*Review*

# MiRNA regulatory functions in farm animal diseases, and biomarker potentials for effective therapies

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**Abstract**

MicroRNAs (miRNAs) are small endogenous RNAs that regulate gene expression post-transcriptionally by targeting either the 3′ untranslated or coding regions of genes. They have been reported to play key roles in a wide range of biological processes. The recent remarkable developments of transcriptomics technologies, especially next-generation sequencing technologies and advanced bioinformatics tools, allow more in-depth exploration of messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs) including miRNAs. These technologies have offered great opportunities for a deeper exploration of miRNA involvement in farm animal diseases, as well as livestock productivity and welfare. In this review, we provide an overview of the current knowledge

of miRNA roles in farm animal diseases with a particular focus on diseases of economic importance. In addition, we discuss the steps and future perspectives of using miRNAs as biomarkers and molecular therapy for livestock disease management as well as the challenges and opportunities for understanding the regulatory mechanisms of miRNAs related to disease pathogenesis.

**Keywords:** Livestock diseases, miRNAs, biomarkers, regulatory networks, mastitis, PRRSV, foot-and-mouth disease, Marek's disease, RNAi therapy.

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## 1. Introduction

MicroRNAs (miRNAs), defined as short non-coding RNA (ncRNA) molecules of about 22 nucleotides in length, regulate a variety of biological processes through post-transcriptional regulation of gene expression. They control the activities of protein-coding genes as well as participate in gene regulation of many cellular processes in animals. MiRNAs often have multiple transcription start sites and regulate gene expression by inhibiting translation initiation or elongation, co-translational protein degradation, and premature termination of translation [1,2]. Besides this, they are also known to form miRNA-mRNA and miRNA-lncRNA pairs, to influence gene regulation and biological activities [1].

From the identification of the first miRNA (lin-4) in 1993 [3], advances in next-generation (NGS) and third-generation sequencing (TGS) technologies in the last decade heralded a new era and ability to identify various classes of small RNA molecules, including miRNA, in different biological samples at unprecedented depth [4]. The advantages offered by multiple sequencing platforms (e.g., Illumina, Ion Torrent, SOLiD, etc.) and bioinformatics data management capabilities support in-depth miRNA sequencing (miRNA-Seq) with the possibility to identify known and novel miRNAs [5,6], mutations [7], and their potential functions[8].

Along with NGS and TGS tools, bioinformatics tools for miRNA sequencing data analyses have progressed quickly from the development of pipelines for processing sequencing data to the inference of miRNA functions. The global processes and tools involved in miRNA discovery in NGS data have been summarized in recent reviews [4,9-11]. Following miRNA discovery, pathways analysis tools (e.g. miRNet [12] or miRPathDB 2.0 [13]) are used to predict their potential functions. Moreover, experimental or wet lab approaches are used for further functional validation of miRNA

target genes and functions. Since the discovery of lin-4 [3], thousands of miRNAs have been identified in humans, mouse, farm animal species and deposited in miRNA databases (Table 1).

**Table 1. Number of detected miRNAs and miRNA related studies in farm animal species***

| Specie | Precursor miRNA | Mature miRNA | Number of studies related to miRNA |
|---|---|---|---|
| Cattle | 1064 | 1025 | 870 |
| Sheep | 106 | 153 | 176 |
| Goat | 267 | 436 | 170 |
| Pig | 408 | 457 | 798 |
| Chicken | 882 | 1232 | 621 |

*Data source: MiRBase release 22 (http://www.mirbase.org/), PubMed data bases (August 2020) with the keywords: 'species name + miRNA.'

Livestock diseases are responsible for huge economic losses to the livestock industry and cause important issues of animal welfare [14,15]. Moreover, many livestock diseases can be transmitted to humans with the potential to cause health issues and even death. The effective control of livestock diseases is a global challenge for the livestock industry requiring multiple layers of control and intervention [16-18]. Many livestock diseases such as mastitis, paratuberculosis and bovine viral diarrhea (BVD) in cattle, porcine reproductive and respiratory syndrome (PRRS) and African swine fever (ASF) in pigs, and Newcastle disease and Avian influenza in poultry, require multi-disciplinary or holistic approaches for effective management and control. Vaccination, therapeutic treatments, and eradication strategies are traditional and routine methods to combat diseases, while modern methods such as genome editing, and RNA interference (RNAi) can lead to the development of alternative strategies for combating disease outbreaks. However, instead of combating diseases, farmers can select animals based on the genetic resistance of health traits, which has been regarded as a sustainable method [14,15]. Together with genetic markers, targeting epigenetic markers and miRNAs have been regarded as a further strategy for combating livestock diseases [19-22].

The potential roles of miRNAs in farm animal diseases have been summarized in a few reviews [23-28]. These reviews however, only provided an overview of the changes in miRNA expression profiles during disease progression. In this review, we present an in-depth and up to date review of miRNA roles in the main farm animal diseases and argue for the potential use of miRNAs as biomarkers for animal disease management.

