

# Exploring the potential of salivary small RNAs as non-invasive biomarkers in pigs

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## Competing interests

No potential conflict of interest relevant to this article was reported.

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## Abstract

Saliva, a non-invasive potential source of circulating microRNAs (miRNAs) and microbiomes, is not well described in pigs. Salivary miRNA expression profiles and the functional significance in pigs were investigated in this study. Saliva samples were extracted from adult female pigs, and small RNA sequencing revealed 26 known and 223 novel miRNAs. The large number of novel miRNAs also demonstrates the differences between salivary miRNAs in pigs and other biological samples. Functional analysis of miRNA target genes indicated enrichments in molecular functions related to transcription regulator activity, cytoskeleton organization, and protein binding, suggesting roles for this interaction in gene expression and physiological control. Moreover, metagenomic analysis revealed microbial sequences representing around 39% of the total reads, with *Corynebacterium* genus, an important member of the oral microbiota, being the most prevalent. Combining miRNA with microbiome data indicates that porcine saliva is rich in molecular information that will be useful for salivary health monitoring and microbiome studies. This study underscores the potential of salivary miRNAs as biomarkers for physiological processes and microbiome interactions in pigs, paving the way for further research into their diagnostic and monitoring applications.

**Keywords:** Pig, Saliva, Transcriptome, MicroRNA, Microbiome, Non-invasive biomarker

## INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs with a length of 22 nucleotides [1]. They regulate the transcription and post-transcription of gene expression by mRNA degradation or translational repression depending on the binding to the 3' untranslated region (UTR) of target mRNA [2,3]. MiRNAs derived from various cell types are secreted into the extracellular space through exosomes, protein complexes, etc. [4], and then transferred to the body fluid. These are commonly known as circulating miRNA, showing potential as biomarkers for disease diagnosis [5]. While most studies on circulating miRNAs have focused on human and livestock blood [6,7], recent studies have identified miRNAs in saliva, leading to an increase in saliva-based research [8,9]. Saliva shares various physiological characteristics and allows for repeated specimen collection, similar to blood. Saliva contains miRNAs, proteins, and hormones, providing insights into its physiological properties and

#### Availability of data and material

Sequence data has been submitted to SRA under accession numbers PRJNA1182251. Data will be made available on request.

#### Authors' contributions

Conceptualization: Jeong GH, Lim KS.  
Data curation: Jeong GH.  
Formal analysis: Jeong GH.  
Investigation: Jeong GH, Lim KS.  
Writing - original draft: Jeong GH.  
Writing - review & editing: Jeong GH, Lim KS.

#### Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

offering further understanding of several biological processes [10]. Blood collection is painful and stressful, whereas saliva sampling does not cause pain and can be easily performed even by untrained individuals [10,11]. Investigating salivary miRNAs provides promising insights for the non-invasive monitoring of health status and related diseases. Additionally, saliva samples may contain diverse microorganisms that can provide further insights into its biological characteristics and relationship with the gut microbiome. Therefore, saliva sampling has attracted considerable interest in human research [8,12,13]. The miRNAs and the microbiome are widely recognized as key indicators of livestock health, productivity, stress, and disease [14–16]. And miRNAs have been used as biomarkers for monitoring health conditions, including inflammation, metabolic disorders, and stress responses, while the microbiome offers insights into gut health, immune function, and overall well-being. In pigs, salivary miRNAs such as miR-19b, miR-27b, and miR-365 have been identified as potential biomarkers for assessing pain and stress, particularly in response to procedures like castration and tail docking, with their expression levels suggesting a viable approach for non-invasive pain monitoring [9]. However, studies on salivary circulating miRNA expression in pigs remain limited.

Therefore, this study aimed to screen salivary miRNAs in pigs and investigate their biological functions. Additionally, we aimed to preliminarily explore the presence and potential role of the salivary microbiome, thereby enhancing our understanding of the biological value of porcine saliva.

## MATERIALS AND METHODS

### Animals and saliva sample collection

Saliva samples were collected using specialized salivary tubes (Salivette, Sarstedt) from two adult female pigs, consisting of a gilt (Duroc, approximately 46-week-old) and a sow (Landrace, approximately 197-week-old). All animals were housed and cared for at the Kongju National University Animal Farm. And the cotton roll was kept in the pig mouth, allowing the animal to chew it for 2–3 min followed by centrifugation of sponge-containing Salivette tubes at 1,660×g for 20 min to collect saliva.

