1 **Title page**

2 **Running Head**

3 Response of sow to *ginseng polysacchari*

4

5 The effect of dietary ginseng polysaccharide supplementation on the immune
6 responses involved in porcine milk-derived esRNAs

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22 **Abstract:** Ginseng and its polysaccharides (GPS) have been well known as an
 23 immune modulator. This study was conducted to investigate the effects of dietary
 24 supplemental GPS on the immune responses involved in sow's milk-derived exosomal
 25 shuttle RNAs (esRNAs) using RNA-Seq and miRNA-Seq. Of the 213 identified
 26 miRNA types, a total of 26 conserved miRNAs were differently expressed in response
 27 to GPS supplementation, including 10 up-regulated and 16 down-regulated miRNAs
 28 in GPS feeding group. In addition, exosomal transcriptome analysis identified 14,696
 29 protein-coding genes in sow's milk exosomes, and 283 genes with 204 and 79
 30 candidates showing up and down-regulation were significantly responded to GPS
 31 supplementation. Integrated analysis of each differently expressed miRNA with
 32 significantly expressed genes further revealed the presence of 51 highly conserved
 33 miRNA-gene interactions that were annotated to be related to immunoregulatory
 34 functions. This work provided an important advance in the functional identification of
 35 dietary GPS supplementation and more fundamental information about how GPS
 36 promoted the immune response and healthy growth of the infant from mothers at
 37 molecular levels.

38

39 **Keywords:** esRNAs; exosome; ginseng polysaccharide; milk; sow

40

41 Introduction

42 Ginseng (*Panax ginseng* C.A. Meyer), one of the most well-known oriental
 43 medicine for several thousand years, has been widely used with mysterious powers as
 44 a tonic, prophylactic and restorative agent, etc. (Sun 2011) The polysaccharide
 45 extracted from the medicinal ginseng root (mostly), stems and leaves was
 46 demonstrated to have many functions, including inhibition of tumors (Li *et al.* 2014),
 47 suppression of bacterial (Fukuyama *et al.* 2012) and viral (Kim *et al.* 2011) activity,
 48 anti-peroxidatic reactions (Luo *et al.* 2008), and innate (Shin *et al.* 2002) or acquired
 49 (Sumiyoshi *et al.* 2011) immune modulation. In recent years, there has been growing
 50 interests in the use of polysaccharides as new, alternative immunologic additives for
 51 agricultural animals. Chen *et al.* (Chen *et al.* 2009) indicated an increase in cellular
 52 and humoral immunities by modulating the production of antibodies, complements
 53 and cytokines in the *Achyranthes bidentata* polysaccharide supplemented weaned
 54 piglets, conferring an important protective role in the non-specific defense against
 55 infections. In our previous study, dietary GPS significantly increased immune enzyme
 56 activity and modified expression of immune genes in shrimp (Liu *et al.* 2011).
 57 However, to date the effects of dietary polysaccharide supplementation on lactating
 58 sows was deficient.

59 In general, breast milk supplied the chief nutrient source during the natural
 60 suckling period of the neonates, and milk liquid with immune-related composition
 61 also provided immunity to the infant and affected the maturation of the infant's
 62 immune system (Admyre *et al.* 2007). Previous research has reported that exosomes
 63 were identified to present in the breast milk (Zhou *et al.* 2012), which were small
 64 membrane vesicles of endocytic origin that were released from the producing cell into
 65 the extracellular environment (Théry *et al.* 2002). And exosomes have been proposed
 66 to signal by binding to the recipient cell surface receptors or by internalisation with
 67 the cell membrane (Valadi *et al.* 2007), potentially donating substantial amounts of
 68 exosomal RNAs such as mRNAs, microRNAs (miRNAs), and other non-coding
 69 RNAs (ncRNAs) to other cells and subsequently affecting the protein production of a
 70 recipient cell (Sato-Kuwabara *et al.* 2005). In addition, previous research has revealed

the ability of human breast milk exosomes to potentially influence the immune system of the infant ([Admyre *et al.* 2007](#)).

Overall, we therefore hypothesized that dietary supplementation with GPS influenced the composition of sow milk, especially for immune-related esRNAs, and further enhanced the immune responses in suckling piglets. This hypothesis was performed by miRNA and RNA sequencing to analyze the porcine breast milk exosomes in response to GPS supplementation.