## 2. MIRNA roles in farm animal diseases

In this section, we present reported changes in miRNA expression profiles during disease pathogenesis in farm animal species including cattle, pig, chicken, sheep, and goat. We also focus on the most important economic diseases of livestock.

### 2.1 Potential regulatory roles of miRNAs in cattle diseases

The first miRNA expression study on bovine adipose, mammary gland, immune-related and embryonic tissues was performed in 2007 [29]. Since then, over 870 studies have characterized about 1,064 precursors and 1,025 mature miRNAs, encoded on all 30 chromosomes, in the *Bos taurus* genome (Table 1). These studies demonstrated crucial regulatory roles of miRNAs in many biological processes in bovine, including mammary gland development and lactation (reviewed in [30]), bovine immunity (reviewed in [31] ) and diseases (review in [23]), and embryo development (reviewed in [32]). This section provides an update on miRNA potential roles in the major bovine infectious diseases, including mastitis, paratuberculosis and bovine viral diarrhea. Important miRNAs for these diseases are listed in Table 2.

**Table 2. Important miRNAs for bovine diseases**

| Diseases | Pathogens | Phenotype or tissue | Changed or potential miRNA biomarkers | References |
|---|---|---|---|---|
| Mastitis | *Streptococcus uberis* | BMEC[2] | miR-200c, miR-210, miR-193a, miR-29b-2, miR-130a, miR-98, let-7b, miR-24-2, miR-128-2, let-7d, miR-128-1, let-7e, miR-185, miR-652, miR-494, miR-2342, miR-29c, miR-29e, miR-29b-2, miR-100, miR-130 | [33] |
| | | BMEC | miR-181a, miR-16 and miR-31, | [34] |

| | | | | |
|---|---|---|---|---|
| | | Milk | miR-27b, miR-152, miR-194, miR-200b, miR-222, miR-379 and miR-18397 | [35] |
| | | Blood | miR-25, miR30e-5p, miR-342, miR-191, miR-399b, miR-451 and miR-486 | [36] |
| | *Staphylococcus aureus* | BMEC | miR-2339, miR-21-3p, miR-423-5p, miR-499, miR-92a, miR-193a-3p, miR-23a, miR-99b, miR-21-3p, miR-193a-3p, miR-365-3p, miR-30c, and miR-30b-5p | [37] |
| | | BMEC | miR-193a-3p, miR-365-3p, miR-184, miR-24-3p, miR-30c, miR-30b-5p, miR-21-3p, miR-148a, miR-423-5p, miR-92a, miR-423-5p, miR-21-3p, let-7a-5p, miR-184 miR-un5, miR-486 and miR-193a-3p | [37] |
| | *Escherichia coli* | BMEC | miR-223, miR-16, miR-136, miR-136, miR-3660, miR-335 and miR-378 | [38] |
| | *Escherichia coli* & *Staphylococcus aureus* | BMEC | miR-144, miR-451 and miR-7863 | [39] |
| | *Streptococcus* agalactiae | milk | miR-21, miR-146a, miR-155, miR-222, and miR-383 | [40] |
| | CMT[1] | milk | let-7i, miR-21, miR-27, miR-99b, miR-146, miR-147, miR-155 and miR-223 | [40] |
| Bovine tuberculosis | *Mycobacterium bovis* | Lung | bta-miR-142-5p, bta-miR-146a and bta-miR-423-3p | [41] |
| Johne's disease | *Mycobacterium avium* subsp. paratuberculosis | Blood | mir-19b, mir-19b-2, mir-1271, mir-100, mir-301a, mir-32, mir-6517 and mir-7857 | [42] |
| | | Ileum | miR-146 b, miR-196 b, miR-2483–5p, miR-133b, miR-1247-5p, miR-184, miR-202, miR-105a, novel-53, miR-433, miR-2400, miR-137, miR-424–3p and miR-138 | [43] |
| | | Serum | miR-1976, miR-873-3p, miR-520f-3p, and miR-126-3p | [44] |
| | | faeces | miR-223, miR-19b, miR-27b, miR-30d, miR-24 and miR-16 | [45] |
| Bovine viral diarrhea | Bovine viral diarrhea virus | serum | miR-423-5p and miR-151-3p | [46] |
| Foot and Mouth disease | Foot and Mouth disease virus | serum | miR-17-5p, miR-31 and miR-1281 | [47] |

