).

97 **MATERIAL AND METHODS**

98 **Care and use of animals**

99 All handling practices involving animals followed the recommendations of Directive
100 2010/63/EU of the European Parliament and the Council on the Protection of Animals
101 used for Scientific Purposes, and the experimental protocols were approved by the IGM-
102 CSIC Animal Experimentation Committee (protocol number 2018-E04). Moreover,
103 according to the three Rs principle (Replace, Reduce and Refine the use of animals under
104 experimental conditions) an *in vitro* protocol with cell cultures (Caco-2) was carried out
105 to test the potential effects of milk exosomes (miRNA) on the immune response of
106 intestinal cells.

107 **Animals, management, and sample collection**

108 All the details of F0 Assaf ewes are fully described in (Santos et al., 2019, 2018c).
109 Briefly, the replacement ewe lambs (F0) were divided into two groups, the first one being
110 fed milk replacer *ad libitum* (F0-ADL) during the suckling period, and the second one
111 (F0-RES) being milk restricted (62.5% of *ad libitum* intake level) during the first 35 days
112 of life. Once weaned, both groups were raised under similar post-weaning conditions
113 (Santos et al., 2018c) and mated to obtain the female progeny (F1-ADL and F1-RES).
114 Then, both groups of newborn Assaf female lambs (F1-ADL and F1-RES) were raised
115 precisely under the conditions described in Andrés et al. (2021). These animals were
116 finally mated, and after lambing, ewes were milked daily. Milk yield was measured after
117 35 days (n=8 per group), and milk samples were collected for each gland separately to
118 determine composition, somatic cell count, and bacteriology. Finally, purification of

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119 microRNAs (miRNAs) of milk-derived exosomes was carried out in three ewes per group
120 (for each gland separately) in order to proceed with miRNome next generation sequence
121 (NGS) profiling as described below.

122 **Milk quality traits, exosome miRNome NGS profiling and pipeline of analysis**

123 Protein, fat, lactose, and total solid content were estimated by infrared spectroscopy
124 (MilkoScan FT6000, Foss Electric, Hillerod, Denmark). Somatic cell count was measured
125 by an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerod, Denmark) and
126 bacteriology by blow cytometry (BactoScan FC, Foss Electric, Hillerod, Denmark). The
127 miRNA extraction of milk-derived exosomes was done using Exoquick™ (SCBI)
128 following the manufacturer's protocol from one mL of milk. Small RNAs, including
129 miRNAs, were isolated with miRNeasy serum/plasma kit (Qiagen Cat No./ID: 217184)
130 according to the manufacturer's protocol. Small RNA libraries were constructed with
131 NEBNext® kit (New England Biolabs, Ipswich, MA, USA). Individually-barcoded
132 libraries were mixed. Pools were size selected on Novex 10% TBE gels (Life
133 Technologies, Carlsbad, CA, USA) to enrich for miRNAs fraction. Sequencing (75 nts
134 single-end) was performed on Illumina NextSeq500 (Illumina, San Diego, CA, USA).
135 The output of NextSeq500 Illumina sequencer was demultiplexed using bcl2fastq
136 Illumina software embedded in docker4seq package (Beccuti et al., 2018). miRNA
137 expression quantification was performed using the workflow previously described (Bardi
138 et al., 2021). In brief, fastq files were quality checked (QC) using FastQC software. Reads
139 shorter than 14 nt were discarded. The QC-passed reads were clipped from the adapter
140 sequences using Cutadapt (Martin, 2011) by imposing a maximum error rate in terms of
141 mismatches, insertions, and deletions equal to 0.15. Sequences were mapped to
142 precursors miRNAs available in miRBase 22.0 (http://www.mirbase.org/). The alignment
143 was performed using BWA (Li and Durbin, 2009) algorithm v. 0.7.12 with the default

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144 settings. Annotation and quantification of miRNAs were done as described (Tarallo et al., 145 2019). The detected counts were organized in a table including all analysed samples. For 146 visualization purposes, only CPM (counts per million reads) were used. Differential 147 expression analysis was evaluated using DESeq2 Bioconductor package (Love et al., 148 2014) implemented in docker4seq package 149 (https://github.com/kendomaniac/docker4seq). Differential expression analysis was done 150 using the above-mentioned counts' table using as threshold an adjusted P-value ≤ 0.1 and 151 an absolute $\log_2$ Fold Change (log2FC) ≥ 1.

