Identification of microRNAs in the Lyme disease vector *Ixodes scapularis*

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Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in many biological processes, including the immune pathways that control bacterial, parasitic, and viral infections. Pathogens probably modify host miRNAs to facilitate successful infection, so they might be useful targets for vaccination strategies. There are little data on differentially expressed miRNAs in the black-legged tick *Ixodes scapularis* after infection with *Borrelia burgdorferi*, the causative agent of Lyme disease in the United States. Small RNA sequencing and qRT-PCR analysis were used to identify and validate differentially-expressed *I. scapularis* salivary miRNAs. Small RNA-seq yielded 133,465,828 (≥18 nucleotides) and 163,852,135 (≥18 nucleotides) small RNA reads from *Borrelia*-infected and uninfected salivary glands for downstream analysis using the miRDeep2 algorithm. Two hundred and fifty-four miRNAs were identified across all datasets, 25 of which were high confidence and 51 low confidence known miRNAs. Twenty-three miRNAs were differentially expressed in uninfected and infected salivary glands: 11 were upregulated and 12 were downregulated upon pathogen infection. This study provides new insights into the miRNAs expressed in *I. scapularis* salivary glands and pave the way for their functional manipulation to prevent or treat *B. burgdorferi* infection.

Keywords: *Ixodes scapularis*, salivary glands, microRNA, RNA sequencing, *Borrelia burgdorferi*

34 ## Introduction

35 Small non-coding RNAs (sncRNAs) regulate genes at the post-transcriptional level in animals,
36 plants, and arthropods including ticks [1-5]. MicroRNAs (miRNAs) are a class of sncRNA between
37 18 and 25 nucleotides in length, and they are now known to be important in arthropod immunity
38 and host-pathogen interactions through their involvement in several cellular processes including
39 development, immunity, and pathogen responses in arthropods [2, 6-10]. In animals, miRNAs
40 regulate post-transcriptional gene expression, most often by binding to the 3'-untranslated
41 region (3'-UTR) of target genes. While perfect complementarity of 2-8 nucleotides at the 5' end
42 of the miRNA (seed region) is necessary for miRNA regulation, the remaining sequence can
43 harbor mismatches or bulges [2, 11, 12]. miRNAs are transcribed as primary miRNA transcripts
44 before processing by Drosha and Pasha proteins into a pre-miRNAs. These are then exported to
45 the cytoplasm and processed by Dicer into mature miRNAs, which are loaded onto the microRNA-
46 induced silencing complex (miRISC) before targeting complementary mRNA for degradation [13,
47 14].

48 Several proteomics and transcriptomics studies have identified differentially expressed
49 transcripts and proteins in uninfected and infected ticks [1, 15-18], but few have investigated the
50 role of pathogens in the differential modulation of the post-transcriptional tick and host
51 machinery. Although there are over 800 tick species and despite their importance as vectors of
52 human and animal diseases, ticks are underrepresented in available miRNA resources. For
53 example, the miRbase database contains 49 *Ixodes scapularis* miRNAs and 24 *Rhipicephalus*
54 *microplus* miRNAs, while MirGeneDB 2.1 contains 64 *Ixodes scapularis* miRNAs. However, small

55 RNA sequencing (RNA-seq) and computational approaches are accelerating the discovery of
56 miRNAs from species with incomplete genome sequencing, assembly, and annotation.

57 *Ixodes scapularis* is a primary vector of human pathogens including the Lyme disease
58 agent *Borrelia burgdorferi*, which infects vertebrates and ticks through evolved complex
59 mechanisms. There is now a good understanding of tick immune pathways and their interactions
60 with *B. burgdorferi* [19-21], but it is still uncertain how *B. burgdorferi* avoids clearance. *B.*
61 *burgdorferi* must traverse tick salivary glands during transmission [71]. Since saliva/salivary gland
62 proteins can enhance *B. burgdorferi* transmission into the vertebrate host, characterization of
63 molecular interactions at the tick bite site and the tick salivary glands is expected to facilitate
64 vaccine development [22, 23], since promoting immunity against tick salivary proteins could
65 neutralize tick bites and pathogen transmission. Once *B. burgdorferi* is acquired by ticks from
66 infected hosts, it resides in the tick gut and only migrates to the salivary gland during subsequent
67 blood feeding, which generally lasts for 3-7 days [72, 73, 74, 75] . While it is known that other
68 tick-borne pathogens such as *Anaplasma marginale* must replicate inside salivary glands for
69 efficient transmission [76], the details of *B. burgdorferi* replication are less well understood.
70 Indeed, borrelial spirochetes invade the tick salivary gland via an unknown mechanism [71] and
71 might be carried to the host dermis via tick saliva. Several salivary gland genes are upregulated
72 in *B. burgdorferi*-infected *Ixodes scapularis* nymphs compared with uninfected ones [24],
73 suggesting a significant role for salivary gland gene regulation in *B. burgdorferi* infection and
74 transmission.

75 To fill the knowledge gap on miRNA expression in *Ixodes scapularis* salivary glands, here
76 we performed miRNA profiling of partially fed *B. burgdorferi*-infected and uninfected tick salivary

77 glands to identify miRNAs that might play a role in *B. burgdorferi* survival, colonization,
78 transmission, and host immunomodulation. In doing so, we detected 254 miRNAs, of which 25
79 were high confidence miRNAs and 51 low confidence miRNAs. Forty-one of the identified miRNAs
80 were present as *I. scapularis* miRNAs in miRBase (v22.1). Gene ontology and network analysis of
81 target genes of differentially expressed miRNAs predicted roles in metabolic, cellular,
82 development, cellular component biogenesis, and biological regulation processes. Several KEGG
83 pathways including sphingolipid metabolism; valine, leucine and isoleucine degradation; lipid
84 transport and metabolism; exosome biogenesis and secretion; and phosphate-containing
85 compound metabolic processes were predicted as targets of differentially expressed miRNAs.

86

87 Materials and Methods

88 Ethics statement

89 All animal experiments were performed in strict accordance with the recommendations in the
90 NIH Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use
91 Committee of the University of Southern Mississippi approved the protocol for blood feeding of
92 field-collected ticks (protocol # 15101501.1).