Materials and Methods

Animals and feeding

The GPS were extracted from the roots of *P. ginseng* according to the protocols described in our previous research ([Liu *et al.* 2011](#)). A total of 20 Large White sows at 90 days of gestation were acquired from WENS breeding pig farm (Qingyuan, Guangdong, China) and allocated into two dietary treatment groups on the basis of age, body weight and parities. Control group (Con) was fed the basal diet, and the diet of treatment group was added with 400 mg/kg GPS until 14th day after delivery. Porcine milk samples were collected at day 1, 3, 7 and 14 after parturition, and kept at -80°C until use.

Milk exosomes collection and sequencing analysis

Preparation of exosomes from porcine milk was operated as our previous descriptions ([Chen *et al.* 2014](#)). Total RNAs were extracted from pelleted exosomes using TRIzol[®] Reagent ([Life Technologies, Carlsbad, CA, USA](#)) by the manufacturer's protocol, and the quantity and purity were analysis using Bioanalyzer 2100 and RNA 6000 Nano Labchip Kit ([Agilent, CA, USA](#)) with RNA Integrity Number (RIN) value ≥ 7.0 . Prior to constructing Illumina-indexed libraries, the total RNAs from four different time points were pooled equally in each treatment group. Subsequently, indexed miRNAome and transcriptome sequencing libraries were constructed with Illumina TruSeq Small RNA Library Preparation Kits and TruSeq

100 RNA Library Preparation Kits ([Illumina, San Diego, CA](#)) respectively, which
101 included size selection of the final library amplicons. Finally, the libraries were
102 sequenced using Illumina HiSeqTM 2000, and the raw reads were demultiplexed and
103 the indexed adapter sequences were trimmed using the CASAVA v1.8.2 software.

104

105 **miRNA analysis**

106 Firstly, the raw reads of control and GPS supplemented group were processed
107 with ACGT101-miR program ([LC Sciences, Houston, Texas, USA](#)) to trim 3' adapter
108 and discard reads with poly N and reads shorter than 17 bp. Then, using BLAST
109 search all clean reads were aligned to porcine miRNA mature and precursor sequences
110 published in miRBase v21.0 (<http://www.mirbase.org/>) to identified conserved
111 miRNAs. The read count of each identified miRNA was firstly normalized to reads
112 per million, and the R v3.0.2 Bioconductor package EDGER v2.4.6 ([Robinson et al.](#)
113 [2010](#)) was applied to identify differentially expressed (DE) miRNAs with p value <
114 0.05 and fold change ≥ 2 between different groups.

115

116 **Align the RNA-seq reads to genome and assemble expressed transcripts**

117 Raw reads of fastq format were firstly processed by removing the low quality
118 reads and the reads that contain adapter or ploy-N. Then, the clean reads from each
119 library were aligned to the Sscrofa10.2 reference genome
120 (<http://hgdownload.soe.ucsc.edu/goldenPath/susScr3/bigZips/susScr3.fa.gz>)
121 downloaded from the the University of California Santa Cruz (UCSC) website with
122 TopHat v2.0.12 ([Trapnell et al. 2009](#)). The mapped reads of each group were
123 assembled by Cufflinks 2.2.1 ([Trapnell et al. 2012](#)), and using Cuffmerge two
124 assemblies were merged to create a single transcriptome annotation with porcine
125 Ensembl's genes generated by UCSC table browser for subsequent protein-coding
126 gene analysis. The gene expression level was analyzed by using RPKM method
127 (Reads per kilobase transcriptome per million mapped reads) ([Mortazavi et al. 2008](#)),
128 and only genes with False Discovery Rate (FDR) value < 0.05 that calculated by
129 Cuffdiff program and $|\log_2 \text{fold change}| \geq 1$ were considered as differently expressed

130 candidates.

131

132 **miRNA target prediction and network construction**

133 Putative targets of DE miRNAs were evaluated using miRanda (Betel *et al.*
134 2010), and only alignments with energies ≤ -20.0 kcal/mol and no mismatch in the
135 seed region (positions 2–8 in the 5' end) were used for further analysis. The
136 construction of interaction networks included three steps: (□) DE mRNAs and DE
137 protein-coding genes were first retained; (□) then mRNA-miRNA negative
138 interactions were predicted by miRanda analysis; (□) potential interactions of
139 targets-miRNA were established and visualized using Cytoscape V3.4
140 (<http://cytoscape.org/>).

