Co-expression of Bovine leukemia virus and Bovine foamy virus-derived miRNAs in naturally infected cattle

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Abstract

MicroRNAs (miRNAs) derived from viruses play a central role in modulating the expression of both viral and host gene, facilitating immune evasion, viral persistence, and pathogenesis. Among the viruses that encode miRNAs and infect cattle such as Bovine leukemia virus (BLV), Bovine foamy virus (BFV), Bovine herpesvirus type 1 (BoHV-1), and Bovine herpesvirus type 5 (BoHV-5), BLV has gained increasing attention due to the critical role that BLV-miRNAs may play in inducing leukemia and lymphoma in infected animals. BLV is highly prevalent in the Americas and has a negatively affects dairy herds, primarily due to restrictions on the commercialization of dairy products from infected animals and a decrease in milk production.

Mixed infections involving BLV and BFV appear to be common in cattle. Considering the ability of foamy viruses to cross species barriers, preventing their presence within the food chain is essential. In this study, through small RNA sequencing, we identified the co-expression of seven BLV-derived miRNAs (*blv-miR-B1-3p*, *blv-miR-B2-5p*, *blv-miR-B2-3p*, *blv-miR-B3-5p*, *blv-miR-B3-3p*, *blv-miR-B4-3p,* and *blv-miR-B5-5p*) and three BFV-derived (*bfv-miR-BF1-5p*, *bfv-miR-BF1-3p*, and *bfv-miR-BF2-5p*) in naturally BLV-infected cattle, while no BoHV-1/5 miRNAs were detected. A differential expression analysis revealed seven bovine miRNAs (*bta-miR-375*, *bta-miR-133a*, *bta-miR-677*, bta-miR-1, *bta-miR-3613a*, *bta-miR-9-5p*, and *bta-miR-95*) with altered expression levels (fold change > |1.5| and q-value < 0.05) between cows with a high BLV proviral load (PVL) and uninfected counterparts. A comprehensive functional analysis of protein-protein interaction networks for genes targeted by both viral and host-derived miRNAs highlighted key pathways implicated in tumorigenesis and the regulation of the immune response. Notably, *bta-miR-375*, previously proposed as an early biomarker for the progression of enzootic bovine leukosis progression, exhibited differential expression in cows with elevated PVL, reinforcing its diagnostic potential. The functional interaction among miRNAs derived from BLV and BFV raises important concerns regarding their potential influence on disease severity and the increased dissemination of BFV. The findings derived from this study offer new perspectives for creating diagnostic and treatment approaches to manage viral persistence and tumor growth in cattle.

Introduction

The study of virus-host interactions has traditionally focused on how viral proteins modulate host proteins to hijack cellular processes in favor of viral replication and pathogenesis (Ruivinho and Gama-Carvalho, 2023). However, like their host cells, many viruses encode regulatory non-coding RNAs, including small RNAs such as microRNAs (miRNAs), which provide new capacities for regulating host gene expression to ensure successful viral infection (Sullivan and Ganem, 2005; Samols et al., 2005; Grundhoff and Sullivan, 2011; Guo and Steitz, 2014). miRNAs are RNA fragments, typically 19–24 nucleotides (nt) long, that fine-tune gene expression at the post-transcriptional level (Bartel, 2004; Griffiths-Jones et al., 2006). The canonical biogenesis of a mature miRNA begins with the transcription of a precursor in the cell

nucleus mediated by RNA polymerase II. This precursor, known as pri-miRNA, is processed by the RNase III enzyme Drosha into a characteristic stem-loop structure called pre-miRNA (approximately 60 nt). Once exported to the cytoplasm by Exportin-5, the loop of the pre-miRNA is cleaved by the RNase III-like enzyme Dicer, yielding a mature miRNA duplex of approximately 20 nucleotides. The regulatory function of miRNAs is primarily mediated by the RNA-induced silencing complex (RISC), which facilitates base-pairing between the mature miRNA´s 2–7 nucleotide seed region and the 3' untranslated region (3'UTR) of the target messenger RNA (mRNA) (Kim, 2005; Bartel, 2009). The miRNA-mRNA interaction induces mRNA degradation by cellular endonucleases or translational repression (He and Hannon, 2004; Bartel et al., 2009). It is estimated that miRNAs may regulate at least 60% of the human coding genome (Friedman et al., 2009; Bartel et al., 2018).

Host-derived microRNAs (miRNAs) regulate viral infections by exhibiting a dual nature that can benefit either the virus or the host. For example, host miRNAs that directly target viral RNA often enhance the viral life cycle. Conversely, host miRNAs can exert an indirect negative effect on the virus by targeting mRNAs that encode essential host factors necessary for the viral cycle or for establishing immune responses and defense mechanisms (Guo and Steitz, 2014; Girardi et al., 2018). Given their high versatility in fine-tuning gene expression and their small coding size, it is not surprising that viruses have evolved the ability to express their own miRNAs (Skalsky and Cullen, 2010). Since the first report of miRNAs encoded by the Epstein-Barr virus (EBV) (Pfeffer et al., 2004), miRNAs encoded by DNA viruses have proven to be the most abundant, particularly those expressed by the Herpesviridae family. In contrast, RNA viruses that express miRNAs are far less common and are typically restricted to those with a DNA intermediate. Additionally, several miRNA-like molecules have been identified in both positive and negative-sense RNA viruses (Pfeffer et al., 2005; Sullivan et al., 2005; Seo et al., 2008; Ghosh et al., 2009; Hussain et al., 2012, 2014; Liang et al., 2014; Kinkaid et al., 2012, 2014; Whisnant et al., 2014; Yao et al., 2014; Teng et al., 2015; Cao et al., 2018; Hashimoto-Gotoh et al., 2020; Aso et al 2021).

Usually, viral miRNAs follow the same biogenesis and effector pathways as host miRNAs. However, certain retroviruses, such as members of the Spumaretrovirinae subfamily (e.g., Simian foamy virus (SFV) and Bovine foamy virus (BFV)) and the Orthoretrovirinae subfamily (e.g., Bovine leukemia virus (BLV)), decouples miRNA transcription from genomic transcription by employing RNA polymerase III instead of RNA polymerase II (Kinkaid et al., 2012, 2014; Whisnant et al., 2014). This mechanism likely prevents the auto-degradation of viral genomes mediated by endonucleases during miRNA biogenesis. (Hartwig, 2014; Aguado and tenOever, 2018).

Generally, the functional role of viral miRNAs involves the up-regulation or down-regulation of viral and host protein expression to regulate the viral life cycle (e.g., promoting or inhibiting cell proliferation and apoptosis), evade the host immune response, and establish latent or persistent infections (Seo et al., 2008; Cullen, 2009; Grundhoff and Sullivan, 2011; Cullen, 2013; Tycowski et al., 2015). This is particularly evident in retroviruses and herpesviruses, where viral protein expression is strongly restricted

during latency. The production of a low-antigenicity regulatory molecule provides a significant advantage for viral persistence while evading host immune detection (Skaslky and Cullen, 2010; Pluta et al 2020).