[1]**CMT, California mastitis test;** [2]BMEC, bovine mammary epithelial cells

### *2.1.1 MiRNA and mastitis*

Mastitis, a common inflammatory disease of the mammary gland can develop into a clinical or subclinical type of infection depending on the causal pathogen [48]. Mastitis infection is caused by diverse pathogens including, but not limited to, *Escherichia coli*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Bacillus spp*, and *Staphylococcus aureus, etc. [49].* Dairy cow intramammary infections due to *S. aureus* have received much attention because of their major economic impact on dairy farms [50,51]. Therefore, more studies have investigated the role of miRNA functions in relation to this pathogen [33,34,37,39,52-58]. Jin et al. [37] performed miRNA expression profiling in MAC-T cells (bovine mammary epithelial cell line) challenged with heat-inactivated *S. aureus* or *E. coli* bacteria at 0, 6, 12, 24, and 48 hrs and reported a pathogen directed miRNA expression pattern whereby four differentially expressed (DE) miRNAs (miR-2339, miR-499, miR-23a and miR-99b) were unique to *S. aureus* while 5 (miR-184, miR-24-3p, miR-148, miR-486, and let-7a-5p) were unique to *E. coli*. Interestingly, the authors also observed a slower initial response of miRNAs to *S. aureus* bacteria (only one DE miRNA reported after 6 hr of infection) compared to *E. coli,* which initiated an earlier miRNA response (six DE miRNAs reported after 6 hr of infection) [37]. Li et al. [59] identified many DE miRNAs (77) between mammary gland tissues from cows infected with *S. aureus* as compared to healthy or control groups and some notable highly expressed miRNAs included miR-223, miR-1246, miR-142-5p (up-regulated) and miR-1, miR-23a, miR-31, miR-23b-3p, miR-26a and miR-145 (down-regulated). By target gene enrichments, the authors indicated potential functions of the DE miRNAs in regulating endocytosis pathway and olfactory transduction pathways involved in cancer [59]. Luoreng et al. [39] identified 305 and 279 DE miRNAs in bovine mammary gland tissues following experimental *E. coli* or *S. aureus* challenge, respectively. Moreover, the results of comparison of DE miRNAs between the *E. coli* and *S. aureus* infected groups identified 197 common DE miRNAs meanwhile, 108 DE miRNAs were specific to *E. coli* while 82 were specific to the *S. aureus* group [39]. Recently, Ma *et al*. [58] reported that miR-378 and miR-185 are important candidate biomarkers of milk infected with *S. aureus*. Numerous lines of evidence indicate that miRNAs regulate the host response to *S. aureus* via different target genes and pathways. For example, miR-223 regulation of *S.*

*aureus* resistance is via the PI3K/AKT/NF-κB pathway [57] while miR-145 modulation of *S. aureus*-induced mastitis is through pathways related to immune cytokines [55]. Moreover, Chen *et al*. [56] reported that miR-15a regulation of the host response to *S. aureus* is through inhibition of Interleukin-1 Receptor-Associated Kinase 2 (*IRAK2*) gene expression.

*S. uberis* is among the most prevalent mastitis-causing pathogens throughout Europe and North America [60]. Using real-time quantitative PCR to examine the expression of 14 miRNAs in bovine mammary epithelial cells (BMECs) challenged with *S. uberis,* Naeem et al. [34] reported significant downregulation (3- to 5-fold) of miR-181a, miR-16 and miR-31 and upregulation of miR-223 (2.5 fold) in infected versus healthy cells. Furthermore, downstream enrichment of miRNAs target genes indicated possible roles of miR-181a in intramammary infections via its regulatory function on Fc-gammaR-mediated phagocytosis, toll-like receptor signaling, and antigen processing and presentation pathways [34]. Lawless *et al*. [33] examined the expression of miRNAs in primary bovine mammary epithelial cells at 1, 2, 4 and 6 hours post-infection with *S. uberis,* and reported 21 miRNAs, including 20 known miRNAs, which were significantly DE post-infection. Furthermore, downregulated miRNA target genes were enriched for pathways related to innate immunity [33]. Ngo et al. [35] profiled circulating miRNAs in cows' milk with naturally occurring mastitis due to different causative agents and identified 26 miRNAs as generic indicators of clinical mastitis, and suggested seven of them (miR-27b, miR-152, miR-194, miR-200b, miR-222, miR-379, and miR-18397) as early mastitis indicators. The authors identified 27 miRNAs unique to *S. uberis* mastitis with emphasis on miR-320a and miR-320b due to roles in the modulation of trained immune activity [35]. Compared to *S. uberis* and *S. aureus*, less attention has been given to miRNA changes during *E.coli* and *S. agalactiae* mastitis. Pu *et al*. [38] identified 35 DE miRNAs in mammary gland tissues from cows with *S. agalactiae*-type mastitis, with regulatory roles in several immune response and signal transduction pathways, such as RIG-I-like receptor signaling pathway, cytosolic DNA sensing pathway and Notch signal pathway. Analyzing miRNA expression profiles from peripheral blood, Li *et al*. [36] identified 173 DE miRNAs between healthy and mastitis Holstein cattle. The authors reported the involvement of several miRNAs (miR-25, miR30e-5p, miR-342, miR-191, miR-399b, miR-451 and miR-486) in biological processes involved in mastitis infection. For example, a potential role of miR-25 in the development of the immune system through targeting of Krüppel-like factor 4 (*KLF4*)

gene was observed [36]. Chen *et al*. [61] validated the functions of 25 miRNAs and genes associated with NF-κB signaling pathway and indicated that NF-κB pathway is active in mastitic cows as compared to healthy cows as a result of decreased inhibition of miRNAs. The authors further suggested miR-16 and miR-223 as new markers for dairy cow mastitis diagnosis [61] . Interestingly, Lai et al. [40] identified five significantly upregulated miRNAs (miR-21, miR-146a, miR-155, miR-222, and miR-383) with the potential to effectively differentiate between California mastitis test positive milk (CMT+) and non-infected milk. In another study, miR-144-5p and miR-130b-5p were significantly downregulated and upregulated, respectively, in mastitis-infected mammary gland tissues compared to healthy tissues [62].