141

142 **Results and Discussion**

143 **Sequence analysis of milk exosomal miRNAs**

144 Solexa sequencing provided a total of 13,256,489 and 11,349,485 raw reads of
145 51 nt from the Con and GPS libraries, respectively. After removing 3' adapter null or
146 5' adapter contaminants, low quality reads, and reads contained poly N or smaller than
147 17 nt, a total of 12,256,073 and 10,654,756 clean reads of 17–31 nt were obtained
148 (Table 1). Subsequently, clean reads were mapped to porcine miRNA mature and
149 precursor sequences published in miRBase 21.0 by BLAST search to identify known
150 miRNAs, and length variation at both 3' and 5' ends and one mismatch inside of the
151 sequences were allowed in the alignment. In total, we identified 213 unique miRNA
152 types aligned to 181 independent pre-miRNA loci with read number more than 10,
153 and 188 known miRNAs were expressed in both Con and GPS groups (Table S1A).
154 The 10 most highly expressed miRNAs in each group accounted for $89.64\% \pm 3.07\%$
155 of the total counts of all unique miRNAs, and four of these miRNAs
156 (ssc-miR-148a-3p, ssc-miR-30a-5p, ssc-miR-21 and ssc-miR-26a) were found in
157 common across two tested groups, indicating that the majority of miRNAs were
158 expressed from very few loci that maybe play very important roles in milk. Recently,
159 similar miRNAs were identified by sequencing of porcine (Chen *et al.* 2014), bovine

(Sun *et al.* 2015) and human (Chen *et al.* 2010) milk exosomes, and miR-148a-3p and miR-30a were also represented as the top 10 miRNAs. Of the identified miRNAs, the most abundant was miR-148a-3p, which represented $56.02\% \pm 5.88\%$ reads across two libraries. The predominance of miR-148a was consistent with its well-established function as a nutritional biomarker corresponding to the protein content of various bovine-derived milk products (Chen *et al.* 2010). Interestingly, feeding studies that have shown exogenous plant (Zhang *et al.* 2012) or milk (Baier *et al.* 2014) miRNAs can be found in the sera and tissues and influence regulation of target genes in recipient animals, suggesting that the enrichment of specific miRNAs derived from milk in our study may also influence the growth and development of neonates. Specifically, miR-148a influenced dendritic cell activation and maturation by down-regulating calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) expression, and finally associated with anti-inflammatory responses (Turner *et al.* 2011). MiR-30a attenuated immunosuppressive functions of IL-1 β -elicited mesenchymal stem cells via targeting transforming growth factor- β -activated kinase 1 binding protein 3 (TAB3) (Hu *et al.* 2015). Also widely studied for possible involvement in the pathogenesis of multiple autoimmune and chronic inflammatory disorders, miR-21 expression was specifically elevated in Th17 cells, and T cell-intrinsic expression of miR-21 was important for effective Th17 differentiation (Murugaiyan *et al.* 2015). In addition, cellular miR-26a suppressed replication of porcine reproductive and respiratory syndrome virus by activating innate antiviral immunity (Jia *et al.* 2015). Even though there were compelling evidences that the most prevalent miRNAs in our study could potentially exert an influence on immune response, the specific functional roles of these miRNAs need further detailed investigations to obtain a thorough understanding of the specific targets and mechanistic effects on consumption of miRNA-loaded porcine milk exosomes by a recipient animal.