Viruses that encode miRNAs and infect cattle include BLV (Kinkaid et al., 2012), BFV (Whisnant et al., 2014), Bovine herpesvirus 1 (BoHV-1) (Glazov et al., 2010), and Bovine herpesvirus 5 (BoHV-5) (Tang et al., 2014). BoHV-1 encodes at least ten miRNAs, two of which have been implicated in maintaining latency both *in vivo and in vitro* (Jaber et al., 2010; da Silva and Jones, 2012). In contrast, the functional roles and target genes of the three BFV-derived miRNAs remain poorly understood (Cao et al., 2020). Among BLV-encoded miRNAs, blv-miR-B4-3p is the most extensevely studied. It shares a seed sequence with miR-29a, an oncomiR that targets tumor suppressors such as peroxidasin homolog (*PXDN*) and HMG-box transcription factor 1 (*HBP1*), as reported in murine tumor-induction studies (Han et al., 2010; Santanam et al., 2010). *In vitro* reporter assays have demonstrated that *blv-miR-B4-3p* exerts a repressive effect on the expression of *HBP1* and *PXDN*. However, a negative correlation between *blv-miR-B4-3p* and *HBP1* expression has been documented, while levels of *PXDN* remained unchanged in ovine primary tumor B cells (Kinkaid et al., 2012; Rosewick et al., 2013). Additionally, recent findings indicate that *PXDN* expression is significantly downregulated in naturally BLV-infected cattle expressing *blv-miR-B4* when compared to their non-infected counterparts. Notably, *miR-29a* expression remains unaffected in both groups (Petersen et al., 2023).

The BLV prevalence reaches up to 85% in North American at farms-level and over 80% in lactating cows of Argentina's main dairy region (VanLeeuwen et al., 2005; 2006; NAHMS, 2007; Gutierrez et al., 2012). International trade restrictions of livestock products from affected herds impact negatively the economy (OIE, 2012), but greater losses stem from reduced milk production and earlier culling of asymptomatic BLV carriers compared to BLV-free herds (Erskine et al., 2012; Bartlett et al., 2013).

Mixed infections involving BLV and BFV appear to be common in cattle (Materniak et al., 2019). Given the ability of foamy viruses (FVs) to cross species barriers and cause zoonotic infections in humans, their presence in the food chain may be a potential risk to both human and animal health. Furthermore, cattle infected with BLV are more susceptible to co-infections with BoHV-1 (de Brun et al., 2023; Mendez et al., 2023; Oviedo-Pastrana et al., 2024).

This study identified the expression of three BFV-derived miRNAs and seven BLV-derived miRNAs in naturally BLV-infected cattle through small RNA analysis sequencing. No miRNAs from BoHV-1/5 were found. Seven bovine miRNAs showed differential expression between cattle infected with BLV/BFV and those uninfected with BLV. A functional analysis of the protein-protein interaction network for genes potentially targeted by both viral and host-derived miRNAs revealed key metabolic pathways associated with tumorigenesis and immune response regulation.

Methods

Selection of Animals

Briefly, adult cows (over 3 years old, >1 lactation) were sampled from the central region of Argentina, an area known for its high dairy farming activity, where the average individual prevalence of BLV exceeds 80% (Gutiérrez et al., 2012). Cows were screened over a 10-month period (T-10, T-5, T-3, T0 months before final sampling) to evaluate both antibody levels and proviral load using anti-BLV ELISA and quantitative PCR (qPCR) assays, respectively (details provided later). All sample collection and handling procedures adhere to the recommendations and guidelines of the Institutional Committee for the Care and Use of Experimental Animals at the Instituto Nacional de Tecnología Agropecuaria.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Fresh blood samples from animals were collected via jugular venipuncture and supplemented with EDTA (225 μM). Peripheral blood mononuclear cells (PBMCs) were isolated on the same day of collection using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, following the manufacturer's protocol. The plasma fraction was kept for anti-BLV ELISA serology. After isolation, PBMCs were preserved in RNAlater solution (Ambion, Austin, TX) and stored at −80°C until further use.

Serology

The anti-BLV ELISA assay, as described by Trono et al. (2001), was employed to identify BLV-infected animals. The whole BLV viral particle served as the antigen for detecting plasma anti-BLV antibodies in each sample. Briefly, a sample-to-positive (S/P) ratio, referred to as the percentage of reactivity (PR), was calculated using the following formula: $S/P = [(OD_{Sample} - OD_{WS-}) / (OD_{WS+1/7} - OD_{WS-})] \times 100$, where OD refers to optical density, WS- indicates the negative serum, and WS+1/7 is the international standard weak positive control serum (diluted 1:7 in negative serum). Samples with a PR greater than 25% were considered positive (BLV(+)), while those below were regarded as negative (BLV(-)).

BLV proviral load (PVL) Quantification

Genomic DNA was extracted from PBMCs using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany) following the manufacturer's instructions. The quality and concentration of genomic DNA from whole blood samples, extracted with the Blood Genomic DNA AxyPrep™ kit (Axygen Biosciences, Union City, USA), were assessed using a microvolume spectrophotometer (NanoDrop™ Technologies, Inc., Wilmington, USA).

A BLV POL gene-based PVL qPCR assay based on the SYBR Green dye detection system was conducted as described by Petersen et al. (2018). Each 25 μL qPCR reaction contained Fast Start Universal SYBR Green Master Mix (2×; Roche), forward and reverse primers (800 nM; BLVpol_5f:5′-

178 CCTCAATTCCCTTTAAACTA-3′ and BLVpol_3r:5′-GTACCGGGAAGACTGGATTA-3′; Thermo Fisher
179 Scientific), and 200 ng of genomic DNA template. Amplification and detection were carried out using a
180 Step One Plus system (Applied Biosystems, Foster City, CA). The specificity of each BLV-positive
181 reaction was confirmed by melting temperature dissociation curve (Tm) analysis. Based on the
182 assumption of a low natural infection rate (1% of BLV-infected cells in the peripheral blood), PVL values
183 below 1,500 copies/µg of total DNA were classified as low, while values above this threshold were
184 classified as high (Hopkins and DiGiacomo, 1997).
185
186 **Small RNA Sequencing**
187 Total RNA was extracted from PBMCs using the miRNeasy kit (Qiagen, Hilden, Germany) according to
188 the manufacturer's protocol. RNA quality (integrity) and concentration were assessed via digital
189 electrophoresis on an Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, US).
190 The QIAseq miRNA Library Kit (Qiagen, Hilden, Germany) was used for miRNA sequencing library
191 construction, which integrates Unique Molecular Indices (UMIs) tags into the adapters during the cDNA
192 synthesis step. These UMI tags help to mitigate PCR amplification biases and sequencing artifacts by
193 allowing identification and collapsing reads with the same amplification origin. A specific 8-nt barcode
194 was assigned to each sample for multiplexing. The multiplexed libraries were pooled to an equal molar
195 concentration and processed in a NovaSeq run (Illumina, San Diego, US) with a 75x50 bp configuration
196 (300–400 million reads pairs per lane).
197
198 **Small RNA sequencing reads processing and deduplication**
199 The quality control of sequencing reads was performed sequentially to ensure reliable reads for
200 unbiased miRNome identification and quantification. The 12-nt UMI pattern was extracted from each
201 read using the umi_tools v1.1.6 software (Smith et al., 2017). Forward (Fw) and reverse (Rv) reads were
202 processed independently before collapsing. Reads with low quality (quality cutoff < 30), a minimum
203 length < 16 nt, the presence of ambiguous bases (N) or adapter sequences were removed using
204 Cutadapt v5.0 (Martin, 2011).
205 Singleton reads resulting were discarded, and only paired Fw and Rv reads were considered. Forward
206 read sequences were aligned to the Rv-complement and merged into a single consensus read using
207 BBMap v25.85 (Bushnell, 2014) with a minimum insert size of 16 nt and a minimum overlap of 18 nt.
208 After merging, Qiagen-specific adapter sequences were removed using Cutadapt v5.0 (Martin, 2011).
209 A stringent mapping strategy was employed for read deduplication using Bowtie2 v2.5.4 (Langmead and
210 Salzberg, 2012). Reads were aligned to the bovine reference genome ARS-UCD1.2 (acc.
211 GCF_002263795.1) and to viral genomes encoding miRNAs that infect cattle, including BLV (acc.
212 NC_001414.1), BFV (acc. NC_001831.1), BoHV-1 (acc. NC_063268.1) and BoHV-5 (acc.
213 NC_005261.3). No mismatches were allowed in the first 18 bases of the read (left end). After alignment