### *2.1.2 MiRNA and Johne's disease*

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD) in cattle, sheep, goats, and other domestic and wild animals [63,64]. JD imposes a substantial economic burden on the dairy industry [63,65], and it is prevalent worldwide [66]. MAP and JD have attracted attention because of their speculated connection to human Crohn's disease [67]. Current JD control strategies are hampered by the lack of accurate and reliable diagnostic tests [68]. Meanwhile, several studies have indicated the potential of miRNAs to serve as diagnostic and prognostic tools of MAP. An initial study comparing the miRNA expression in serum from experimentally MAP-challenged calves with age-matched controls, and from cattle defined as seropositive for anti-MAP antibodies with seronegative cattle, found no DE miRNAs between the groups [69]. More recently, Malvisi *et al*. [42] identified 7 upregulated (mir-19b, mir-19b-2, mir-1271, mir-100, mir-301a, mir-32, and one novel miRNA) and two downregulated miRNAs (mir-6517 and mir-7857) in the blood of MAP positive animals compared with unexposed animals. Studying the ileum, a target tissue for MAP infection, Liang et al., [43] reported 14 DE miRNAs when comparing infected and control ileum tissues from calves. Via integrated downstream analysis of miRNA and mRNA transcriptome data, the authors suggested that the DE miRNAs potentially regulate host responses to MAP infection through several routes; such as the potential role of miR-196b in the proliferation of endothelial cells, the role of miR-146b in bacteria recognition, and involvement of bta-miR-146b in the regulation of the inflammatory response [43]. Recently, Gupta et al. [44] developed a model using a combination of 4 miRNAs (miR-

1976, miR-873-3p, miR-520f- 3p and miR-126-3p), which distinguished moderate and severely infected JD animals from non-infected animals. Ileum and lleal lymph node are important tissues during MAP infection as they are the sites of MAP and host interaction and where the host initiates immune responses to the pathogen. Wang et al., [70,71] reported involvement of a different set of miRNAs in the regulation of MAP response in the ileum and ileal lymph node. In the ileal lymph node, Wang *et al*. [70] reported 37 DE miRNAs (18 up-regulated and 19 down-regulated) when comparing the miRNA expression of MAP infected cows relative to MAP negative cows. The authors highlighted possible roles of miR-100, miR-330 and miR-2447 via the Th17 cell differentiation pathway during MAP infection in ileal lymph node [70]. MiRNAs seem to be involved in many other processes rather than immune related pathways in response to MAP infection in the ileum [71]. For instance, the authors reported that DE miRNAs such as miR-370 and miR-383 are associated with lipid metabolism in this tissue [71]. Moreover, Shaughnessy *et al*., demonstrated the utility of a number of miRNAs in bovine feces in differentiating healthy animals from those with late-stage JD, thereby providing potential biomarkers for MAP infection and disease progression [45].

#### *2.1.3 MiRNA and other cattle diseases*

MiRNA expression is also changed during the progression of some other notable infectious diseases of bovine including Bovine viral diarrhea (BVD), foot and mouth disease (FMD) and tuberculosis, etc. Like JD, the symptoms of BVD virus (BVDV) infections in cows are subclinical and difficult to detect. Only one study has examined miRNA expression in relation to BVD [46]. The authors investigated miRNA profiles of BVDV infected colostrum from five neonate Holstein calves inoculated with BVDV at different time points: before infection (day 0) and at 4-, 9-, and 16-days post-challenge and reported two DE miRNAs (miR-423-5p and miR-151-3p) between BVDV challenged and control groups across time points. However, both miRNAs demonstrated inconsistent expression patterns whereby miR-423-5p increased until day 4 post-challenge and decreased to control level by day 16 post BVDV exposure. At the same time, miR-151-3p remained similar to the control level until day 9 before increasing in BVDV challenged animals compared to control animals on day 16. [46]. Thus, more studies are required to identify miRNA roles in BVDV and miRNA biomarkers of BVDV. FMD is a highly contagious disease of domestic and wild cloven-hoofed animals [72], and its