152 **Cell culture for cytokine expression**

153 To observe the effect of milk-derived exosomes on IL-2 and IL-9 secretion, we carried 154 out an *in vitro* test on intestinal CaCo-2 cells cultured with Gibco™ DMEM/F-12, 155 HEPES, 10% FBS. The cells were seeded in a 96-well plate (10,000 cells/well), and the 156 exosomes extracted from the milk samples using the Total Exosome Isolation Reagent 157 (from other body fluids) (Invitrogen™) were added at 100 µg/well, with two replicates 158 per sample. The cells were incubated for 72h at 37°C and 5% CO_2. After this time, the 159 plates were centrifuged, and the supernatants were removed to carry out the analysis of 160 IL-9 and IL-2 in a MACSQuant® cytometer using the MACSPlex Cytokyne 12 kit, 161 human (Miltenyi Biotec™), following the manufacturer's instructions.

162 **Statistical analysis**

163 The milk yield of both glands at the peak of lactation (35 days after lambing) was 164 summed for each ewe. The resulting data were subjected to a one-way analysis of variance 165 using the GLM procedure of SAS; diet was included in the model as a fixed effect. Milk 166 composition and quality traits were also subjected to analysis of variance, the model 167 including the fixed effects of treatment (feed restriction –F1-RES– *vs. ad libitum* –F1-168 ADL–), gland and their interaction (treatment × gland); the model also included the

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169 random effect of animal nested to the diet and the interaction gland by animal nested to
170 diet as residual error. The impact of diet was contrasted against animals nested to the diet
171 and the effects of gland or gland by diet against gland by animal nested to the diet.
172 Differences between least square means were determined with the SAS PDIFF option.
173 Somatic cell counts (SCC*1000/ml), individual bacteria count measured like impulse
174 bacteria count (IBC*1000/ml), and colony forming units (CFU*1000/ml) data were
175 previously log-transformed before the analyses.

176 **RESULTS**

177 **Milk production, quality traits and exosome miRNome NGS profiling**

178 Milk production during the peak of lactation was not different due to nutritional
179 programming events in F1 ewes caused by early feed restriction of F0 mothers (1.60 vs
180 1.61 kg for F1-ADL and F1-RES ewes, respectively; RSD=0.750; P=0.972). Chemical
181 composition and quality traits of milk samples obtained during the peak of lactation of
182 F1-ADL and F1-RES dams are shown in Table 1. Only the SCC showed differences
183 between treatments, being significantly reduced in the milk of F1-RES ewes when
184 compared to F1-ADL ewes.

185 As far as miRNome is concerned, and after removing two outtiers, the principal
186 component analysis of the abundances corresponding to the miRNA sequences obtained
187 from the milk exosomes of F1-ADL and F1-RES dams revealed to clear clusters (Figure
188 1) related to the treatment (F1-RES and F1-ADL). Moreover, the differential expression
189 analysis with DESeq2 detected 5 differentially expressed miRNAs in the exosomes of the
190 milk obtained from both groups of ewes (Table 2) . Thus, oar-miR-150, oar-miR-221,
191 oar-miR-23a, oar-miR-27a, oar-miR-376c were all down modulated in F1-RES when
192 compared to F0-ADL.

193 **Effect of milk-derived exosomes on IL-9 and IL-2 secretion by Caco-2 cells**

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194 Incubation of these Caco-2 tumoral cells with milk exosomes (100 µg/well) of F1-
195 ADL and F1-RES ewes for 72 h produced no significant differences in the amount of the
196 cytokines secreted (IL-2 and IL-9) to the culture medium (Figure 2). Therefore, milk
197 exosomes of F1-RES ewes did not seem to exert a role in modulating the production of
198 IL-9 and IL-2 by Caco-2 intestinal cells when compared to the response observed for the
199 F1-ADL group.

200 **DISCUSSION**

201 Epigenetic marks in the germline of early feed-restricted lambs (F0) may be
202 responsible for the intergenerational inheritance (F1) of altered phenotypes (Andrés et al.,
203 2021). In this study, we are focused on the differences observed in the milk of the
204 offspring (F1) born from early feed-restricted dams (F0). According to the results, milk
205 yield during the peak lactation period was not reduced in F1-RES ewes. Additionally,
206 counts on bacteriology (IBC or CFU) and SSC confirm that no animals with clinical
207 mastitis were observed in either of the F1 groups. Therefore, the lower significant values
208 on SCC for F1-RES dairy sheep in both left and right glands might be explained by other
209 factors, such as a reduced exfoliation of mammary epithelial cells (MEC; Herve et al.,
210 2019).