214 to the reference sequences, reads were deduplicated based on their mapping coordinates and the
215 previously extracted UMI tags using UMICollapse v1.0.0 (Liu, 2019).
216
217 **miRNAs identification and quantification**
218 Deduplicated reads were mapped to the reference precursor miRNA sequences for bovine, BLV, BFV,
219 BoHV-1 and BoHV-5 obtained from miRBase v22.1 (Kozomara et al., 2019). Mature miRNA expression
220 was quantified using miRDeep2 v0.1.3 (Friedländer et al., 2012) with default parameters, generating a
221 count matrix of miRNA expression for each sample.
222
223 **Differential miRNA Expression analysis**
224 The raw count matrix for all samples was analyzed using DESeq2 v1.44.0 (Love et al., 2014) to assess
225 differential miRNA expression between BLV-infected and non-infected animals. Based on miRNA
226 expression profiles, an exploratory analysis of variation patterns between infected and non-infected
227 animals was performed using principal component analysis (PCA). To ensure homoskedasticity of the
228 data, raw miRNA counts were transformed using the Variance Stabilizing Transformation (VST) method
229 implemented in DESeq2 v1.44.0. Pairwise distances between samples were calculated based on the
230 root-mean-square deviation of miRNA expression levels.
231 To test for differential miRNA expression, the DESeq2 model adjusts miRNA counts by accounting for
232 sample-specific differences, such as library size, sequencing composition, and miRNA-specific biases,
233 using negative binomial models. Under the null hypothesis, the model assumes that a miRNA has the
234 same mean expression across the two conditions (BLV(-) and BLV(+) groups). The statistical
235 significance of the Log_2 fold change (Log_2FC) between conditions was assessed using the Wald test.
236 Correction for multiple comparisons was performed using the Benjamini-Hochberg false discovery rate
237 (BH-FDR) method (Benjamini and Hochberg, 1995). The miRNAs with BH-FDR-adjusted p-values (q) <
238 0.05 and a $Log_2FC > |1.5|$ were considered statistically significant. The PCA and Volcano plots were
239 performed under the R environment (R Core Team, 2024).
240
241 **miRNAs gene target prediction**
242 Significant differentially expressed bovine and viral-derived miRNAs between BLV(+) and non-infected
243 cows were used to predict potential bovine gene targets *in silico* (miRNA:mRNA interactions) using
244 miRanda v3.3 with default parameters (Enright et al., 2003). The analysis utilized 3´-UTR sequences of
245 21,400 bovine genes from the ARS-UCD1.2 annotation (Rosen et al., 2020). The miRanda algorithm
246 evaluates potential gene targets based on seed-site alignment and the thermodynamic stability of RNA-
247 RNA duplexes. Predicted targets were filtered using a score threshold of ≥140 and a free energy ≤−20
248 kcal/mol.
249
250 **Gene ontology and graph-based pathway analysis of miRNA target genes**

251 The functional annotation of the potential target genes was carried out using STRINGdb (Szklarczyk et
252 al., 2021) with Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG)
253 pathways (Kanehisa and Gotoh, 2000). Next, a protein-protein interaction (PPI) network of the identified
254 target genes was constructed using STRINGdb. This platform integrates curated and predicted
255 interactions from multiple sources, including experimental evidence, computational predictions, scientific
256 literature, co-expression data and primary public databases. Highly interconnected protein clusters within
257 the network were identified using the Markov Cluster Algorithm (MCL) with default parameters (inflation
258 parameter = 2.5). Only clusters with more than 15 nodes (genes) were considered (Dongen et al., 2008).
259 GO terms and KEGG pathways overrepresentation analysis was performed on the identified clusters
260 using Fisher's exact test based on the hypergeometric distribution, using a term similarity threshold > 0.7
261 (Tavazoie et al., 1999). Wordclouds and Treemaps plots of significant GO terms grouped by semantic
262 similarity were obtained using the rrvgo package with default parameters (Sayols, 2023).
263

264 **Results**

265 In this study, four animals consistently exhibited both high antibody reactivity across all sampling times
266 (5671 = 178.6 ± 28.4; 5841 = 191.3 ± 32.8; 6021 = 169.8 ± 42.7 and 6097 = 183.2 ± 25.4) and high
267 proviral loads at T-3 and T0 (average proviral load: 5671 = 63,148.0; 5841 = 44,234.3; 6021 = 52,902.4
268 and 6097 = 91,255.4). These cows were classified as BLV(+). In contrast, three animals that consistently
269 tested negative for both anti-BLV ELISA and qPCR were classified as BLV(-)
270 The quality of extracted total RNA was assessed using the RNA Integrity Number (RIN), which ranged
271 7.8 to 9.6 across all samples. Small RNA sequencing libraries were then constructed by incorporating
272 adapters with UMIs tags. A multi-step processing pipeline was performed to obtain high-quality, non-
273 redundant reads for unbiased downstream analyses. A descriptive characterization of each sample and
274 the number of sequencing reads before and after quality control is summarized in Table 1.
275 Demultiplexed reads were mapped to the reference miRNA sequences of bovine, BLV, BFV, BoHV-1
276 and BoHV-5 (n = 1060) to identify and quantify the miRNAs present in each sample. On average, 351
277 miRNAs (with mapped reads ≥ 5) derived from both bovine and viral sources were identified and
278 quantified across all samples (Supp. Table S1). A multivariate analysis of sample distances based on the
279 normalized miRNA expression matrix revealed that BLV(-) samples clustered together, forming a distinct
280 group separate from BLV(+) samples. PC1 explains 62% of the variance in the expression matrix, while
281 PC2 accounts for 17% (Figure 1).
282 In the BLV(+) group, a total of 10 viral-derived miRNAs were consistently expressed, including 7 from
283 BLV: *blv-miR-B1-3p* (avgexp = 27,681.1), *blv-miR-B2-5p* (avgexp = 59,058.0), *blv-miR-B2-3p* (avgexp =
284 112.5), *blv-miR-B3-5p* (avgexp = 309.6), *blv-miR-B3-3p* (avgexp = 49,312.2), *blv-miR-B4-3p* (avgexp =
285 65.1), and *blv-miR-B5-5p* (avgexp = 951.7). Additionally, 3 BFV-derived miRNAs were identified: *bfv-*
286 *miR-BF1-5p* (avgexp = 6,844.7), *bfv-miR-BF1-3p* (avgexp = 8,257.3), and *bfv-miR-BF2-5p* (avgexp =