outbreaks incur enormous economic, political and social ramifications. A recent study has suggested a role for miRNAs in FMD virus (FMDV) infection in cattle [47]. The authors performed an expression profiling of 169 miRNAs in bovine serum collected during acute, persistent and convalescent phases of FMDV infection and reported potential roles of miR-17-5p in acute infection, miR-31 in FMDV persistence, and miR-1281 in both acute and persistent infection [47]. Vegh et al. [41] performed miRNA expression profiling in alveolar macrophages isolated from lung of bovine infected with *Mycobacterium bovis*, the causative agent of bovine tuberculosis, and identified different sets of DE miRNAs at different time points post-infection. The identified DE miRNAs were suggested to play a crucial role in a tightly controlled balance between pathogen survival strategies and the host immune response [41]. Through a functional validation experiment, Want et al., [73] indicated that miRNA-199a play important roles in *M. bovis* infection via inhibition of cellular autography and downregulation of IFN-β expression. To identify biomarkers for bovine tuberculosis, Sawera *et al*. [74] characterized the expression of four miRNAs (miR-146a, miR-29c, miR-155 and miR-99b) in milk whey and identified miR-146a as a potential biomarker for rapid diagnosis of *M. bovis* infection.

### 2.2. Potential regulatory roles of MiRNA in pig diseases

The first set of porcine miRNAs identified through sequence homology search with known human miRNAs belonged to the miR17-92 cluster and included miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a [75]. Since then, a total of 408 precursors and 457 mature porcine miRNAs have been reported and deposited in miRNA databases (Table 1). The studies on miRNA functions have focused on specific diseases or pathogens, mostly using *in vivo* challenge experiments. A number of important miRNAs for pig diseases are listed in Table 3.

**Table 3. Important miRNAs for pig diseases**

| Disease | Pathogens | Tissues/cells | MIRNAs | References |
|---|---|---|---|---|
| **Porcine reproductive and** | Porcine reproductive and | Porcine alveolar macrophages | miR-30a-3p, miR-132, miR-27b, miR-29b, miR-146a and miR-9-2 | [76] |

| | | | | |
|---|---|---|---|---|
| **respiratory syndrome** | respiratory syndrome virus | Blood monocytes and porcine alveolar macrophages | miR-181 | [77] |
| | | | miR-125b | [78] |
| | | | miR-23, miR-378, and miR-505 | [79] |
| | | MARC-145 cell | miR-145, miR-127 | [80] |
| | | Lung | miR-183, miR-219, miR-28-3p and miR-143-3p | [81] |
| | | Lung | miR-26 | [82] |
| | | Lung | miRNA-30c | [83] |
| | | Lung | miR-22 | [84] |
| | | Lung | miR-373 | [85] |
| | | Alveolar macrophages | miR-140, miR-92b, miR-545, miR-1306, miR-374b and miR-199b | [86] |
| | | Alveolar macrophages | miR-10a-5p | [87] |
| | | Blood | miR-125b, Ssc-miR-145-5p | [88] |
| **Swine influenza infection** | Influenza A virus | *In silico* | miR-124a, miR-145 | [89] |
| | | Influenza A virus subtype H1N2 | miRNAs miR-15a, miR-21, miR-146, miR-206, miR-223 and miR-451 | [90] |
| Multiple diseases | Salmonella species | Whole blood | miR-155 | [91] |
| | | Intestines | miR-29a | [92] |
| | Lawsonia intracellularis | | miR-486, miR-500, miR-127, miR-215, miR-194b-5p and miR-122 | [93] |
| | *Escherichia coli* F18 | | miR-143, let-7f, miR-30e, miR-148a, miR-148b, miR-181a, miR-192, miR-27b, miR-15b, miR-21, miR-215 and miR-152 | [94] |
| | | Duodenum | miR-196b, miR-499-5p and miR-218-3p | [95] |
| | *Trichuris suis*. | Serum | let-7d-3p | [96] |
| | *Actinobacillus pleuropneumoniae* | Lung | miR-664-5p, miR-451 and miR-15a | [97] |
| | Porcine cytomegalovirus | Macrophages | miR101, miR-7, miR-128, miR155-5p, miR-196-5p, miR-18a, miR-19b, miR-24-3p | [98] |
| | African swine fever virus | Spleen and submandibular lymph node | miR-126-5p, miR-92c, miR-92a, miR-30e-5p and miR-500a-5p, miR-125b, miR-451 and miR-125a | [99] |

| | | | | |
|---|---|---|---|---|
| | Influenza A virus | Lung | miR-15a, miR-18a, miR-21, miR-29b, and miR-590-3p | [100] |