### Small RNA extraction and sequencing

Small RNA was extracted from the saliva using a XENOPURE™ Plasma/Serum Small RNA Purification Kit (Xenohelix). RNA quality was assessed using an Agilent Technologies TapeStation 2200 (Agilent Technologies). Sequencing was performed using 100 base single-end reads on a NovaSeq 6000 sequencer (Illumina).

### Salivary small RNA processing

Raw reads were quality-checked using FASTQC (v0.12.1) [17], and adaptor and low-quality sequences were trimmed using Cutadapt (v4.7) [18]. Trimmed reads ( $\geq 15$ nt) were then aligned to the porcine reference genome (*Sus scrofa* 11.1.106, GCA\_000003025.6) using Bowtie1 (v1.3.1) [19] for detecting miRNAs. Novel and known miRNAs were predicted using miRDeep2 with miR databases for various species, including *Sus scrofa*, *Bos taurus*, *Equus caballus*, *Ovis aries*, *Canis familiaris*, and *Homo sapiens* [20]. Separately from this, the trimmed reads were also aligned using STAR (v2.7.11b) to the presence of other types of small RNA transcripts with splicing events in porcine saliva [21].

### Target gene prediction and functional annotation

To further explore the potential biological functions and processes of miRNAs in porcine saliva,

the potential target genes of miRNAs were predicted using the TargetScan (v8.0) tool [22]. For the predicted target genes of salivary miRNAs, the biological process, cellular component, and molecular function from gene ontology (GO) were used to annotate functions using the Database for Annotation, Visualization, and Integrated Discovery [23] using the default options. A GO bubble plot was generated using the SRplot tool [24].

### Taxonomic classification of microbial small RNAs

Taxonomic classification of salivary small RNA sequences in pigs was performed using Kraken2 [25], and the taxonomic composition was visualized using Krona [26].

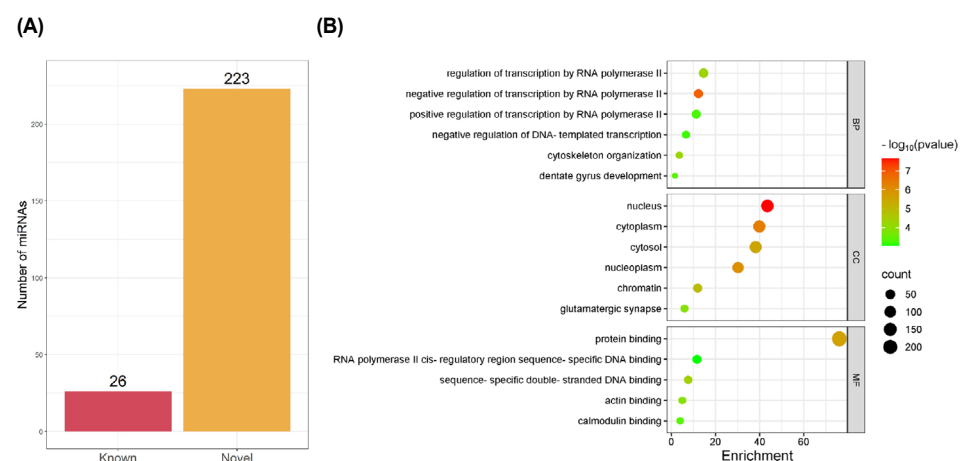
## RESULTS AND DISCUSSION

### Identification of known and novel miRNAs in porcine saliva

Saliva was collected from adult female pigs with well-developed salivary glands, and 26 known miRNAs were detected via small RNA sequencing (Fig. 1A and Table 1). Among these, miR-19b has been highlighted in a previous study as a potential biomarker of inflammatory responses in piglets after tail docking and castration [9]. Additionally, 223 novel miRNAs were identified based on miRNA sequence databases of other species. This result may be related to the relatively low number of annotated miRNAs in pigs in miRBase 22.1 [27], compared with that in humans and other livestock species [28], which likely explains the high number of novel miRNAs annotated. Alternatively, these novel miRNAs could be saliva-specific. The sizable detection of novel miRNAs supports the potential of saliva as a specimen for investigating miRNA profiles and their biological processes in pigs. Saliva is a body fluid sample that can be easily collected by anyone through a noninvasive method, and it has various molecular characteristics, which may provide valuable insight as potential biomarkers for disease diagnosis and evaluating individual disease resilience.