93 Ticks and tissue dissections

94 Ticks were purchased from the Oklahoma State University Tick Rearing Facility. Adult male and
95 female *I. scapularis* were kept according to standard practices [25] and maintained in the
96 laboratory as described in our previously published work [26, 27]. Unfed female adult *I. scapularis*

97 were infected with laboratory grown *B. burgdorferi* strain B31.5A19 using the capillary feeding
98 method at Tulane National Primate Research Center [28]. *Borrelia* infected and uninfected ticks
99 (n=45 in each group) were placed on each ear of a rabbit host for tick blood-feeding. Blood-fed
100 adult female *I. scapularis* were dissected within 60 min of removal from the rabbit. Tick tissues
101 were dissected and washed in M-199 buffer as described previously [77]. Salivary glands and
102 midguts from individual *I. scapularis* were stored in RNAlater (Life Technologies, Carlsbad, CA,
103 USA) at −80°C until use.

104 **RNA isolation, cDNA synthesis, and PCR-based *B. burgdorferi* detection in tick tissues**

105 The TRIzol method was used for RNA extraction from individually-dissected midgut tissues, and
106 cDNA was synthesized as described previously [29, 78]. *B. burgdorferi* was detected in tick
107 midguts using the *flaB* gene in a PCR assay [29, 30]. After testing for *B. burgdorferi* infection in
108 tick midguts, the corresponding salivary gland tissues from the same uninfected/infected ticks
109 (n=10 salivary glands from each group) were pooled in separate tubes and RNA isolated using the
110 TRIzol method [78] .

111 **Small RNA sequencing (RNA-seq)**

112 Small RNA libraries were made using the Illumina TruSeq Kit following the manufacturer's
113 protocol (Illumina, San Diego, CA). Briefly, short adapter oligonucleotides were ligated to each
114 end of the small RNAs in the sample, cDNA made with reverse transcriptase, and PCR used to add
115 sample-specific barcodes and Illumina sequencing adapters. The final concentration of all
116 sequencing libraries was determined using a Qubit fluorometric assay (Thermo Fisher Scientific,
117 Waltham, MA), and the DNA fragment size of each library was assessed using a DNA 1000 high-

118 sensitivity chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). After
119 purification by polyacrylamide gel electrophoresis, sample libraries were pooled and sequenced
120 on an Illumina NextSeq 500 (single end 36 base) using the TruSeq SBS kit v3 (Illumina) and
121 protocols defined by the manufacturer. Four small RNA libraries of clean and *B. burgdorferi*-
122 infected, partially fed, and pooled salivary glands were sequenced. RNA library preparation and
123 indexing were performed at the UMMC sequencing facility.

124 **Data analysis**

125 A schematic of the experimental plan and data analysis is shown in **Supplementary Figure S1**.
126 miRDeep2 v.2.0.0.8 [31, 32], was used to process RNA-seq data. To predict novel miRNAs, the
127 reads from all samples were combined. The mapper function of miRDeep2 first trims the adapter
128 sequences from the reads and converts the read files from FASTQ to FASTA format. Reads shorter
129 than 18 bases were discarded and the remaining reads mapped to the *I. scapularis* reference
130 genome using default miRDeep2 mapper function parameters. Reads mapping to the genome
131 were used to predict novel miRNAs. The *Drosophila melanogaster* genome was also used as
132 another reference genome, and mapped reads were aligned to available miRNAs of *D.*
133 *melanogaster* in miRBase (v22) and quantified. Reads were mapped to the reference genomes of
134 *D. melanogaster* and *I. scapularis* and locations of potential miRNA read accumulations
135 identified. The regions immediately surrounding the mapped reads were examined for miRNA
136 biogenesis features including mature miRNAs, star and precursor reads, and stem-loop folding
137 properties. miRDeep2 models the miRNA biogenesis pathway and uses a probabilistic algorithm
138 to score compatibility of the position and frequency of sequencing reads with the secondary
139 structure of the miRNA precursor.

140 For miRNA expression, a count table was generated using bedtools multicov, which counts
141 alignments from indexed BAM files that overlap intervals in BED files provided from the
142 miRDeep2 analysis.

143 **ISE6 cell culture, infection with *B. burgdorferi*, and validation of differentially expressed**
144 **miRNAs by qRT-PCR**

145 Differentially expressed miRNAs in RNA-seq data were validated by qRT-PCR in the ISE6 cell line
146 derived from *I. scapularis* embryos cultured and maintained as suggested by Munderloh and
147 Kurtti [33]. The *B. burgdorferi* (strain B31) isolate was a kind donation from Dr. Monica Embers
148 (Tulane University, Covington, LA). Cultures were grown and maintained as suggested previously
149 [28]. Once ISE6 cells reached confluency, they were infected with *B. burgdorferi* as described
150 previously [34]. Briefly, ISE6 cells were inoculated with the supernatant of a log phase *B.*
151 *burgdorferi* culture at a multiplicity of infection of 50 and incubated for 24 hours before
152 harvesting. RNA was isolated using the TRIzol method and the expression of miRNAs analyzed by
153 qRT-PCR with the Mir-X miRNA qRT-PCR TB Green kit (Takara Bio Inc, Kusatsu, Shiga, Japan;
154 catalog #638316). qRT-PCR conditions were an initial denaturation at 95°C for 10 mins then 40
155 cycles of 95°C for 5 secs, 60°C for 20 secs.

156 **Normalization, differential expression (DE), and statistical analysis of miRNAs between**
157 **uninfected and infected salivary glands**

158 Differential expression (DE) analysis of identified miRNAs was performed using the interactive
159 web interface DeApp [35]. Low expression genetic features were removed after alignment if the
160 counts per million (CPM) value was ≤1 in less than two samples. Sample normalization and a

161 multidimensional scaling (MDS) plot are shown in **Supplementary Fig. S2**. DE analysis was
162 performed with edgeR with a false discovery rate (FDR) adjusted *p*-value of 0.05 and minimum
163 fold-change of 1.5. DeApp displays a dispersion plot showing the overall DE analysis along with
164 statistical significance (*p*-value, FDR adjusted *p*-value) and a volcano plot corresponding to the
165 specified parameters and cutoff values.

166 ***In silico* mapping of *B. burgdorferi*-infected small RNA sequences to the *Borrelia burgdorferi***
167 **genome**

168 *In silico* mapping of small RNA sequences of *B. burgdorferi*-infected salivary glands to the *Borrelia*
169 *burgdorferi* genome (GCF_000181575.2_ASM18157v2_genomic.fna) detected 18,165 *Borrelia*
170 *burgdorferi* sequences, but no miRNAs of *B. burgdorferi* origin were predicted by miRDeep2
171 analysis.