### *2.2.1 MiRNA and porcine reproductive and respiratory syndrome virus infection*

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important viral pathogens in the swine industry [101]. The first miRNA study in relation to PRRSV infection used Illumina deep sequencing to construct small RNA expression profiles from cultured PRRSV-infected porcine alveolar macrophages [102]. The authors detected 40 DE miRNAs within the first 48 hours post-infection (hpi) while the expression of six miRNAs (miR-30a-3p, miR-132, miR-27b, miR-29b, miR-146a and miR-9-2) were altered at more than one time point. Furthermore, a miR-147 mimic experiment indicated that PRRSV replication was negatively impacted by the high expression level of miR-147 [102]. In a subsequent study, it was reported that miR-181 could directly impair PRRSV infection *in vitro* through specific binding to a highly (over 96%) conserved region in the downstream region of *ORF4* (open reading frame 4) of the viral genomic RNA [77]. Furthermore, miR-181 can downregulate the PRRSV receptor *CD163* in blood monocytes and porcine alveolar macrophages [77]. Moreover, downregulation of *CD163* led to the inhibition of PRRSV entry into porcine alveolar macrophages and subsequent suppression of PRRSV infection [77]. Wang et al. [78] reported that miR-125b can inhibit the activation of NF-κB, consequently preventing PRRSV replication. Reports of important functions of miR-23, miR-378, and miR-505 in the regulation of PRRSV infection have also emerged. Zhang et al. [79] reported that miR-23 was capable of inducing type I interferon (**IFN**-$\alpha$) expression during PRRSV infection through IRF3/IRF7 activation, which might further lead to the inhibition of virus infection. Zhou et al. [80] observed that miR-145 was strongly induced by PRRSV infection, whereas miR-127 expression was significantly reduced at all infection time points in MARC-145 cells challenged with PRRSV. Results of miRNA expression profiles of lung tissues from Tongcheng or Landrace pigs infected with a highly pathogenic PRRSV strain indicated that miR-183, miR-219, miR-28-3p and miR-143-3p were upregulated significantly at 3, 5, 7 day post infection (dpi) in both breeds [81]. Li et al. [82] screened 15 miRNAs implicated in innate immunity or antiviral functions and observed that over-expression of the miR-26 family strongly inhibited PRRSV replication *in vitro* via downregulated expression of ***IFN***-$\alpha$ and IFN-stimulated genes (*MX1* and *ISG15*) during PRRSV infection. It was demonstrated that miRNA-30c modulates IFN-$\alpha$ responses to

facilitate PRRSV infection by targeting Janus kinase 1 (JAK1) pathway [83]. Meanwhile, Xiao et al. indicated that miR-22 promoted PRRSV replication by targeting the Heme oxygenase-1 (*HMOX1*) gene of the host cells [84]. The authors suggest that PRRSV could modify antiviral host factor expression and enable viral replication via the cellular miRNAs [84]. Chen et al. [85] demonstrated that miR-373 promotes the replication of PRRSV by inhibiting the production of beta interferon (IFN-β) via targeting nuclear factor IA (*NFIA*), *NFIB*, interleukin-1 receptor-associated kinase 1 (*IRAK1*), *IRAK4* and interferon regulatory factor 1 (*IRF1*). Twenty-three upregulated DE miRNAs, and 25 downregulated DE miRNAs were identified when comparing the miRNA expression profiles of alveolar macrophages of indigenous Chinese Tongcheng pigs infected with PRRSV and control groups [86]. The authors reported the epigenetic roles of miRNAs (miR-19a-3p, miR-29a-3p, miR-29c-3p and miR-342-3p) through their downregulation of methylation-related genes during PRRSV infection [86]. A role for miR-10a-5p inhibition of replication of PRRSV by targeting the host factor signal recognition particle 14 (*SRP14*) was demonstrated [87]. Results of an examination of the effect of PRRSV infection on the expression of 89 miRNAs yielded candidates with potential anti- and pro-viral functions; such as predicted ability of miR-125b to limit PRRSV viral levels and miR-145-5p to cause alternative macrophage priming [88]. Therefore, highly pathogenic type 2 strain - PRRSV infection affects host homeostasis through changes in miRNA expression and influence on host immune, metabolic, and structural pathways [88]. Zhang et al. [103] demonstrated that PRRSV infection induced miR-c89 expression and that miR-c89 could regulate the expression of the host factor porcine retinoid X receptor β (RXRB) gene to inhibit PRRSV replication.

### *2.2.2. MiRNA and swine influenza infection*

Influenza is a zoonotic viral disease that represents a health and economic threat to humans and animals worldwide [104]. In mammals, influenza viruses replicate mainly in the respiratory tract, usually accompanied by clinical signs, whereas in avian species, the major replication site is the intestinal tract without clinical symptoms [105]. Several studies with different approaches have investigated the implication of miRNAs in swine influenza infection [90,106,107]. Using computational procedures, initial research identified 36 pig miRNAs having putative target genes in swine influenza viral sequences isolated in a period of 38 years, which indicated that putative target