211 Contrary to our data, feed restriction of dairy cows has been associated with an
212 elevated rate of MEC exfoliation and decreased milk yield (Herve et al., 2019). However,
213 it must be considered that our data were measured on the filial generation (F1) obtained
214 from the ewes (F0) having undergone feed restriction; hence, the observed results in F1
215 may be caused by transmitting epigenetic marks through the germline and not directly
216 through the feed restriction *per se*. Accordingly, two hypermethylated genes (e.g.,
217 *MAP2K3* and *MAPK10*) involved in the senescence and apoptosis of MEC, respectively
218 (Yu et al., 2007) were found in F1-RES lambs (Andrés et al., 2021). Therefore, it is

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219 assumed that, as a consequence of this hypermethylation, the expression of both genes
220 was reduced in the MEC of F1-RES ewes. This circumstance might explain, at least
221 partially, the decreasing numbers of SCC in this group. Therefore, our results point
222 towards the role of the intergenerational transmission of nutritional programming events
223 (driven through epigenetic marks of the germline) on the individual variation of SCC
224 observed in a healthy dairy sheep herd.

225 A decreased senescence and/or apoptosis *per se* in the MEC of F1-RES ewes would
226 have promoted an increased milk production of these animals. Nevertheless, milk
227 production was not different in F1-RES ewes, probably because other factors, such as
228 MEC's metabolic activity, were also modified (Herve et al., 2019). In fact, several
229 indicators of metabolic disorders during the replacement period of F1-RES ewes have
230 been reported previously (Andrés et al., 2021), thus in agreement with the numerically
231 lower levels of urea and acetone (together with increased values of BHB, an indicator of
232 ketosis) in the milk during the lactation period of F1-RES ewes (Table 1).

233 Regarding the milk exosomes, the differential abundance of miRNA sequences
234 reaveled two diffential clusters mainly related to the treatment of the progenie (F1-ADL
235 and F1-RES ewes, Figure 1). More concisely, five miRNA amounts were down-
236 modulated in F1-RES dams. Thus, it has been described that oar-miR-150 promotes
237 apoptosis of ovine ovarian granulosa cells by targeting the *STAR* gene (Zhou et al., 2019),
238 so the down-modulation of this miRNA might be an indicator of decreased apoptosis in
239 F1-RES ewes. Remarkably, these results seem to align with a reduced apoptosis of MECs.
240 Moreover, both oar-miR-150 and oar-miR-221 have been related to the reproductive traits
241 of sheep (e.g., follicular and luteal phases and progesterone production; Chen et al., 2022;
242 Zhou et al., 2019); therefore, the down-modulation observed for both miRNAs might also

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243 be in agreement with the impaired reproductive traits observed for F1-RES ewes during
244 the replacement period (Andrés et al., 2021).

245 Some other miRNAs (e. g., miR-23a and oar-miR-27a) have been related to the
246 regulation of mRNA expression of genes associated with milk lipid synthesis in the MEC
247 of dairy goats and cows (Duman et al., 2022; Lin et al., 2013). Thus, our higher numerical
248 values for fat content in the milk of F1-RES ewes, together with the down-regulation of
249 oar-miR-27a, agree with the results described by Duman et al. (2022) in dairy cows, who
250 mentioned that miR-27a inhibits milk fat synthesis by targeting the PPARG gene in
251 bovine MECs.

252 These miRNAs have been associated with milk production, with controversial results
253 in this last case. Thus, Duman et al. (2022) found that oar-miR-23a was upregulated in
254 the low lactating-yield group, whereas Hao et al. (2021) established no differences for
255 miR-23a expression levels in the mammary gland of high lactating-yield sheep when
256 compared to the low lactating-yield sheep. Our results regarding oar-miR-27a neither
257 corroborate those observed by Duman et al. (2022) who described negative correlations
258 with milk protein and lactose content in sheep. Nevertheless, the metabolic syndrome and
259 insulin resistance described previously for F1-RES lambs during the replacement phase
260 (Andrés et al., 2021) seem to be following the down-modulation of oar-miR-376c in this
261 group since it is well-known that oar-miR-376c potentially target the 3′ untranslated
262 region (3'UTR) of IGF1R (Insulin-like Growth Factor-I Receptor; Pokharel et al., 2018).