172 **Prediction of target genes, proteome re-annotation, gene ontology (GO), and KEGG enrichment**
173 **analyses**

174 TargetSpy [36], MIRANDA [37], and PITA [38] were used in miRNAconsTarget from sRNAtoolbox
175 to predict genes regulated by tick salivary gland miRNAs up- or downregulated in the *in silico*
176 analysis [39]. Targets common to all three programs were further considered. *In silico* target
177 prediction provided a high number of false positives, but cross-species comparisons and
178 combinatorial effects reduced this number [40]. Target gene networks and KEGG pathways
179 significantly enriched for target genes were extracted using the STRING [41] output. PANNZER2
180 [42] was used to functionally re-annotate the proteome of up- or downregulated genes (targets

181 of detected miRNAs), and WEGO [43] was used to analyze and plot gene ontology (GO)
182 annotations.

183

184 Results and Discussion

185 Profile characteristics of small RNA libraries

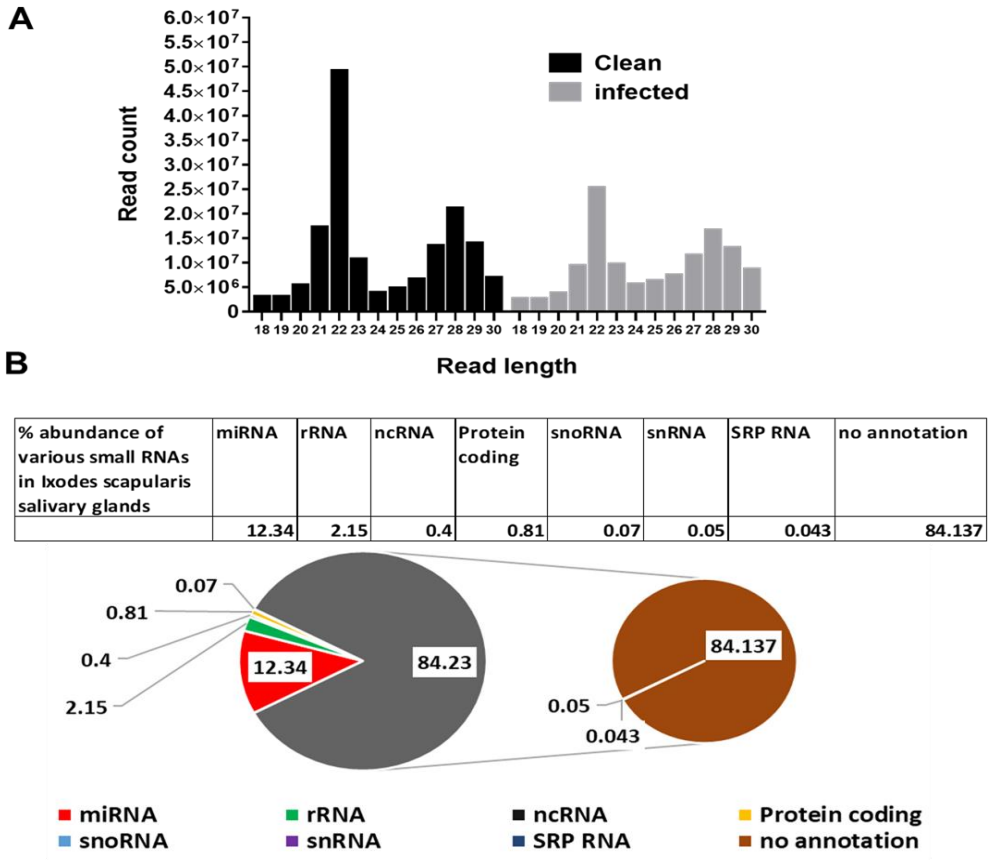
186 There were 216,292,174 raw small RNA reads from *B. burgdorferi*-infected salivary glands and
187 212,542,697 from uninfected salivary glands. After adapter trimming and removal of short reads
188 (≤18 nucleotides (nt)), 133,465,828 small RNA reads were available from *B. burgdorferi*-infected
189 samples and 163,852,135 from uninfected samples for downstream analysis. The read length
190 distribution shows the types of small RNAs present in *B. burgdorferi*-infected and uninfected
191 salivary gland samples. Two main peaks at 22 nt (miRNAs/siRNAs) at 29 nt (piRNAs) were
192 distinguishable in *B. burgdorferi*-infected and uninfected samples (**Figure 1A**). There were ~5 x
193 10^7 and ~2.5 x 10^7 22 nt miRNA sequences in uninfected and infected salivary gland samples,
194 respectively. The read length distribution in uninfected samples was comparable to infected
195 samples.

196 Other small RNA categories

197 A summary of reads matching various small RNAs categories from *B. burgdorferi*-infected and
198 uninfected salivary glands are shown in **Figure 1B**. Other small RNAs include signal recognition
199 particle rRNAs, ncRNAs, protein-coding RNAs, snoRNAs, snRNA SRP RNAs, and not annotated
200 small RNAs. Of the total small RNA reads from *Ixodes scapularis* salivary glands, 12.3% were

201 miRNAs, 2.2% rRNAs, 0.4% ncRNAs, 0.8% protein coding, 0.07% snoRNAs, 0.05% snRNAs, 0.04%

202 SRP RNAs, and 84.3% reads were not annotated.



% abundance of various small RNAs in Ixodes scapularis salivary glands	miRNA	rRNA	ncRNA	Protein coding	snoRNA	snRNA	SRP RNA	no annotation
	12.34	2.15	0.4	0.81	0.07	0.05	0.043	84.137

203

204 **Figure 1.** (A) Small RNA sequence length distribution in uninfected (clean) and *Borrelia*
205 *burgdorferi*-infected *Ixodes scapularis* salivary glands. MicroRNAs are twenty-four (22)
206 nucleotides in length. Clean, uninfected salivary glands; infected, *Borrelia burgdorferi*-infected
207 salivary glands. (B) Summary of the abundance of combined small RNA reads from *Ixodes*
208 *scapularis* salivary glands matching various small RNA categories. Reads from uninfected and
209 *Borrelia burgdorferi* infected samples are combined together for abundance calculation.
210