287 14,920.6). In contrast, viral-derived miRNAs expression was low (avgexp < 30.0) or undetectable in the
288 BLV(-) group, except for *blv-miR-B2-5p* (avgexp = 67.4) and *blv-miR-B3-3p* (avgexp = 49.8) (Table 2).
289 Differential expression analysis of host miRNAs, identified a total of seven bovine miRNAs (bta) with a
290 fold change (FC) > |1.5| and a q-value < 0.05 were considered differentially expressed (BTA-miRNAs-
291 DE) between the BLV(+) and BLV(-) groups: *bta-miR-375* (FC = 8.87, q-value = 0.00003), *bta-miR-133a*
292 (FC = 4.14, q-value = 0.0007), *bta-miR-677* (FC = 8.22, q-value = 0.01), bta-miR-1 (FC = 7.31, q-value =
293 0.01), *bta-miR-3613a* (FC = 3.83, q-value = 0.01), *bta-miR-9-5p* (FC = 3.83, q-value = 0.04) and *bta-*
294 *miR-95* (FC = 5.09, q-value = 0.04) (Figure 2; Supp. Table S2).
295 To evaluate the potential functional impact of these seven differentially expressed bta-miRNAs and the
296 ten viral-derived miRNAs, a target gene prediction study was conducted, identifying a total of 1,911
297 potentially regulated genes. Of these, 379, 1,046 and 315 were exclusively targeted by BTA-miRNAs-
298 DE, blv-miRNAs, and bfv-miRNAs, respectively (Supp. Table S3). A low level of overlap was observed
299 among the potential target genes, with only seven genes shared across all three miRNA groups:
300 Synaptotagmin (*SYT12*), N-alpha-acetyltransferase (*NAA60*), G-protein-coupled receptor kinase 5
301 (*GRK5*), Potassium inwardly rectifying channel subfamily J member 9 (*KCNJ9*), Zinc finger and BTB
302 domain containing 38 (*ZBTB38*), Methyl-CpG binding protein 2 (*MECP2*), and Paternally expressed
303 gene 10 (*PEG10*). Meanwhile, 69, 20, and 75 genes were shared between BTA-DE – BLV, BTA – BFV,
304 and BLV – BFV, respectively (Figure 3 and Supp. Table S3).
305 Functional annotation of the 1,911 potential target genes was conducted using Gene Ontology (GO)
306 terms and metabolic pathways, revealing 94 significantly overrepresented GO biological process (BP)
307 terms, 18 GO molecular function (MF) terms and 37 GO cellular component (CC) terms. The word cloud
308 visualizations for the enriched GO terms in the GO BP and GO MF categories are shown in Figure 4, the
309 most frequent occurring words for the overrepresented processes and functions included "Regulation,"
310 "Process," "Response," "Binding," "Activity," and "Kinase," among others. The overrepresentation
311 analysis of KEGG and Reactome metabolic pathways revealed five significant terms: "*Ras signaling*
312 *pathway*" (bta04014, q = 0.04), "*Pathways in cancer*" (bta05200, q = 0.02), "*Oxytocin signaling pathway*"
313 (bta04921, q = 0.024), "*Morphine addiction*" (bta05032, q = 0.02), and "*Cushing syndrome*" (bta04934, q
314 = 0.04). Additionally, the terms "*Signal Transduction*" (BTA-162582, q = 0.0002) and "*Immune System*"
315 (BTA-168256, q = 0.01) were significant in Reactome pathways analysis (Supp. Table S4).
316 A protein-protein interaction (PPI) network was developed, comprising 1,911 nodes (genes) and 9,198
317 edges (interactions) (Supp. Figure S1). From this network, five highly interconnected protein clusters
318 (C1, C2, C3, C4, and C5) were identified (PPI p-value < $1.0e^{-16}$), which potentially represent hubs of
319 specialized biological function (Figure 5). Functional overrepresentation analysis for these clusters
320 revealed key cellular processes involved in immune response modulation, cell signaling, infection
321 persistence, and mechanisms related to cancer development (Supp. Table S4).
322 For example, Figure 6 illustrates the grouping based on the semantic similarity of significantly
323 overrepresented GO BP terms for C1 (q < 0.05), whit representative terms including "*Cell surface*

receptor signaling pathway" "*Extrinsic apoptotic signaling pathway in absence of ligand*" "*Regulation of leukocyte proliferation*" "*Anatomical structure morphogenesis*", "*Cell activation*", "*Regulation of cell migration*", "*Cellular developmental process*" and "*Positive regulation of immune system process*". For C2, the enriched GO BP terms were primarily link to immune system processes, including "*Cytokine-mediated signaling pathway*", "*Regulation of T cell activation*", "*Regulation of interferon-gamma production*", "*Positive and negative regulation of cytokine production*" and "*Regulation of leukocyte differentiation*" among others. In C3, mostly associated with transcriptional regulation through chromatin modification, including "*Histone deacetylation*", "*Histone modification*", "*Positive regulation of transcription, DNA-templated*" and "*Chromatin remodeling*". Finally, the enriched biological processes in C4 and C5 were releated to signaling pathways, such as the "*Regulation of Wnt signaling pathway*" and the "*G protein-coupled receptor signaling pathway*" for C4 and C5, respectively.

Significantly enriched KEGG pathways for C1 included: "*Pathways in cancer*" (bta05200, q-value = $3.2e^{-09}$), "*Apoptosis*" (bta04210, q-value = $6.4e^{-06}$), "*MicroRNAs in cancer*" (bta05206, q-value = 0.0006), "*Human T-cell leukemia virus 1 infection*" (bta05166, q-value = 0.0004) and "*Regulation of actin cytoskeleton*" (bta04810, q-value = 0.001). For C2, significant pathways included "*Viral protein interaction with cytokine and cytokine receptor*" (bta04061, q-value = 8.99e-13), "*JAK-STAT signaling pathway*" (bta04630, q-value = 6.58e-09) and "*Chemokine signaling pathway*" (bta04062, q-value = 1.44e-05). Additionally, for C4 and C5, the enriched pathways were "*Wnt signaling pathway*" (bta04310, q-value = 5.64e-21) and "*Apelin signaling pathway*" (bta04371, q-value = 1.58e-10), respectively.

Discussion

This study, conducted a comprehensive characterization of circulating miRNAs in the peripheral blood of naturally BLV-infected and non-infected cattle was performed. Noteworthy, all BLV-infected cows had a high PVL, which has been associated with progression of the BLV pathogenesis (Mirsky et al., 1996; Jimba et al., 2010), and with an increase in viral dissemination within herds (Mammerickx et al., 1987). Following the identification of BLV-miRNAs in persistently infected cell lines (Kinkaid et al., 2012), their expression was assessed using next-generation sequencing for complete miRNA profiling and RT-qPCR to test the expression of candidate miRNAs. BLV-miRNAs were identified in primary leukemic B-cells and B-cell lymphomas isolated from BLV-infected ovine/bovine tumors (Rosewick et al., 2013), experimentally infected cattle (Gillet et al., 2016), and naturally BLV-infected cattle (Frie et al., 2018; Casas et al., 2020; Petersen et al., 2023).

On the other hand, BFV-miRNAs have been identified in both persistently and recently infected MDBK cells, as well as in BFV experimentally challenged cattle (Whisnant et al., 2014). However, to our knowledge, this is the first report of the co-expression of miRNAs derived from two distinct viruses (BLV