genes and host miRNA (miR-124a, ssc-miR-136 and miR-145) interactions were maintained almost throughout virus evolution [89]. Analyzing miRNA, mRNA and protein expressions in lung tissues of pigs challenged with influenza A virus subtype H1N2 at different time points (0h, 24 h, 72 h, and 14 dpi), Skovgaard et al. [90] identified six DE miRNAs (miR-15a, miR-21, miR-146, miR-206, miR-223 and miR-451) in one or more time points compared with control pigs and suggested that besides functions in innate immunity, DE miRNAs may be involved in controlling acute influenza infection in pigs [90]. Jian *et al*. [108] compared miRNA expression in pulmonary alveolar macrophages from piglets during H1N1 SwIV infection with a control sample and reported 214, 204 and 207 miRNAs DE at post-infection day 0, 4 and 7 compared to control samples, respectively. Núñez-Hernández *et al*. [99] analyzed miRNA and innate immune mRNA expression in blood samples before (day 0) and after (1, 3, and 14 days) Influenza A virus challenge of pigs and identified 20 DE miRNAs that potentially regulated genes involved in apoptosis and cell cycle regulation. In another study, Brogaard et al. found 27 DE miRNAs in lung tissues of pigs on day 3 following challenge with influenza A viruse H1N2 and suggested five miRNAs (miR-15a, miR-18a, miR-21, miR-29b and miR-590-3p) as potential modulators of viral pathogen recognition and apoptosis [100]. Zhang *et al.* [109] reported that swine influenza virus H1N1/2009 infection could modulate the expression of host miRNAs (miR-204 and miR-4331) to facilitate its replication in the host.

### *2.2.3 MiRNA and other pig diseases*

Salmonella species infect many vertebrate species, including pigs. Pigs colonized with *Salmonella enterica serovar Typhimurium* are usually asymptomatic, making their detection in carrier pigs difficult. Variable fecal shedding of Salmonella is an important cause of foodborne illnesses in humans. Huang *et al*. [91] performed the first analysis of the whole blood transcriptional response induced by Salmonella. Comparing the miRNA expression in two groups of pigs identified as either low Salmonella shedding (LS) or persistent Salmonella shedding (PS), Huang *et al*. [91] demonstrated decreased expression of miR-155 in pigs of PS phenotype [91]. Hoeke *et al*. [92] examined the interactions between miRNAs and their target mRNAs in Salmonella-infected piglet intestines using microarray analysis and showed that miR-29a regulates intestinal epithelial cell proliferation by targeting caveolin-2. Li *et al*. [93] analyzed the miRNA expression profiles in Lawsonia intracellularis-

infected porcine intestines and found 83 DE miRNAs compared to controls, out of which 53 were upregulated, and 30 were downregulated. Enrichment analyses indicated that the DE miRNAs could target genes involved in pathways related to the immune response, amino acid metabolism, and cell communication/growth/motility [93]. Huang et al. [110] compared the whole blood miRNA transcriptomes of pigs (Duroc × Landrace × Yorkshire) at 2-days post-inoculation and before Salmonella infection and identified 29 DE miRNAs, including miR-146a-5p, miR-125a and miR-129a-5p with roles in Salmonella infection and immunology signaling pathways. Following validation by real time quantitative PCR, the authors concluded that miR-146a-5p in peripheral blood could significantly increase the fecal bacterial load [110].

*E.coli* F18 is one of the main causal pathogen of post-weaning diarrhea in piglets. Ye *et al*. [94] reported 58 DE miRNAs, of which 46 were increased, and 12 were decreased in *E.coli* F18-sensitive pigs. After downstream analyses, the authors suggested 12 candidate miRNA disease markers, including 11 miRNAs (miR-143, let-7f, miR-30e, miR-148a, miR-148b, miR-181a, miR-192, miR-27b, miR-15b, miR-21, and miR-215) with increased expression and one (miR-152) with decreased expression. Ye et al. [94] also identified 24 DE miRNAs (including 15 upregulated and 9 downregulated) demonstrating more than 2-fold differential expression between the F18-resistant and F18-sensitive piglets. Using the Meishan piglet as a model animal to test their susceptibility to *E. coli* F18, Wu *et al*. [95] identified miR-196b, miR-499-5p, and miR-218-3p as candidate miRNAs involved in *E. coli* F18 infection. Furthermore, the authors noted that miR-218-3p might regulate Discs Large MAGUK Scaffold Protein 5 (*DLG5*) gene in *E. coli* F18-resistant pigs [95]. *Clostridium perfringens* (*C. perfringens*) type C causes piglet diarrhea with serious economic consequences to the swine industry. Want *et al*. [111] identified 53 DE miRNAs in the ileum when comparing control (IC) pigs with pigs susceptible (IS) or resistant (IR) to *C. perfringens*. Furthermore, it was suggested that the identified miRNAs targeted specific genes to control the infection status. For example, Nuclear Factor Of Activated T Cells 4 (*NFATC4*), ETS-Like Gene 1 (*ELK*1)/Heat Shock Protein Family A (*Hsp70*)/Member 2 (*HSPA2*)/Interleukin 7 Receptor (*IL7R*) and Cardiotrophin Like Cytokine Factor 1 (*CLCF1*) were the target genes of miR-7134-5p, miR-500 and miR-92b-3p, respectively [111].