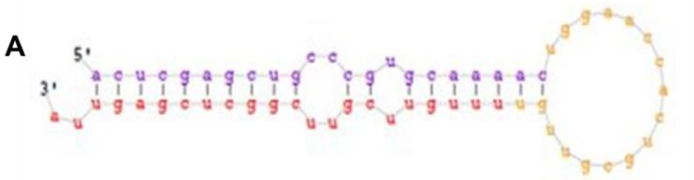
211 **MicroRNA profiling of infected and uninfected salivary glands and identification of novel tick**

212 **miRNAs**

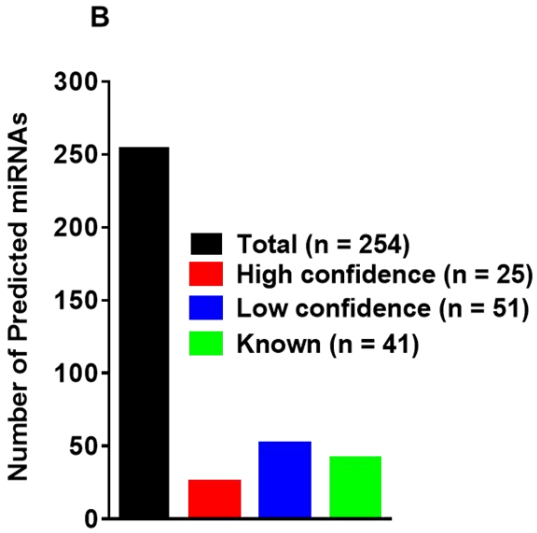
213 **Fig. 2A** shows the basic hairpin-loop structure of an miRNA and other parameters (Dicer cut

214 overhangs, total read count, mature read count, loop read count, total read count, randfold

215 score, and total score) used to determine whether a hairpin-loop structured RNA is an miRNA.
216 Using miRDeep2, 254 miRNAs were predicted in the salivary gland libraries. By adopting a
217 conservative approach, miRNAs were categorized as high (n=25) and low confidence (n=51)
218 miRNAs (**Fig. 2B**) based on standard criteria [79]. Of 49 *Ixodes scapularis* miRNAs present in
219 miRBase, 41 were detected in our data. Of the 254 *Ixodes scapularis* miRNAs, several had
220 homologs present in *D. melanogaster.* The details of the identified miRNAs and their miRDeep2
221 scores are presented in **Supplementary Table S1**.



Provisional ID	nDS_002860020.1_1808
Score total	485654.1
Score for star read(s)	3.9
Score for read counts	485643.7
Score for mfe	1.8
Score for randfold	1.6
Score for cons. Seed	3
Total read count	952581
Mature read count	949093
Loop read count	15
Star read count	3488



222

223 **Figure 2**. (A) Basic stem-loop structures of predicted microRNAs. miRDeep2 was used to identify
224 potential miRNA precursors based on nucleotide length, star sequence, stem-loop folding, and
225 homology to the *Ixodes scapularis* reference genome. Shown are the predicted stem-loop
226 structures (yellow), star (pink), and mature sequences of predicted miRNAs (red) in the salivary
227 glands of *Ixodes scapularis* ticks. (B) Annotation of predicted miRNAs in partially fed uninfected
228 (clean) and *Borrelia burgdorferi*-infected *Ixodes scapularis* salivary glands. 254 microRNAs were
229 predicted in infected and uninfected salivary gland tissues. 25 were categorized as high
230 confidence (n = 25) and 51 as low confidence (n=51) based on standard criteria and a conservative
231 approach. Out of 49 *Ixodes scapularis* miRNAs available in miRbase (v22.1), 41 were detected in
232 this study.

In silico DE analysis of miRNAs in *B. burgdorferi*-infected salivary glands

In DE analysis, 11 miRNAs were upregulated and 12 were downregulated in infected salivary glands compared with uninfected salivary glands (**Figure 3A, B; Table 1**). Several of the identified tick miRNAs were conserved in *D. melanogaster* (dme-miR-375-3p, dme-miR-993-5p, dme-miR-12-5p, dme-bantam-3p, dme-miR-100-5p, dme-miR-8-3p, and dme-miR-304-5p). miRNAs downregulated in *B. burgdorferi*-infected salivary glands relative to uninfected salivary glands were isc-miR-153, isc-miR-1 and isc-miR-79, nDS_002871802.1_376, nDS_002633080.1_31953, nDS_002549652.1_43448, nDS_002537755.1_45711, nDS_002537755.1_45739, nDS_002861213.1_1750, nDS_002763926.1_14582, and nDS_002548557.1_43749, while upregulated miRNAs were isc-miR-317, isc-miR5310, isc-miR-2001, isc-miR-5307, isc-miR-71, isc-miR-87, nDS_002784743.1_12165, nDS_002664372.1_28027, nDS_002716687.1_21278, nDS_002620414.1_33611, and nDS_002680650.1_26098.