360 and BFV) in naturally infected cattle. We identified and quantified seven BLV-derived and three BFV-
361 derived miRNAs. No miRNAs from BoHV-1 and -5 were detected.
362 Coinfections of BLV and BFV are prevalent among cattle (Materniak et al., 2019), akin to the
363 occurrences in cats infected with Feline foamy virus (FFV) and Feline leukemia virus (FeLV) (Cavalcante
364 et al., 2018), as well as in baboons with Simian foamy virus (SFV) and Simian T-cell leukemia virus
365 (STLV) (Alais et al., 2018). While BFV infections in cattle are generally regarded as mild and
366 asymptomatic (Whisnant et al., 2014; Materniak-Kornas et al., 2019), there is evidence to suggest that
367 co-infections with BFV and BLV could amplify pathogenicity and impair the bovine immune system,
368 thereby aiding in the transmission and spread of BFV (Amborski et al., 1989; Jacobs et al., 1992;
369 Materniak et al., 2013; 2019). Infectious BFV has been extracted from raw milk, and considering that
370 foamy viruses can cross species barriers, there is rising concern over the zoonotic potential of BFV in
371 humans (Romen et al., 2007; Pinto-Santini et al., 2017). Moreover, increased pathogenic effects have
372 been noted in mixed infections of macaques with SFV and Simian immunodeficiency virus (SIV)
373 (Choudhary et al., 2013), as well as in cats coinfected with Feline immunodeficiency Virus (FIV) and FFV
374 or FeLV and FFV (Cavalcante et al., 2018; Powers et al., 2018).
375 The seven miRNAs derived from BLV comprised no more than 8% of the total miRNA sequencing reads
376 in all analyzed samples (data not provided), consistent with the expression levels reported by Casas et
377 al. (2020) for natural BLV infections. In contrast, Rosewick et al. (2013) found that all ten mature BLV-
378 derived miRNAs accounted for about 40% of total miRNAs present in B-cell lymphomas from sheep
379 infected with BLV. Similarly, Ochiai et al. (2021) noted that BLV-derived miRNAs made up 38% of total
380 miRNAs in Japanese black cattle diagnosed with enzootic bovine leukosis (B-cell lymphoma).
381 Additionally, Kincaid et al. (2012) reported that BLV miRNAs were found in greater quantities than
382 cellular miRNAs in the BL3.1 cell line persistently infected with BLV. Although the relative expression
383 levels of individual BLV miRNAs differ across studies, *blv-miR-B4-5p* and *blv-miR-B5-3p* appear to be
384 either weakly expressed or undetectable. The dominant BLV-miRNA may change depending on the
385 disease stage or phase of infection analyzed. Overall, these findings suggest that BLV miRNAs are
386 highly expressed in *ex vivo* cell cultures, persistently infected cell lines, primary B-cell tumors, and cattle
387 affected by enzootic bovine leukosis. In cattle with subclinically BLV-infections, their expression levels
388 might vary based on several factors, including the infection route (natural or experimental) and the
389 animal's age (whether calf or adult cow), among other factors (Kincaid et al., 2012; Rosewick et al.,
390 2013; Gillet et al., 2016; Frie et al., 2018; Casas et al., 2020; Ochiai et al., 2021). Conversely, the other
391 BLV transcripts show little to no expression *in vivo*, highlighting the essential function of BLV miRNAs in
392 viral persistence (Lagarias and Radke, 1989; Radke et al., 1992).
393 Likewise, three BFV-derived miRNAs (*bfv-miR-BF1-5p*, *bfv-miR-BF1-3p*, and *bfv-miR-BF2-5p*) have
394 been identified in MDBK cells that are persistently and recently infected with BFV, showing higher
395 expression levels during persistent infections. Furthermore, two BFV-miRNAs were found in the
396 peripheral blood leukocytes of cattle experimentally infected with BFV, utilizing a qPCR assay (Whisnant

397 et al., 2014). This research is the first to document the expression of these three BFV-derived miRNAs in
398 natural setting infections.
399 A previous study compared BLV seropositive and seronegative cattle but without testing for BLV
400 genomic material (Casas et al., 2020). The detection of BLV-miRNAs in seronegative cows suggests a
401 previous exposure to the virus, likely resulting in an inadequate immune response that did not generate a
402 positive result in the anti-BLV ELISA test. However, a longitudinal analysis of seroconversion was not
403 performed. This study found that all ten virus-derived miRNAs were consistently and highly expressed in
404 the BLV (+) group, whereas only a few showed weak expression in the BLV(-) group. Although
405 undetected infections cannot be entirely excluded, it is improbable that adult animals over two years old,
406 which tested negative via qPCR and ELISA at two intervals (three months apart), were unrecognized
407 cases. The potential for BLV- or BFV-miRNAs to function as xeno-miRs and be transmitted from infected
408 to uninfected animals remains a question for future research.
409 Notably, the expression profiles of BLV(+) / BFV-miRNAs(+) and BLV(-) samples distinctly differentiate
410 the two groups of cows groups, as shown in the PCA plot. This indicates a shift in the host miRNA
411 expression profile between them.
412 Among the significant differentially expressed bovine miRNAs, *bta-miR-375* was identified, which has
413 been previously reported to be associated with BLV infection in cattle (Casas et al., 2020; Ochiai et al.,
414 2021; Ma et al., 2022), and suggested it as an early biomarker for diagnosing enzootic bovine leukosis
415 (EBL). Moreover, its levels effectively distinguished EBL-affected cattle from asymptomatic cattle with
416 high sensitivity and specificity (Murakami et al., 2024).
417 In a similar manner, *bta-miR-133a* was found to be differentially expressed in the serum of BLV-
418 seropositive cows compared to seronegative cows (Ma et al., 2022). The other significantly differentially
419 expressed miRNAs (*bta-miR-677*, *bta-miR-95*, *bta-miR-9-5p*, *bta-miR-3613a*, and *bta-miR-1*) have not
420 been previously reported in relation to BLV and/or BFV infections.
421 To date, efforts to assign biological functions to BLV- and BFV-derived miRNAs have focused on
422 predicting potential target genes for specific BLV- and BFV-miRNAs, in addition to selecting candidate
423 target genes for functional assays (Kinkaid et al., 2012; Rosewick et al., 2013; Gillet et al., 2016; Frie et
424 al., 2018; Casas et al., 2020; Cao et al., 2020; Petersen et al., 2023). In this study, we evaluated the
425 functional interaction (protein-protein interaction network) of potential target genes of BLV-miRNAs, while
426 also considering target genes of BFV-miRNAs and differentially expressed bovine-miRNAs.
427 It has been demonstrated that BLV fails to induce leukemia/lymphoma in sheeps (oncogenicity
428 suppressed) challenged with an isogenic BLV provirus lacking the miRNA genomic region. Additionally,
429 BLV-miRNAs are crucial in promoting efficient viral propagation and disease progression in cattle (Gillet
430 et al., 2016; Safari et al., 2020).
431 The analysis of overrepresented metabolic pathways for the complete potential target gene set identified
432 "*Pathway in cancer*" as one of the significant KEGG pathways. This pathway involves a series of cellular
433 signaling pathways that activate crucial hallmarks of tumorigenesis, including tissue invasion and

metastasis, evading apoptosis, genomic instability, cell proliferation, genomic damage, and insensitivity to anti-growth signals (Weinberg, 2007). Supporting this, the grouping by semantic similarity of the overrepresented GO BP terms for the most interconnected protein cluster (biological function hub) C1 included terms associated with cellular processes that, when disrupted, could be linked to tumor development events. These terms include "*Regulation of cell migration*," "*Extrinsic apoptotic signaling pathway in the absence of ligand*", "*Chromatin remodeling*", "*Regulation of leukocyte proliferation*", and "*Cell surface receptor signaling*". Interestingly, other overrepresented KEGG terms in C1 included the "*Ras signaling pathway*", "*Rap1 signaling pathway*", "*PI3K-Akt signaling pathway*", "*MAPK signaling pathway*", "*Regulation of actin cytoskeleton*" and Reactome terms such as "*RAF/MAP kinase cascade*" and "*Signaling by Receptor Tyrosine Kinases*". This signaling pathways are amplified and propagated intracellularly by various kinases, ultimately affecting how transcription factors and histone-modifying complexes control downstream gene expression (Suganuma and Workman, 2013). In this context, a crucial signaling cascade most altered in human cancers is the mitogen-activated protein kinase (MAPK) pathway, which includes the RAS–RAF–MAPK kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway (Gimple and Wang, 2019; Bahar et al., 2023). Using the ovine BLV pathogenesis model, sheeps were experimentally challenged with an isogenic BLV-miRNA deletion mutant, and global transcriptome analysis revealed that BLV-miRNAs primarily promote the proliferation of BLV-infected B-cells by inhibiting immune response and cell signaling pathways (Safari et al., 2020). In turn, in a reporter assay, the FBJ murine osteosarcoma viral oncogene homolog (*FOS*) was identified as a direct target of BLV-miRNAs. The *FOS* gene is a component of the activator protein-1 (AP-1) complex, which is involved in the primary response to B-cell receptor signaling and is frequently downregulated in many cancers, including breast carcinomas (Huo and Rothstein, 1995; Gillet et al., 2016).