189 Expression analysis on porcine milk exosomal transcriptome

190 The single-end RNA reads generated from sequencing of the exosomal libraries
191 were trimmed to remove adapter sequences and then filtered. After filtering
192 procedures, a total of 77,106,888 and 82,953,484 high-quality reads were considered
193 for further analysis, resulted in ~6.46 and ~6.95 gigabases (Gb) from Con and GPS
194 libraries, respectively. We aligned all these high-quality reads onto the porcine
195 Sscrofa10.2 reference genome, and found that over $63.59\% \pm 0.25\%$ of the reads were
196 mapped to the genome, including $58.31\% \pm 0.23\%$ of the mapped reads that were
197 aligned uniquely in each library (Table S2A). Transcripts assembled with mapped
198 reads using cufflinks revealed a total of 14,696 protein-coding genes across the two
199 libraries, and Con specific units accounted for 95.30% of all identified genes, as well
200 as 94.55% existed in the GPS library (Table S2B), consistent with previous study
201 indicating that 19,320 mRNA transcripts were present in bovine milk exosomes
202 (Izumi *et al.* 2015). In addition, the top 100 highly expressed genes in sequencing
203 libraries accounted for $72.91\% \pm 0.96\%$ of the total expression levels calculated by
204 RPKM value, and a total of 98 genes were common to both groups. We then
205 performed Gene Ontology (GO) enrichment analysis of these highly expressed genes
206 using the DAVID bioinformatics resource, which employs a Fisher's Exact Test with
207 Benjamini–Hochberg correction. A total of 82 enriched GO categories were derived
208 using a P-value cut-off of $P < 0.05$, including 56 Biological Process (BP), 19 Cell
209 Component (CC) and 7 Molecular Function (MF) categories (Table S2C), respectively.
210 These categories were mainly focused on translational elongation, ribosome
211 biogenesis, rRNA processing and rRNA metabolic process, clearly indicating that the
212 exosomal RNAs were enriched in rRNA-family species (Jenjaroenpun *et al.* 2013).
213 Jenjaroenpun *et al.* found that cellular exosomes contained various classes of RNA
214 moleculars with the major class represented by fragmented ribosomal RNA (rRNA),
215 in particular 28S and 18S subunits. The observation in our and previous studies
216 suggested that the majority of exosomal rRNAs were fragmented, and these results
217 may explain why the 28S and 18S rRNA cannot be detected in exosomal total RNAs
218 (Gu *et al.* 2012).

219

220 **miRNA and their target expression in response to GPS supplementation**

221 After normalization, miRNAs that changed more than 2-fold and p value less
222 than 0.05 were considered to be differently up or down regulated in response to GPS
223 supplementation. In total, there were 10 miRNAs up-regulated in the milk exosomes
224 of GPS group compared with control, as well as 16 down-regulated miRNAs ([Table](#)
225 [S1B](#)). To identify the potential function of these DE miRNAs, target prediction was
226 performed using miRanda software. Prediction analyses yielded a total of 3,525
227 unique genes potentially regulated by DE miRNAs. This resulted in 4,902
228 miRNA-target interactions; 3,271 of these were targeted by up-regulated miRNAs,
229 and 1,631 were targeted by down-regulated miRNAs ([Table S1C](#)). The KEGG
230 pathway analysis of the predicted targets by DAVID Bioinformatics Resources
231 revealed 22 unique KEGG terms with P value < 0.05, which had been previously
232 implicated in immune and disease. These pathways included the top 5 statistical
233 enrichment, namely Pancreatic cancer, MAPK signaling pathway, B cell receptor
234 signaling pathway, T cell receptor signaling pathway and Fc gamma R-mediated
235 phagocytosis ([Table S1D](#)), suggesting the functions of GPS in immune enhancement
236 and regulation.

237 Previous reports have documented the functional connection between RNA
238 editing and miRNA-mediated post-transcriptional gene silencing ([Bartel et al. 2009](#)).
239 To characterize the potential effects of RNA editing on miRNAs in porcine milk
240 exosomes, we first profiled porcine milk exosomal transcriptome between Con and
241 GPS groups. Of 14,696 identified protein-coding genes, a total of 283 exosomal genes
242 were further considered to be differently expressed in response to GPS
243 supplementation based on $|\log_2 \text{fold change}| \geq 1$, $\text{FDR} < 0.05$ and $\text{gene coverage} \geq 60\%$,
244 including 204 up-regulated and 79 down-regulated genes in GPS groups ([Table S2D](#)).
245 Next, we aimed to compute all possible interactions of each DE miRNA with
246 significantly expressed genes. Generally, an up-regulation of a target gene indicates a
247 decrease activity of the corresponding miRNA; therefore, a miRNA-mRNA
248 interaction pair means anti-regulation of a miRNA and a corresponding gene. In final,
249 miRanda analysis revealed the presence of 51 highly conserved miRNA-mRNA