263 All these miRNAs contained in exosomes are nucleic acids (DNA, mRNA, miRNA,
264 lncRNA, and ncRNA) that can be delivered to the intestine of the offspring (F2) being
265 fed this F1-milk. Therefore, they can alter gene expression and regulate the immune
266 response by producing and releasing cytokines (Ayyar and Moss, 2021) and the integrity
267 barrier function. Accordingly, and aligned with the three Rs principle, an *in vitro* assay

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268 was attempted to check whether exosomes extracted from the milk of F1 ewes could
269 affect IL-9 and IL-2 levels secreted by Caco-2 cells. Caco-2 tumor cells exhibit
270 enterocyte-like characteristics and provide a suitable *in vitro* model to conduct
271 experiments to test enterocyte's local immunological responses. For example, IL-9 plays
272 a role during inflammation by influencing the properties of the intestinal tissue barrier
273 through different tight junction molecules (Gerlach et al., 2015), so it has been related to
274 inflammatory bowel disease. Moreover, IL-9 has a negative impact on the intestinal
275 epithelial barrier function in Caco-2 cells (Li et al., 2018). On the other hand, low doses
276 of IL-2 have been demonstrated to attenuate both the pro-inflammatory cytokine
277 production and disruption of epithelial barrier integrity by restoring tight junction proteins
278 and mucin production and suppressing apoptosis in induced colitis tissues (Lee et al.,
279 2020). Other studies have observed an increase of IL-2 in the intestinal mucosa of patients
280 with inflammatory bowel disease (Niessner and Volk, 2008). In any case, the levels of
281 these cytokines were not altered by the exosomes extracted from the milk of F1-RES ewes
282 when compared to those of the F1-ADL group, thus suggesting a lack of effect on the
283 intestinal cells, and also on offspring's gut.

284 **Conclusions**

285 The results of the present study suggest a role of the intergenerational transmission of
286 nutritional programming events on the individual variation of somatic cell counts
287 observed in the milk of a healthy dairy sheep herd. Moreover, some miRNAs related to
288 biological functions such as development, apoptosis, muscle differentiation,
289 reproduction, or milk production were down modulated in the milk exosomes of the
290 offspring (F1-RES) obtained from early feed restricted dams (F0) as a consequence of
291 nutritional programming events (early feed restriction of F0). However, these exosomes
292 (F1-RES) did not seem to exert a modulation of immune response in intestinal cells.

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299 **Repository with the miRNA sequences uploaded**

300 Primary data of miRNA obtained from the exosomes of milk are deposited on GEO
301 database (GSE266266).

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428 **Table 1.** Chemical composition and quality traits of milk samples obtained during the peak of lactation of F1-ADL and F1-RES ewes

	F1-ADL		F1-RES				*P*-value		
	Left gland	Right gland	Left gland	Right gland	RSD 1	RSD 2	Treatment	Gland	Treat × Gland
Fat (%)	4.79	4.68	4.94	4.94	1.514	0.177	0.827	0.609	0.629
Protein (%)	5.23	5.32	5.22	5.15	0.737	0.046	0.852	0.684	0.038
Lactose (%)	4.70	4.89	5.05	4.97	0.237	0.105	0.183	0.427	0.092
TS (%)	15.8	16.0	16.3	16.1	0.936	0.159	0.586	0.786	0.092
Urea	499	511	397	396	156.8	34.16	0.297	0.794	0.758
Acetone	0.127	0.193	0.080	0.090	0.2356	0.0275	0.611	0.074	0.149
SCC ln(1000 /ml)	5.75	5.07	4.42	4.51	0.43	0.88	0.019	0.594	0.491
IBC ln(1000/ml)	2.86	2.35	2.62	2.23	0.78	0.442	0.710	0.153	0.816
CFU ln(1000/ml)	1.90	1.53	1.66	1.52	0.446	0.338	0.643	0.268	0.586

429 TS: total solids, SCC: somatic cells count; IBC: impulse bacteria count; CFU: coloning forming units

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431 **Table 2.** Differentially expressed miRNA (milk exosomes of F1-ADL and F1-RES ewes) passing the threshold $P_{adj} \leq 0.1$ and |log2FC| >= 1

	Base Mean	log2FoldChange	lfcSE	stat	*P*-value	P_{adj}
oar-miR-150	318.6	1.35	0.323	4.178	2.94E-05	0.006
oar-miR-221	38.0	1.19	0.306	3.874	> 0.001	0.011
oar-miR-23a	4725.8	1.06	0.286	3.718	> 0.001	0.014
oar-miR-27a	125.0	1.21	0.346	3.480	> 0.001	0.027
oar-miR-376c	5.50	1.38	0.421	3.277	0.001	0.044