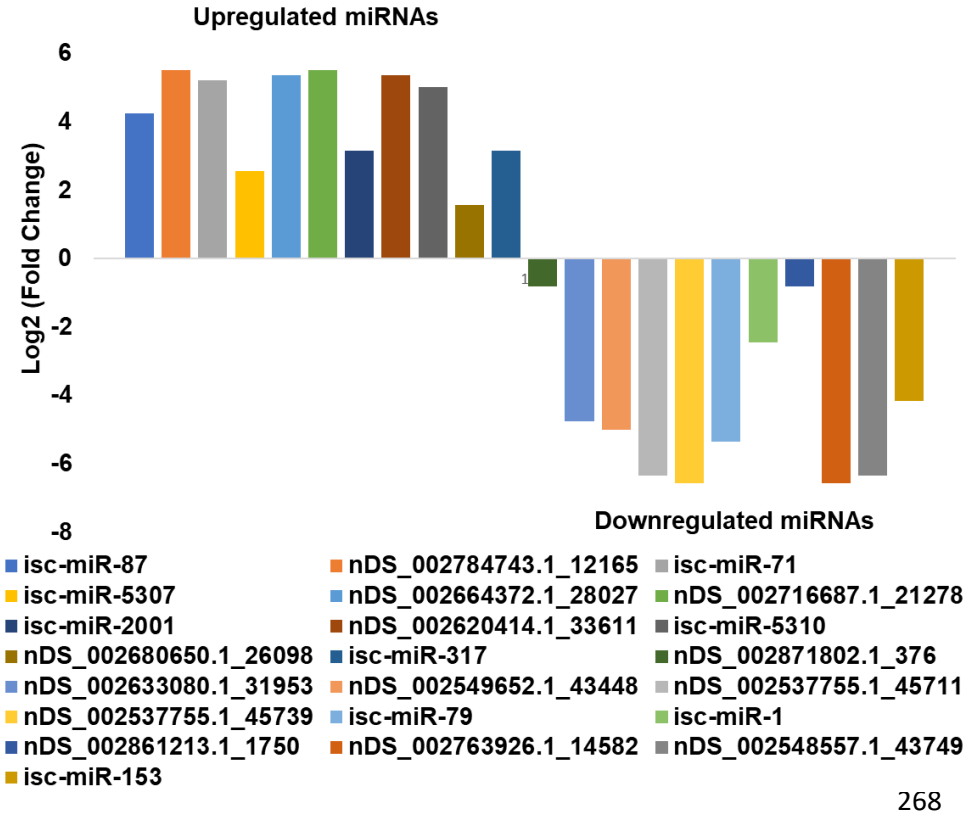


Figure 3. *In silico* differential expression of predicted miRNAs in *Borrelia burgdorferi*-infected, partially fed salivary glands relative to partially fed clean salivary glands. EdgeR was used for differential expression analysis. 12 predicted miRNAs were down-regulated, 11 were upregulated, while 39 were unaffected. miRNAs with a log2 fold-change expression > |1| and FDR ≤ 0.1 were considered significantly differentially expressed (see Table 1). Also, in Table 2, putative roles and targets of these DE conserved miRNAs have been provided based on available studies in other arthropods.

276 **Table 1.** *In silico* differential expression of *Ixodes scapularis* miRNAs in salivary glands after
277 *Borrelia burgdorferi* infection compared with uninfected salivary glands. miRNAs with a log2 fold-
278 change expression > |1| and FDR ≤ 0.1 were considered significantly differentially expressed.
279 Values highlighted in **red** indicate significant upregulation and values highlighted in **green** indicate
280 significant downregulation.
281

Differentially expressed miRNA	Log_2FC	logCPM	LR	P-value	FDR
isc-miR-87	4.245604	11.83289	33.20241	8.30E-09	4.65E-08
nDS_002784743.1_12165	5.511035	11.48887	29.7299	4.97E-08	1.55E-07
isc-miR-71	5.226443	11.95849	48.94389	2.63E-12	3.69E-11
isc-miR-5307	2.570363	12.34313	24.71399	6.65E-07	1.55E-06
nDS_002664372.1_28027	5.362184	11.43227	26.80077	2.26E-07	5.84E-07
nDS_002716687.1_21278	5.511153	11.48887	29.72059	4.99E-08	1.55E-07
isc-miR-2001	3.146271	11.78845	19.75379	8.81E-06	1.64E-05
nDS_002620414.1_33611	5.361301	11.43227	26.76848	2.29E-07	5.84E-07
isc-miR-5310	5.009416	11.31195	20.86089	4.94E-06	1.06E-05
nDS_002680650.1_26098	1.567661	15.43032	103.3589	2.80E-24	7.83E-23
isc-miR-317	3.146271	11.78845	19.75379	8.81E-06	1.64E-05
nDS_002871802.1_376	-0.80359	15.73883	19.22785	1.16E-05	1.91E-05
nDS_002633080.1_31953	-4.7431	11.95812	10.11663	0.001469	0.001959
nDS_002549652.1_43448	-4.9974	12.11011	12.13051	0.000496	0.000731
nDS_002537755.1_45711	-6.34111	13.07343	30.24285	3.81E-08	1.52E-07
nDS_002537755.1_45739	-6.57147	13.2637	36.56262	1.48E-09	1.03E-08
isc-miR-79 dme-miR-9a-5p	-5.35587	12.34273	15.64359	7.65E-05	0.000119
isc-miR-1 dme-miR-1-3p	-2.44072	12.67077	10.50794	0.001189	0.001664
nDS_002861213.1_1750	-0.80359	15.73883	19.22785	1.16E-05	1.91E-05
nDS_002763926.1_14582	-6.57147	13.2637	36.56262	1.48E-09	1.03E-08
nDS_002548557.1_43749	-6.34111	13.07343	30.24285	3.81E-08	1.52E-07
isc-miR-153	-4.15091	11.64621	6.563386	0.01041	0.013249

282

283 miR-2001 was upregulated in *B. burgdorferi*-infected salivary glands compared with
284 controls. miR-2001 is not present in the *Drosophila* genome but is an evolutionarily conserved

285 **Table 2.** List of differentially expressed microRNAs detected in *Borrelia burgdorferi* infected tick

286 salivary glands and their putative roles

microRNA	Role of ortholog microRNA	Target genes	Reference
isc-miR-153	Development, immune response		[82]
isc-miR-1	Stress response, immunity, development, facilitating infection	*HSP60, HSP70, GATA4*	[49, 50, 51, 52, 83, 84, 85, 86, 87]
isc-miR-79	Innate immunity, differentiation, apoptosis	*Roundabout protein 2 pathway (robo2), DRAPER, HP2 (Hemolymph protease), P38, pvr, puc*	[55, 56, 57, 88, 89, 90, 91]
isc-miR-317	Negative influencer of Toll pathway, host homeostatic response (targeting Gap junctions, TRP channels), mitotic regulation, Fecundity	*Dif-Rc, Thioredoxin peroxidase-2, STAT*	[45, 91, 92, 93, 94]
isc-miR-2001	Under extreme environmental conditions		[95]
isc-miR-5307	Propagation of Powassan virus inside vero cells		[96]
isc-miR-71	Stress response, development, facilitates WSSV infection	*cap-1 , riok1, Actin1, myD88, IML3*	[91, 97, 98, 99, 100]
isc-miR-87	Pathogen survival (disruption of Toll and IMD pathway)	*Serine/Threnine Kinase, Toll 1A, Putative TLR 5b, FADD*	[60, 62, 63, 91, 101, 102]
isc-miR-5310	Pathogen survival by modulating signaling pathways, Feeding behavior		[64-68, 81, 103]

287

288 miRNA in ticks [44]. It has previously been shown to play a role in host immunomodulation

289 required for pathogen survival. miR-2001 has been detected in *I. ricinus* tick saliva [45] and *H.*