The functional annotation of the proteins within hub C2 revealed that immune system-related terms, such as "Cytokine-mediated signaling pathway", "*Regulation of T cell activation*" and "*Regulation of interferon-gamma production*" were significant overrepresented, along with the Reactome term "*Cytokine Signaling in immune system*". Additionally, enriched GO BP terms across C3 included "*Histone modification*", "*Positive regulation of cell population proliferation*", and "*Histone deacetylation*" among others; as well as the Reactome terms "*Regulation of TP53 Activity through Acetylation*" and "*Chromatin modifying enzymes*". The transcription factor *TP53*, a tumor suppressor activated by DNA damage, plays a critical role in maintaining genomic integrity (Aubrey et al., 2016). The expression of two transcription factors, B-lymphocyte-induced maturation protein 1 (*BLIMP1*) and B-cell lymphoma 6 (*BCL6*), is negatively correlated with BLV-miRNA expression in BLV(+) cows. These transcription factors play a pivotal role regulating B-cell differentiation, antibody affinity and T-cells immune function. Particularly, an important function of *BCL6* is to enable GC B-cells to proliferate in response to T-cell antigens by specifically repressing gene expression related to DNA damage sensing. This repression allows for the tolerance of DNA breaks induced during immunoglobulin class rearrangement and somatic hypermutation (Nutt et al., 2007; Swaminathan et al., 2014; Frie et al., 2018).

471 The functional role of BFV-miRNAs remains poorly investigated. Predicted gene targets for *bfv-miR-BF2-*
472 *5p* were Ankyrin repeat domain-containing protein (*ANKRD17*) and Bax-interacting factor 1 (*BIF1*) (Cao
473 et al., 2020). *BIF1* plays a key role in activating the pro-apoptotic Bax protein in the intrinsic apoptosis
474 pathway as well as in autophagy and autophagosome formation, thus acting as a tumor suppressor
475 (Coppola et al., 2008; Xu et al., 2016). *ANKRD17,* on the other hand, is involved in DNA replication and
476 cell cycle progression. It also interacts with genes that are responsible for sensing viral RNAs and trigger
477 immune responses (Deng et al., 2009; Wang et al., 2012).
478 Among the genes targeted by BLV-miRNAs, BFV-miRNAs, and BTA-miRNAs-DE, we identified G-
479 protein-coupled-receptor-kinase-5 (*GRK5*), which is a member of the G protein-coupled receptor kinases
480 (GPCRKs). The GRK proteins use ATP to phosphorylate serine and threonine residues in specific
481 receptor locations, initiating their desensitization and down-regulation (Black et al., 2016). Additionally,
482 two genes associated with DNA methylation binding, methyl-CpG binding protein 2 (*MECP2*) and Zinc-
483 finger-and-BTB-domain-containing-38 (*ZBTB38*), act as epigenetic readers of DNA, by binding to
484 methylated DNA and regulating transcription. Cytosine methylation is crucial for silencing exogenous
485 retroviruses, and *MECP2* seems to mediate this transcriptional silencing (Challita and Kohn, 1994;
486 Lorincz et al., 2001; Vuu et al., 2023). Remarkably, another identified target gene is *PEG10*, an
487 oncogene involved in cell proliferation, apoptosis, and metastasis in B-cell lymphocytic leukemia (Xie et
488 al., 2018).
489 Regarding the differentially expressed BTA-miRNAs, *miR-1*, *miR-133a*, and *miR-375* are recognized as
490 tumor suppressors and are downregulated in various types of cancer, exerting their effects through
491 distinct mechanisms (Yoshino et al., 2011; Hua et al., 2021). For instance, *miR-1* interacts with the proto-
492 oncogene B-cell lymphoma 2 (*BCL-2*), an anti-apoptotic gene. Overexpression of *miR-1* inhibits cell
493 proliferation, migration, and invasion while promoting apoptosis in breast cancer (Peng et al., 2020).
494 Similarly, *miR-133a* modulates biological processes such as proliferation, apoptosis, and autophagy
495 (Hua et al., 2021). On the other hand, *miR-375* is significantly downregulated in several cancers and
496 considered a biomarker of poor prognosis (Mathé et al., 2009; Avissar et al., 2009; Yan et al., 2014; He
497 et al., 2017), but it is overexpressed in breast cancer (de Souza Rocha Simonini et al., 2010). Similarly,
498 *miR-95-3p* and *miR-9-3p* act as prognostic markers, promoting the progression of cervical and breast
499 cancers, respectively (Li et al., 2020; Feng et al., 2022).
500 Conversely, the differentially expressed bovine miRNA *bta-miR-677* enhances the production of type I
501 interferons (IFN-I) and interferon-stimulated genes (ISGs) (Zhong et al., 2022). Similarly, inhibiting *miR-*
502 *3613-3p* decreases the expression of IFN-α and IFN-β, thereby affecting the anti-hepatitis B activity of
503 interferons (Zhao et al., 2019). Moreover, *miR-3613-3p* has been recognized as a tumor suppressor,
504 with its deletion associated with poor prognosis in estrogen receptor-positive breast cancer patients
505 (Chen et al., 2021).
506

Conclusion

In this study, we report for the first time the co-expression of seven miRNAs derived from BLV and three from BFV in cattle naturally infected with BLV. Several bovine BTA-miRNAs were found to be differentially expressed (DE) between cows with BLV high proviral load (PVL) and non-infected cows. Among them, *bta-miR-375* was previously identified as a potential early biomarker for the progression of enzootic bovine leukosis, and this study further emphasizes its potential utility in identifying high PVL cows alongside other candidate miRNAs.

The functional analysis of protein-protein interaction networks involving BLV-, BFV-, and BTA-DE miRNAs-targeted genes identified key metabolic pathways that potentially underlie tumorigenesis and immune response modulation, including cellular signaling pathways, regulation of T cell activation, apoptosis, chromatin remodeling, and cytoskeleton regulation. These findings emphasize the interplay between viral miRNAs and host cellular processes in promoting disease progression. The co-expression of miRNAs derived from BLV and BFV raises concerns about whether their interaction may exacerbate disease pathogenesis or facilitate the dissemination of BFV in light of the zoonotic potential that BFV presents. Ultimately, the identified miRNAs and associated metabolic pathways offer promising opportunities for developing diagnostic tools and therapeutic strategies aimed at controlling viral persistence and tumorigenesis in cattle.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Formal analysis: MIP, HAC.

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Methodology: HAC.

Visualization: HAC.

Writing – original draft: HAC.

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Roles as defined by: CRediT (contributor role taxonomy)

Availability of data

Raw small sequencing reads are available in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1219522.

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Table 1. Sample characterization and small RNA sequencing output

ID	PR (SD)[1]	PVL	RIN	Raw reads (n)	Reads after QC (n)
5671	178.6(28.4)	63,148.0	9.6	8,090,674	4,243,257
5841	191.3(32.8)	44,234.3	9.6	11,869,092	4,038,887
6021	169.8(42.8)	52,902.4	7.8	15,487,902	6,148,370
6097	183.2(25.4)	91,255.4	9.3	12,511,860	4,744,543
5830	<25	-	9.0	11,903,095	5,115,654
6493	<25	-	9.3	10,871,552	4,312,484
6962	<25	-	9.0	11,545,292	4,950,884

[1] Anti-BLV enzyme-linked immunosorbent assay % of reactivity; % of reactivity values < 25.0 indicated negative samples

Abbreviatures: ID = sample identification; PR = % of reactivity; SD = standard deviation; RIN = RNA integrity number; PVL = T-3 and T0 BLV average proviral load; QC = quality control.