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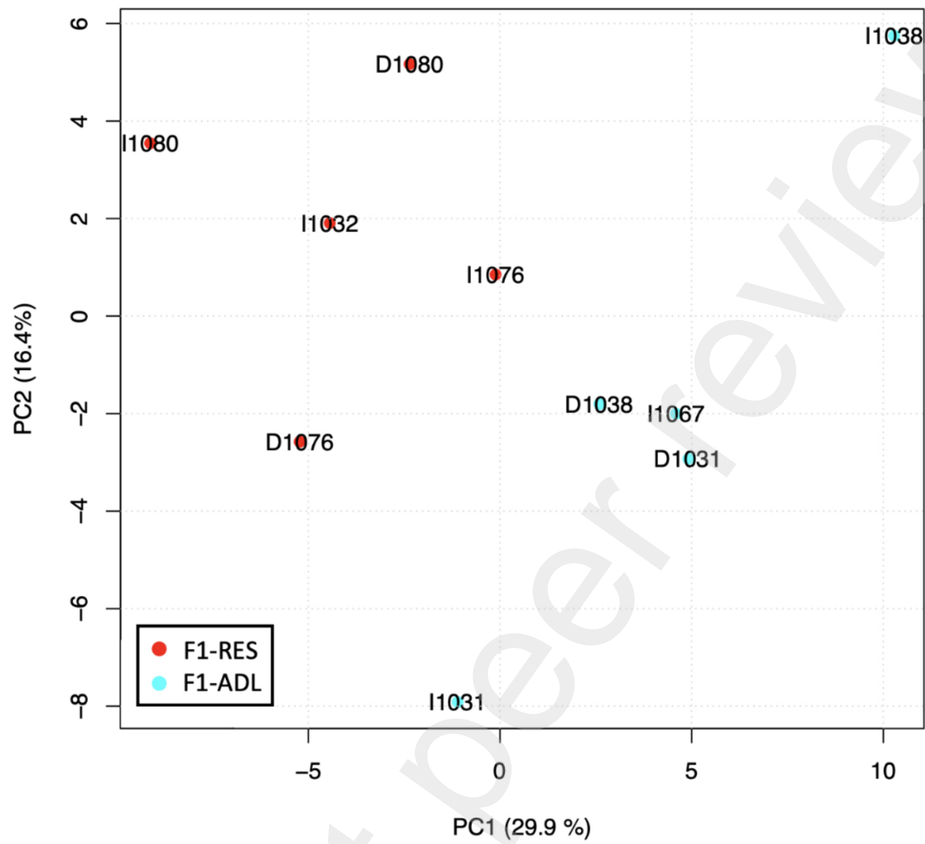
434 **Figure 1.** Principal component analysis of the abundances corresponding to the miRNA

435 sequences obtained from the milk exosomes of F1-ADL and F1-RES ewes.



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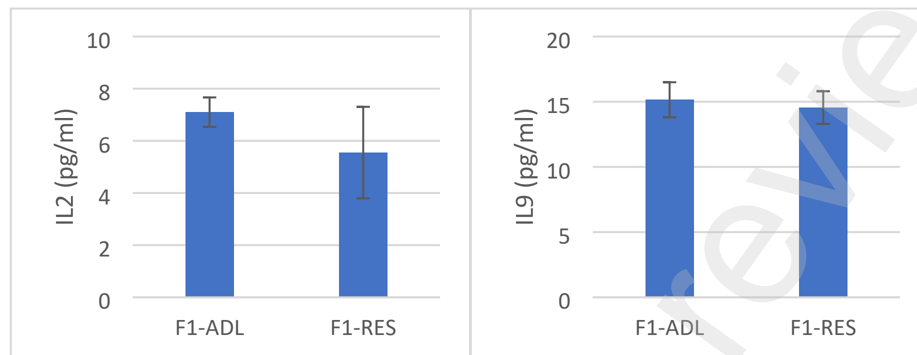
437 **Figure 2.** Impact of exosome addition obtained from the milk of F1-RES and F1-ADL
438 ewes on IL-9 and IL-2 secretion by Caco-2 cells. After 72 h in the presence of exosomes,
439 culture supernatants were collected and the levels (pg/ml) of IL-9 and IL-2 were
440 determined by flow cytometry.

441



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