290 *longicornis* tick saliva extracellular vesicles (EVs) [46] along with several other miRNAs, suggesting

291 that EVs containing these miRNAs could be transferred to the host to modulate host cellular
292 functions to facilitate tick and pathogen survival [45, 46]. EVs are involved in the intercellular
293 transfer of miRNAs, lipids, and proteins and the disposal of unnecessary cell contents [46, 47].
294 The discovery of EVs in the excretory-secretory products of ectoparasites suggests that EVs are
295 probably taken up by host cells, deliver their cargoes to the host, and favor immunomodulation,
296 pathogen survival, and disease progression [48, 80]

297 miR-1 was downregulated in *B. burgdorferi*-infected tick salivary glands compared with
298 uninfected salivary glands. miR-1 belongs to a family of miRNAs including miR-7 and miR-34
299 conserved across fruit flies, shrimps, and humans, where it modulates similar pathways
300 (development, apoptosis) and is upregulated during stress insults [49]. In mosquitoes, miR-1 is
301 upregulated during *Plasmodium* infection [50] and also facilitates or promotes West Nile virus
302 infection [51]. miR-1 has been shown to be generally upregulated in response to infection, while
303 we detected its downregulation in *B. burgdorferi*-infected SGs. In *Listeria*-infected macrophages,
304 miR-1 promotes IFN-γ-dependent activation of the innate immune response [52].

305 Tick saliva and salivary gland extracts reduce IFN-γ and IL-2 production in T cells and
306 inhibited T cell proliferation [53, 54] suggesting immune suppression, a possible survival
307 mechanism for tick pathogens. mir-79 was also downregulated in *Borrelia burgdorferi* infected
308 salivary glands, and mir-79 has been shown to participate in immunity and other processes such
309 as cellular differentiation, neurogenesis, and apoptosis. In *Rhipicephalus haemaphysaloides*, mir-
310 79 was downregulated upon lipopolysaccharide (LPS) induction in female and male ticks,
311 suggesting a role for mir-79 in LPS-mediated stimulation of the innate immune response [55].
312 The JNK pathway is an immune response pathway against Gram-negative bacteria [56], and mir-

313 79 is known to disrupt JNK signaling by targeting its component genes *pvr* (CG8222) and *puc*
314 (CG7850) [57]. Our detection of the downregulation of mir-79 in *B. burgdorferi*-infected salivary
315 glands is probably due to stimulation of the JNK pathway as a tick immune response against *B.*
316 *burgdorferi*. Although *B. burgdorferi* is described as an atypical Gram-negative bacterium due to
317 its double membrane, it lacks classical lipopolysaccharide (LPS). It also has a different cellular
318 organization and membrane composition to other diderms [58]. However, surprisingly in *A.*
319 *phagocytophilum* (an intracellular Gram-negative bacterial pathogen)-infected ticks, mir-79 was
320 upregulated to facilitate infection by targeting the Roundabout protein 2 pathway (Robo2) [59],
321 suggesting different roles for mir-79 in ticks when infected with different bacterial pathogens.
322 *Borrelia burgdorferi* (Bb) is extracellular and a Gram-negative bacterial pathogen, but *A.*
323 *phagocytophilum* is a well-known intracellular a Gram-negative bacterial pathogen. Do
324 extracellular and pseudo-Gram-negative status of *B. burgdorferi* make it a different bacterial
325 pathogen than Gram negative or positive? More clarity of tick immune response is required in
326 case of *B. burgdorferi* infection.

327 In *Drosophila [35]*, miR-317 negatively regulates Toll pathway signaling, and its
328 upregulation in *B. burgdorferi*-infected salivary glands may suggest a similar role to facilitate *B.*
329 *burgdorferi* survival inside tick salivary glands. *In silico*, miR-317 targets Dif-Rc, an important
330 transcription factor in the Toll pathway in *Drosophila* [35] and STAT in JAK-STAT signaling in
331 *Manduca sexta [60]*. An *in silico* study in *I. ricinus* suggested a combinatorial effect of tick salivary
332 miRNAs on host genes important for maintaining host homeostasis and tick-host interactions,
333 including miR-317 targeting gap junctions and TRP channels, which play significant roles in host
334 homeostatic responses [45]. miR-71 was upregulated in *B. burgdorferi*-infected salivary glands,

335 and its predicted targets include *MyD88*, which is activated when ligands bind to the Toll-like
336 receptor (TLR), interleukin 1 receptor (IL-1R), or IFN-γR1 and trigger MyD88-mediated signaling
337 and pro-inflammatory cytokine responses. Another miR-71 target is IML3, an arthropod
338 immunolectin that recognizes LPS on Gram-negative bacteria as a part of arthropod immune
339 defenses. Immunolectins are also predicted targets of miR-87, -276, -9a, and -71 [60]. We
340 hypothesize that miR-71 disrupts tick immune pathways and protects *B. burgdorferi* in the
341 salivary gland. It has also been shown to prolong the life and regulate stress responses in
342 nematodes, being upregulated in the Dauer larval stage when food or other life-sustaining
343 resources are scarce [61].

344 miR-87 was upregulated in *B. burgdorferi*-infected salivary glands. Previous studies in
345 other arthropods such as *Manduca sexta* and *Aedes albopictus* have suggested a role in
346 disrupting innate immunity, particularly via IMD and Toll signaling pathways [60, 62, 63]. In *Aedes*
347 *albopictus*, miR-87's predicted targets are Toll pathway signaling Ser/Thr kinase, Toll-like
348 receptor Toll1A, class A scavenger receptor with Ser-protease domain, galectin [63], and TLR5b
349 [62], while in *Manduca sexta* its predicted target is FADD, an adaptor protein involved in DISC
350 formation [60]. Our *in silico* data also showed upregulation of miR-5310 in *B. burgdorferi*-infected
351 salivary glands. miR-5310 is a tick-specific miRNA [44], and a recent study demonstrated its
352 downregulation in *Anaplasma phagocytophilum*-infected nymphs compared with unfed
353 uninfected nymphs [64]. Previous studies have also indicated modulation of signaling events via
354 miR-5310 upon *A. phagocytophilum* infection [64-68, 81] In *B. burgdorferi* infection, we speculate
355 that might modulate signaling events and protect *B. burgdorferi* in the tick salivary glands. miR-

5310 might also be involved in tick feeding, as it was found to be downregulated in *Rhipicephalus microplus* tick larvae upon exposure to host odor but not being allowed to feed [44].