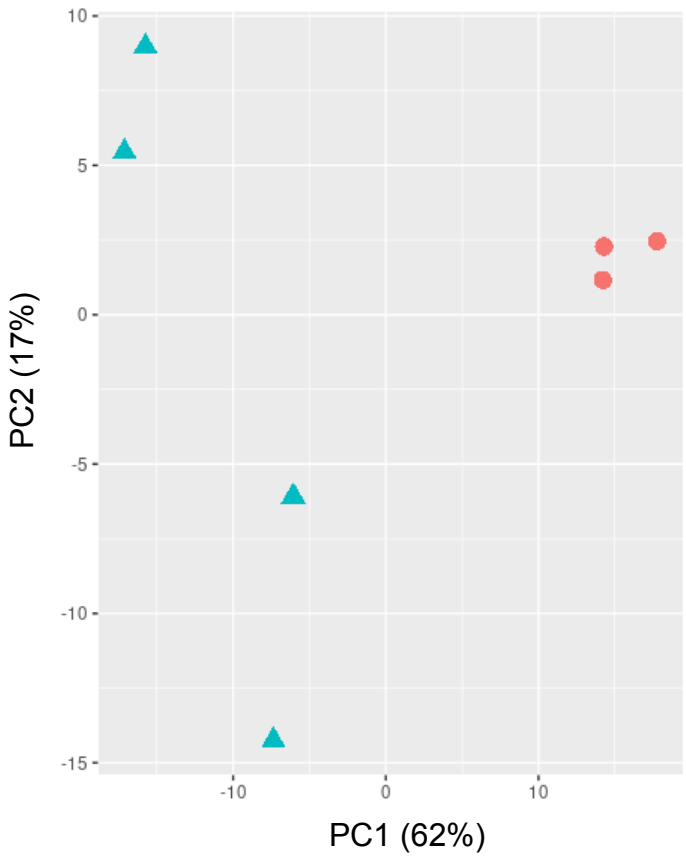


Figure 1. Principal component analysis of miRNAs expression matrix. Blue triangles and Red circles represent BLV(+) and BLV(-) samples, respectively. PC = principal component. PC1 and PC2, represent 62% and 17% of variance contained in the expression matrix data, respectively.

Table 2. Viral derived-miRNAs expression

Condition	BLV(+)					BLV(-)			
miRNA/ID	5671	5841	6021	6097	avgexp(SD)	5830	6493	6962	avgexp(SD
bfv-miR-BF1-5p	8,973.2	719.9	14,813.3	2,872.6	6,844.7(6,359.3)	15.6	5.8	15.4	12.3(5.6)
bfv-miR-BF1-3p	12,947.2	1,329.6	15,826.4	2,925.9	8,257.3(7,204.2)	7.1	11.6	8.6	9.1(2.3)
bfv-miR-BF2-5p	17,694.3	1,735.7	33,383.7	6,868.5	14,920.6(13,991.2)	0.0	15.4	17.1	10.8(9.4)
bfv-miR-BF2-3p	2.8	0.0	6.3	6.2	3.8(3.0)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B1-5p	0.0	0.0	0.0	0.0	0(0.0)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B1-3p	7,556.4	13,726.1	46,243.5	43,198.3	27,681.1(19,875.4)	28.3	24.1	24.0	25.5(2.5)
blv-miR-B2-5p	30,941.9	35,856.1	88,113.4	81,320.4	59,057.9(29,825.4)	60.9	69.4	71.9	67.4(5.7)
blv-miR-B2-3p	34.2	47.4	171.3	197.0	112.5(83.6)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B3-5p	262.0	371.8	259.8	344.7	309.6(57.3)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B3-3p	37,074.5	33,573.4	61,540.1	65,060.6	49,312.2(16,278.9)	39.7	55.9	53.9	49.8(8.8)
blv-miR-B4-5p	1.4	0.0	0.0	1.0	0.6(0.7)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B4-3p	39.9	69.9	79.8	70.8	65.1(17.4)	0.0	0.0	0.0	0.0(0.0)
blv-miR-B5-5p	864.3	972.1	1,218.4	752.0	951.7(199.2)	0.7	1.0	0.9	0.8(0.1)
blv-miR-B5-3p	0.0	1.2	0.8	2.1	1.0(0.9)	0.0	0.0	0.0	0.0(0.0)

Abbreviatures: ID = Sample ID; avgexp = normalized mean expression; SD = Standard deviation

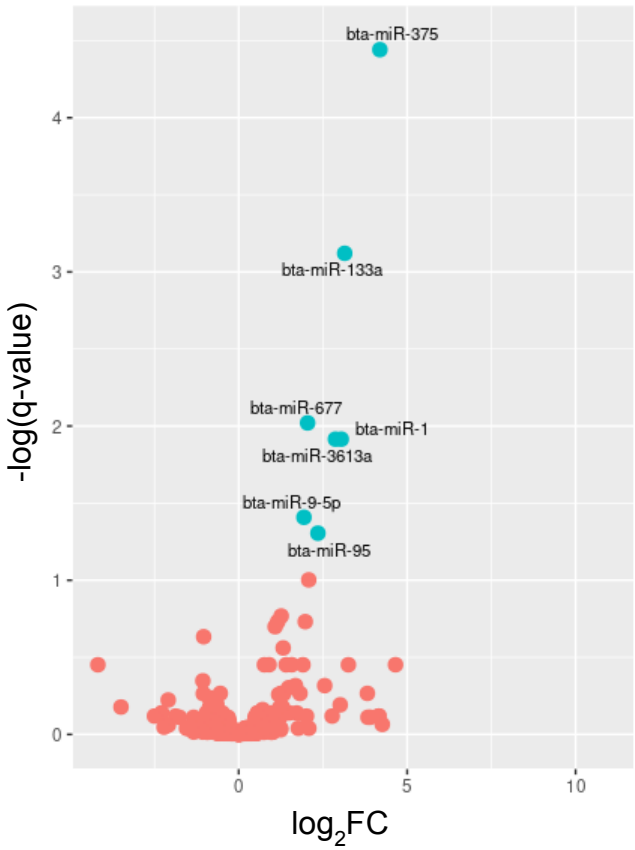


Figure 2. Volcano plot. Differential expression analysis of bovine miRNAs between BLV(+) and BLV(-) groups. Each circle represents a bovine miRNA. Red circles indicate miRNAs with FC < |1.5| and non-significant, while blue circles represent miRNAs with FC > |1.5| and q < 0.05 (-log(q-value) > 1.3). Viral-derived miRNAs, primarily quantified in BLV(+) samples, were excluded from the plot.