interactions. Of these negative interactions, a total of 9 down-regulated genes appeared to be targeted by 8 up-regulated miRNAs in GPS group, and 17 up-regulated genes was targeted by 12 down-regulated miRNAs (Figure 1). By applying a cut-off criterion of FDR value < 0.05, GO enrichment analysis of these target candidates revealed a few important terms those were significantly enriched in the functions of immunity, such as neutrophil chemotaxis (CCL3L1, FCER1G, TGFβ2), phagocytosis (FCER1G, BIN2), cell chemotaxis (CXCL2, BIN2) and chemokine-mediated signaling pathway (CCL3L1, CXCL2) (Table S2E). In details, CCL3L1 dramatically influenced cell-mediated immunity (Dolan *et al.* 2007) and was targeted by ssc-miR-19a in our paper. Phagocytosis-related molecule FCER1G played a role in uptake of antigens bound to immunoglobulin E (Landi *et al.* 2011), and cytokine TGFβ2 acted an integral role in regulating immune responses (Rautava *et al.* 2011), these implying the biological functions of their anti-corresponding miR-22-3p and miR-374b-5p in porcine milk exosomes. In addition, kidney-specific miR-192 (Liang *et al.* 2007) significantly involved in immune-related gene expression (Wu *et al.* 2012), consistent with our results in negative regulation of BIN2, a membrane sculpting protein that influenced leucocyte podosomes, motility and phagocytosis (Sánchez-Barrena *et al.* 2012). And mast cell and macrophage chemokine CXCL2, a target gene of miR-30a-3p, controlled the early stage of neutrophil recruitment during tissue inflammation (De Filippo *et al.* 2013). Taken together, we found that the expression of several key miRNAs and genes in porcine milk-derived exosomes were induced or decreased under GPS supplementation in porcine basal diets, and these miRNA-mRNA interactions were reported to be significantly related to immunoregulatory functions. Meanwhile, previous research has showed that exosomal mRNAs or miRNAs, termed esRNAs, can be delivered to another cell and take effect on the new location (Valadi *et al.* 2007). Therefore, the findings suggested the functions of dietary GPS supplementation in enhancing the immune-related esRNAs expression in milk-derived exosomes, and these candidates may be further transferred to suckling piglets for immune system development and healthy growth.

In conclusion, we detected 26 miRNAs and 283 genes in porcine milk-derived

280 exosomes that showed differential expression in response to GPS supplementation in
281 sow's basal diets. Integrated analysis and functional annotation revealed 51 highly
282 conserved miRNA-mRNA interactions that were significantly related to
283 immunoregulatory functions. This work provided an important advance in the
284 functional identification of dietary GPS supplementation and more fundamental
285 information about how GPS promoted the immune response and healthy growth of the
286 infant at the molecular level.

287

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294 interests.

295

296 **Conflict of Interests**

297 The authors have declared that no conflict of in-terest exists.

298

299 **Supporting information available**

300 Known miRNAs identified in both Con and GPS groups were showed in Table
301 S1A; differently expressed miRNAs between Con and GPS groups in response to
302 GPS supplementation were showed in Table S1B; miRNA target gene prediction were
303 showed in Table S1C; KEGG analysis of the target genes of DE miRNAs were
304 showed in Table S1D. Summary of RNA-seq dates were showed in Table S2A;
305 Protein-coding genes identified in both Con and GPS groups were showed in Table
306 S2B; Gene Ontology (GO) enrichment analysis of highly expressed genes were
307 showed in Table S2C; Differently expressed genes between Con and GPS groups in
308 response to GPS supplementation were showed in Table S2D; GO enrichment
309 analysis of target candidates presented in miRNA-mRNA interaction network were

showed in Table S2E.

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425

426 **FIGURE CAPTIONS**

427

428 **Figure 1.** miRNA-mRNA correlation networks

429 Note: Circular nodes represented targets and triangular nodes represented miRNAs.

430 Red nodes represented an up-regulation and green represented a down-regulation

431 relative to match in GPS group. The size of each node represented for average

432 expressed level of each gene between two groups, and the significant levels between

433 different groups were associated with the color of node deepening.

434

435

436

Table 1 Summary of small RNA sequencing data

Type	Con		GPS	
	Count	Percent(%)	Count	Percent(%)
Total reads	13256489	100%	11349485	100%
3' adapter NULL	193785	1.46%	39610	0.35%
Insert NULL	147791	1.11%	106849	0.94%
5' adapter contaminants	14380	0.11%	4129	0.04%
Removed inferior quality	95210	0.72%	61994	0.55%
Smaller than 17 nt	549207	4.14%	482024	4.25%
Poly-A/T/C/G/N	43	0.00%	123	0.00%
Clean Reads	12256073	92.45%	10654756	93.88%

Figure 1