Prediction of target genes and gene ontology (GO) and functional enrichment analyses of the target network

Target proteins were used to build a high-confidence interaction network (interaction scores >0.9).

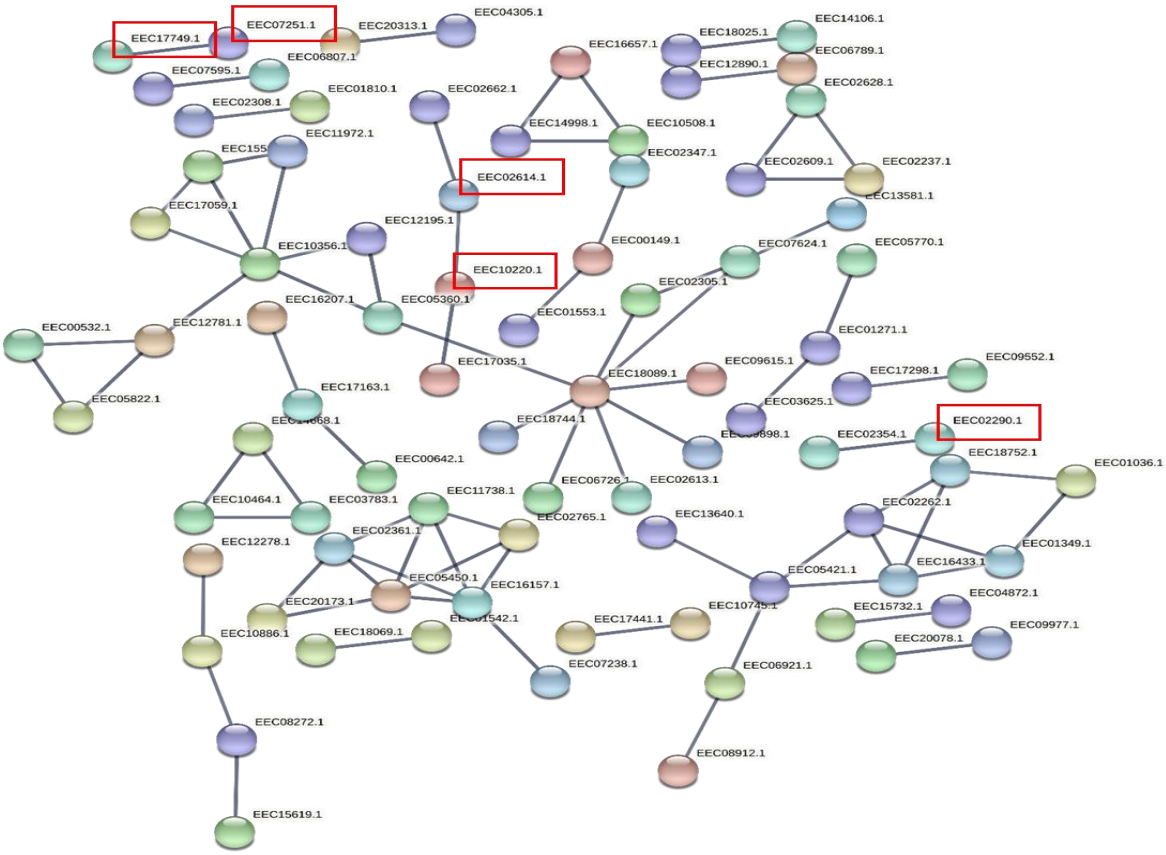


Figure 4. A network built exclusively from *Ixodes scapularis* proteins targeted by *in silico* differentially expressed miRNAs in *Borrelia burgdorferi*-infected, partially-fed salivary glands relative to partially fed clean salivary glands. Red boxes indicates the proteins involved in significant KEGG pathways such as sphingolipid metabolism (EEC07251.1, KEGG:R02541); valine, leucine, and isoleucine degradation (EEC10220.1, KEGG:R04188) ; lipid transport and metabolism (EEC02614.1, KEGG:R01178) ; exosome biogenesis and secretion (EEC07251.1, EEC17749.1, KEGG:R02541) ; and phosphate-containing compound metabolic process (EEC02290.1, KEGG:R00004)/

371
372 STRING web analysis (**Figure 4**) showed that the target proteins of 23 DE miRNAs (11
373 upregulated and 12 downregulated) had similar interactions to those expected for a random set
374 of proteins of similar size sampled from the *I. scapularis* genome (nodes = 687, edges = 79,
375 average node degree = 0.23, average local clustering coefficient = 0.0966, expected number of
376 edges = 77, PPI enrichment *p*-value = 0.411). This does not necessarily mean that these selected
377 proteins are not biologically meaningful, rather that these tick proteins may not be very well
378 studied and their interactions might not yet be known to STRING.

379 Many target genes were identified for the DE miRNAs using the sRNAtoolbox
380 miRNAconsTarget program [39]. To minimize false-positive targets, we chose only those targets
381 predicted by all three miRNA target prediction algorithms (TargetSpy, MIRANDA, and PITA).
382 Forty-one KEGG pathways were enriched for target genes (proteins) of DE miRNAs
383 (**Supplementary Table S2**) and included sphingolipid metabolism; valine, leucine, and isoleucine
384 degradation; lipid transport and metabolism; exosome biogenesis and secretion; and phosphate-
385 containing compound metabolic process (**Figure 4**). Gene ontology (GO) analysis indicated that
386 most target genes of DE miRNAs play significant roles in cellular processes, metabolic processes,
387 biological regulation, developmental processes, and responses to stimuli (**Figure 5**). Surprisingly,
388 immune response genes were one of the least affected functions.