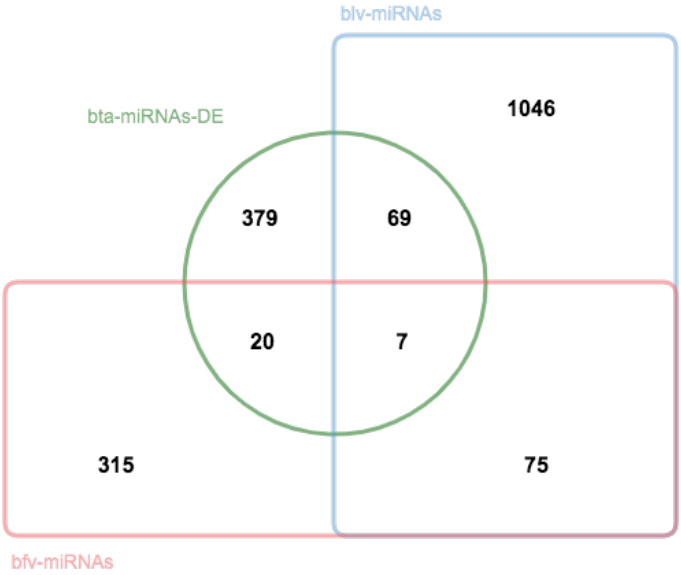
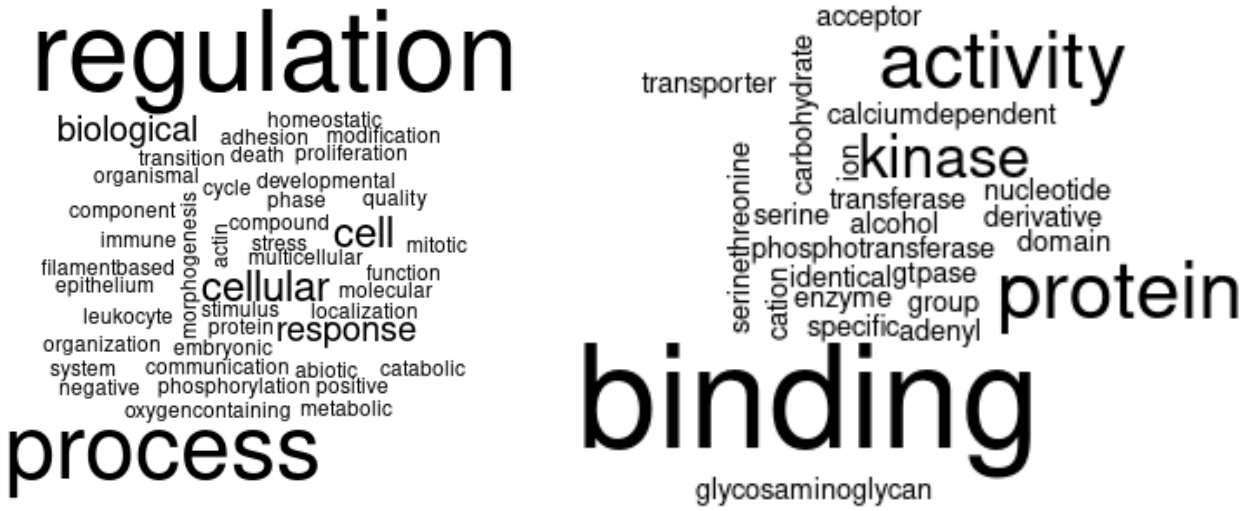


Figure 3. Venn diagram of number of predicted gene target for bta-miRNAs-DE, blv-miRNAs and bfv-miRNAs.



A) GO Biological process B) GO Molecular function

Figure 4. Word cloud for GO term overrepresented in target genes for biological process A) and molecular fuction B) categories.

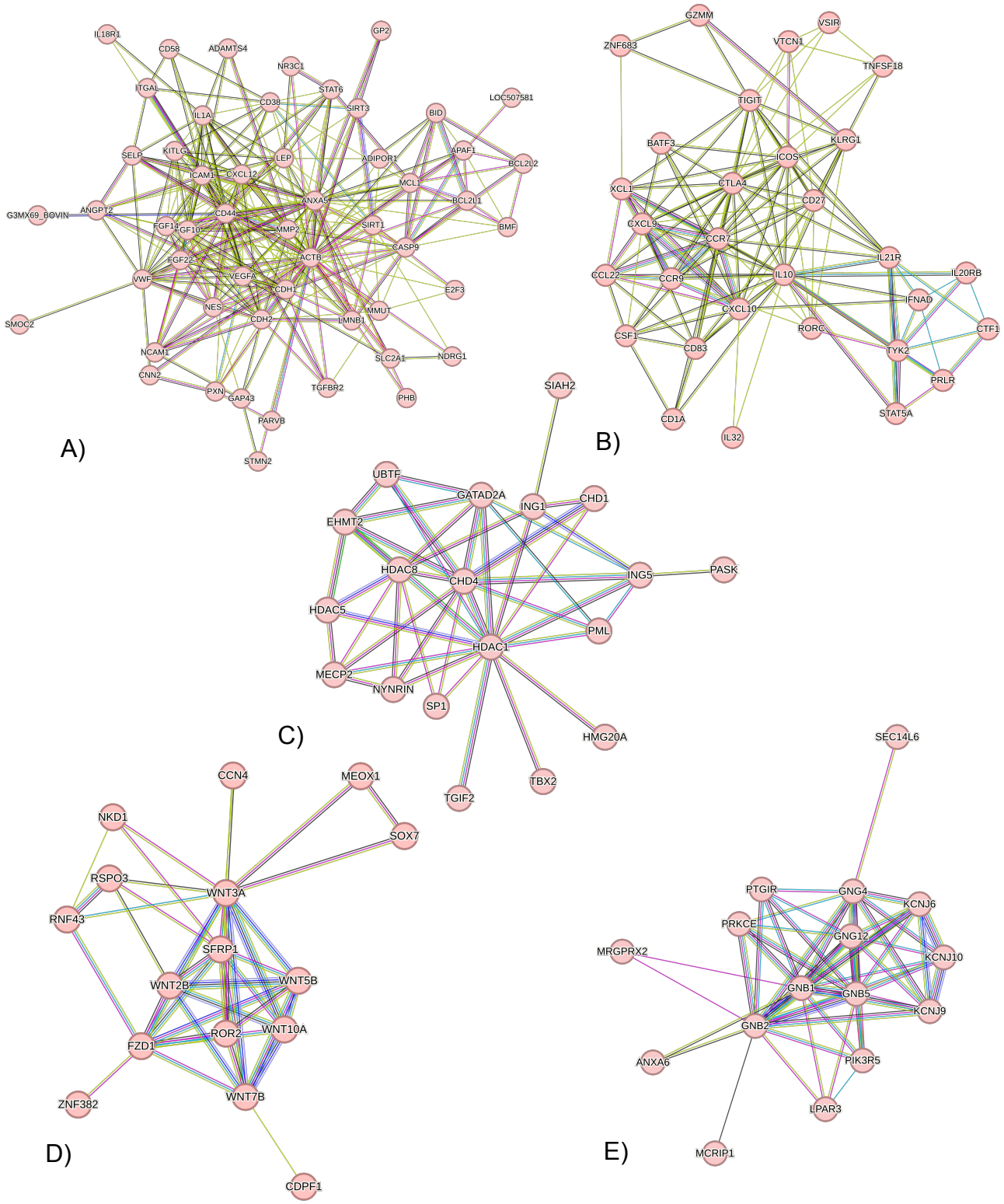


Figure 5. Protein-protein interaction (PPI) cluster analysis. Five cluster (> 15 nodes) were identified A) Cluster 1 (C1): Nº nodes: 53 - Nº edges: 282, avg. node degree: 10.6; B) Cluster 2 (C2): Nº nodes: 30 – Nº edges: 130, avg. node degree: 8.67; C) Cluster 3 (C3): Nº nodes: 19 – Nº edges: 45, avg. node degree: 4.74; D) Nº nodes: 16 – Nº edges: 42, avg. node degree: 5.25; E) Nº nodes: 16 – Nº edges: 53, avg node degree: 6.62. Nodes represent proteins encoded by a predicted miRNA gene target. Edges represent protein-protein interactions as determined by STRINGdb evidence, including: known interactions (curated databases and/or experimentally validated), predicted interactions (gene fusions, gene co-occurrence), text mining, co-expression, and protein homology.

Figure 6. Treemaps plot for GO BP enriched in C1 cluster. The terms were grouped (colored) based on semantic similarity, where the term with the lowest q-value is considered representative of the group.