### Functional analysis of microRNAs target genes

GO analysis of the target genes of known miRNAs revealed their involvement in key biological processes, such as transcriptional regulation, cell structure maintenance, and neural development, primarily functioning within the nucleus and cytoplasm (Fig. 1B). In particular, RNA polymerase



**Fig. 1.** The number of salivary microRNAs (miRNAs) in pigs (A) and bubble plot for gene ontology (GO) analysis of their candidate target genes (B).

**Table 1.** List of Known miRNAs and their sequence in porcine saliva

No.	miRNA	miRNA sequence
1	ssc-let-7a	UGAGGUAGUAGGUUGUAUAGUU
2	ssc-let-7c	UGAGGUAGUAGGUUGUAUGGUU
3	ssc-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
4	ssc-let-7g	UGAGGUAGUAGUUUGUACAGUU
5	ssc-let-7i-5p	UGAGGUAGUAGUUUGUGCU
6	ssc-miR-1	UGGAAUGUAAAGAAGUAUGUA
7	ssc-miR-103	AGCAGCAUUGUACAGGGCUAUGA
8	ssc-miR-107	AGCAGCAUUGUACAGGGCUAUGA
9	ssc-miR-1343	CUCCUGGGGCCCGCACUCUCGC
10	ssc-miR-142-5p	CAUAAAGUAGAAAGCACUACU
11	ssc-miR-191	CAACGGAUCCCAAAGCAGCUG
12	ssc-miR-19b	UGUGCAAUCCAUGCAAAACUGA
13	ssc-miR-200b	UAAUACUGCCUGGUAUAGUAGC
14	ssc-miR-205	UCCUUAUCCACCGGAGUCUG
15	ssc-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
16	ssc-miR-23a	AUCACAUUGCCAGGGAUUUCC
17	ssc-miR-23b	AUCACAUUGCCAGGGAUUACCA
18	ssc-miR-24-1-5p	GUGCCUACUGAGCUGAAACACAGU
19	ssc-miR-24-2-5p	GUGCCUACUGAGCUGAUUACAGU
20	ssc-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
21	ssc-miR-30e-5p	UGUAAACAUCUUGACUGGAAGCU
22	ssc-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
23	ssc-miR-7-5p	UGGAAGACUAGUAAUUUGUUGUU
24	ssc-miR-92a	UAUUGCACUUGUCCCGGCCUGU
25	ssc-miR-92b-5p	AGGGACGGGACGCGGUGCAGUGUU
26	ssc-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG

II is the key enzyme responsible for protein-coding genes transcription [29], and miRNAs can regulate gene expression in both positive and negative ways [30]. Their interactions with RNA polymerase II suggest that miRNAs may act as significant regulators of gene expression. The cytoskeleton organization process is crucial for maintaining the structural stability of cells. MiRNAs involved in this process can regulate the expression of genes that control the assembly and dynamics of the cytoskeleton. According to previous studies, miRNAs are an important role in regulating cytoskeletal proteins [31–33], which affects cell signaling and structural integrity. Furthermore, the dentate gyrus development term, which is essential for neurogenesis and neuronal development [34], was significantly enriched in the GO analysis. This implies that miRNAs can regulate the expression of genes involved in dentate gyrus development [35]. In addition, the high enrichment of ‘protein binding’ suggests that salivary miRNAs may influence various physiological functions in pigs by regulating protein-protein interactions. These results suggest that porcine saliva contains functional miRNAs with important roles in the physiological regulation of gene expression and various physiological functions in pigs.

### Microbial composition of porcine saliva

The STAR (29.4%–33.5%) and Bowtie (2.04%–3.42%) mapping rates differed notably (Table 2), likely due to ability of STAR to recognize splicing events [21]. Overall, the relatively low mapping

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**Fig. 2.** Krona plot for taxonomic abundance of salivary small RNAs in pigs.

considerable in further studies to improve the accuracy and reliability of saliva samples.

## CONCLUSIONS

In this study, we identified both known and novel miRNAs in porcine saliva, highlighting its potential as a valuable sample for miRNA exploration and microbiome analysis. The diverse microbial presence, including known oral microbiota, such as *Corynebacterium*, suggests that saliva is a promising sample for noninvasive monitoring of microbiome diversity and physiological states in pigs. However, the origin of the unmapped reads remains unclear and requires further investigation. This study lays the groundwork for future research on the diagnostic potential of miRNAs and unidentified components in porcine saliva, offering new possibilities for noninvasive health monitoring in pigs.

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