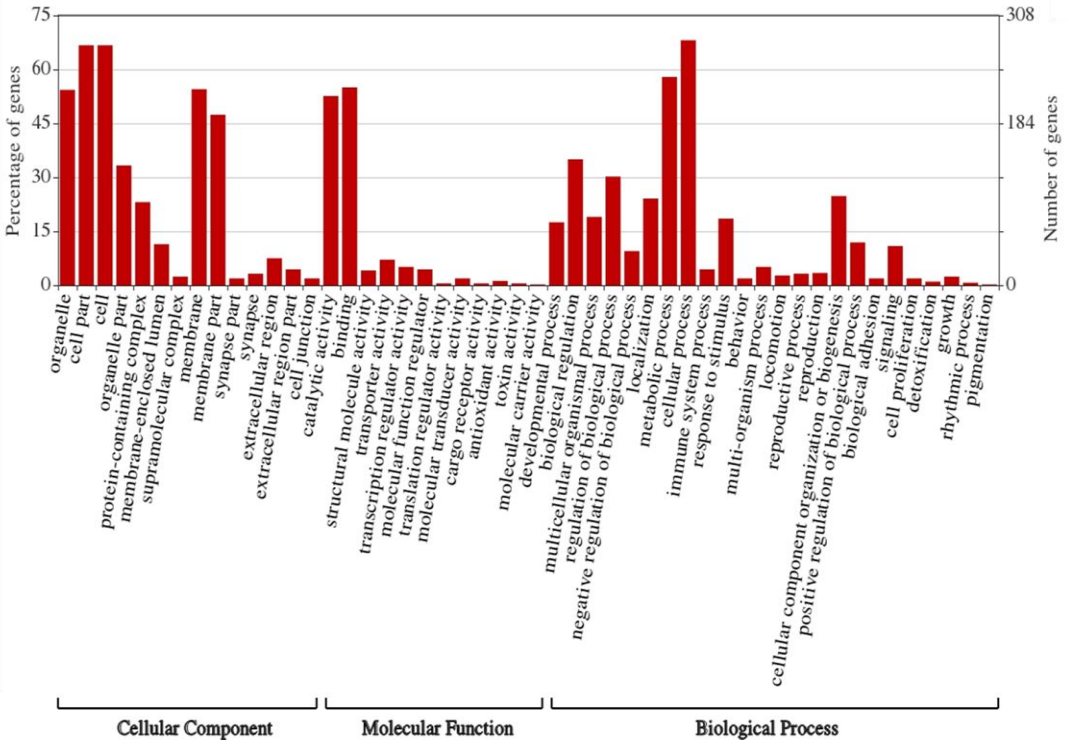
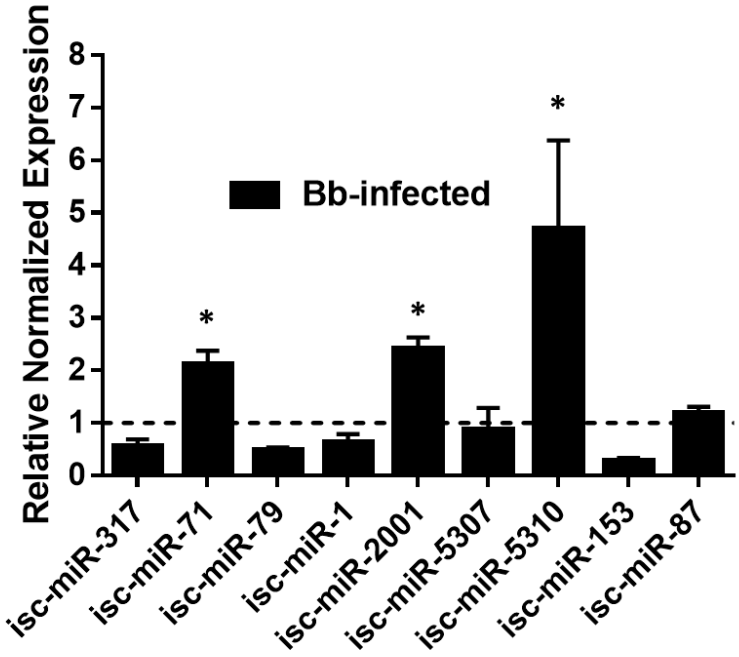


Figure 5. Gene ontology (GO)-derived biological processes related to genes targeted by differentially expressed miRNAs in partially fed *B. burgdorferi*-infected salivary glands relative to partially fed uninfected salivary glands from *Ixodes scapularis* ticks.

Lipid metabolism was one of the main KEGG pathways detected by Pannzer and STRING analyses and was predicted to be regulated by tick salivary gland miRNAs miR-1, miR-5310, miR-71, and miR-79. It has previously been shown that binding of *B. burgdorferi* to host glycosphingolipid can contribute to tissue-specific adhesion of *B. burgdorferi*, and the inflammatory process in Lyme borreliosis might be affected by interactions between *B. burgdorferi* and glycosphingolipid [69]. Therefore, we hypothesize that tick miRNAs (via saliva) promote sphingolipid synthesis inside hosts to promote *Borrelia* adhesion, and indeed there is evidence that its infection affects lipid metabolism in hosts [70].

403 **Validation of DE miRNAs by qRT-PCR**

404 The expression of DE miRNAs were validated in *B. burgdorferi*-infected and uninfected ISE6 cells
405 by qRT-PCR (**Figure 6; Supplementary Table S3**), which closely mirrored the RNA-seq data for
406 many targets, although some differences were not statistically significant by qRT-PCR and isc-
407 miR-317 was downregulated rather than upregulated. These differences could be due to the use
408 of different methodologies to quantify miRNA expression [10].



409

410 **Figure 6.** qRT-PCR validation of differentially expressed miRNAs detected in *Borrelia burgdorferi*-infected
411 ,partially-fed salivary glands relative to partially-fed uninfected salivary glands from *Ixodes scapularis*
412 ticks. qPCR validation was performed in *Borrelia burgdorferi* infected and clean ISE6 cells. Expression of
413 miRNAs was normalized to clean ISE6 cells (indicated as 1 on the y-axis). Statistical significance for qRT-
414 PCR-based differential expression was determined by the 2-tailed Student's *t*-test, where * is $p<0.05$.

415

416 ## Conclusions

417 This is the first comprehensive miRNA profiling study of *Ixodes scapularis* salivary glands with and
418 without *Borrelia burgdorferi* infection. Here we identified several potential miRNAs targets in tick
419 salivary glands which might play a significant role in *Borrelia* colonization, survival, transmission,
420 and host immunomodulation. Functional validation of these miRNAs is now required. Further
421 characterization of tick salivary gland miRNAs would contribute to a better understanding of the
422 mechanisms underpinning *Borrelia* transmission and propagation inside hosts, not least due to
423 its special status as an extracellular spirochaete and atypical Gram-negative organism that might
424 exploit different survival mechanisms. The impact of *B. burgdorferi* on miRNA expression must
425 also be studied in other tick tissues and hosts to understand cues of its vector competence in
426 ticks and immunomodulation in vertebrates.

427

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