Studying miRNA involvement in porcine whipworm (*Trichuris suis)* infection, Hansen and colleagues [96] reported significant increase in the expression level of one circulating miRNA, let-7d-

3p, in the serum of pigs 8 weeks post-infection with *T. suis.* However, the use of this miRNA as a potential biomarker needs further verification since the pre-patent period of *T. suis* is 6–7 weeks. In another study, Podolska *et al*. [97] identified miR-664-5p, miR-451 and miR-15a as promising candidate miRNAs involved in pig response to *Actinobacillus pleuropneumoniae* infection. Liu et al. [98] identified a large number (130) of DE miRNAs between control and *Porcine cytomegalovirus* infected porcine macrophages. Núñez-Hernández *et al*. [99] identified 12 DE miRNAs seven dpi and three dpi (including 4 upregulated miRNA: miR-451, miR-145-5p, miR-181a and miR-122 and 8 down regulated miRNAs: miR-92a, miR-23a, miR-92b-3p, miR-126-5p, miR-126-3p, miR-30d, miR-23b and miR-92c) in both spleen and submandibular lymph node tissues from pigs experimentally infected with a virulent (E75) African swine fever virus. The authors also identified 8 DE miRNAs (miR-126-5p, miR-92c, miR-92a, miR-30e-5p, miR-500a-5p, miR-125b, miR-451 and miR-125a) when comparing the miRNA expression in spleen and lymph node tissues from pigs challenged with virulent E75 with those challenged with its attenuated strain (E75CV1) [99]. Zhang *et al*. [112] identified 21 DE miRNAs between lung tissues from Porcine circovirus type 2 (PCV2)-infected and PCV2-uninfected Laiwu pigs, as well as 7 DE miRNAs between PCV2-infected and PCV2-uninfected Yorkshire × Landrace crossbred pigs. The authors further observed that miR-122 represses the protein expression and viral DNA replication of PCV2 by down-regulating the expression of *NFAT5* (nuclear factor of activated T-cells 5) and *NPEPPS* (aminopeptidase puromycin sensitive) in PK15 cells [112]. Li *et al*. [113] analyzed the expression profiles of miRNAs in PCV2-infected cells and non-infected cells and reported 44 DE miRNAs (16 upregulated and 28 downregulated) with potential roles in cellular inflammatory responses and cytokine dysfunction.

### 2.3. Potential regulatory roles of miRNAs in poultry diseases

The first study on chicken miRNAs identified 25 miRNAs from chicken embryos and adult chicken tissues ( cerebrum, cerebellum, heart, lung, liver, kidney, and spleen) through small RNA cloning and sequencing [114]. Since then, many studies have explored the miRNA expression in relation to both production and disease traits in chickens. About 882 precursors and 1232 mature miRNAs have been reported for chicken (Table 1). Potential miRNA biomarkers of some poultry diseases, including

Marek's disease, Avian Leukosis Virus, Infectious Bursal Disease Virus, and other diseases are listed in Table 4.

**Table 4. Important miRNAs for chicken diseases**

| Disease | Pathogen | Tissue | Changed or potential miRNA biomarkers | References |
|---|---|---|---|---|
| Marek's Disease | Gallid herpesvirus 2 | Spleen and liver | miR-221, miR−140, miR−199, miR-181a, miR−146b, miR−146c and miR−26a | [115] |
| | | Spleen | miR-15, miR-456 and let-7i | [116] |
| | | Spleen | miR-21 | [117] |
| | | Spleen | miR-26a | [118] |
| | | Spleen and liver | miR-103 | [119] |
| | | Spleen and liver | miR-219b | [120] |
| | Marek's disease virus | Bursa samples | mir-30a, mir-1662, mir-9-1, mir-9-2, mir-499, mir-193b and mir-1684a, | [121] |
| Avian Leukosis | Avian leukosis virus | Liver | mir-221, mir-222, mir-1456, mir-1704, mir-1777, mir-1790, mir-2127, let-7b, let-7i, mir-125b, mir-375 and mir-458 | [122] |
| | | Liver | miR-375 | [123] |
| | | Liver | miR-221, miR-193a, miR-193b and miR-125b | [124] |
| | | Liver | miR-221, miR-222, | [125] |
| | | Liver | miR-23 | [126] |
| | | Liver | mir-34b-5p | [127] |
| | | Liver | let-7b and let-7i | [128] |
| | | chicken embryo fibroblasts | miR-184-3p, miR-146a-3p, miR-146a-5p, miR-3538 and miR-155, | [129] |
| Bursal disease | Bursal disease virus | DF-1 cells | miR-9 | [130] |
| | | DF-1 cells | miR-2127 | [131] |
| | | DF-1 cells | miR-130b | [132] |
| Avian influenza | Avian influenza viruses | Lung and trachea | miR-146, miR-15, and miR-21 | [133] |
| | | Lung | miR-34a, miR-122–1, miR-122–2, miR-146a, miR-155, miR-206, miR-1719, miR-1594, miR-1599 and miR-451, | [134] |
| | | Embryo fibroblasts | miR-146c, miR-181a, miR-181b, miR-30b, miR-30c, miR-30e, miR-455, miR-1599 and miR-1416 | [135] |
| Chronic respiratory diseases | Mycoplasma gallisepticum | lung | miR-8 family, miR-499 family and miR-17 family | [136] |
| | | cell (DF-1) | miR-99a | [137] |
| | | cell (DF-1) | miR-101-3p | [138] |

| | | | |
|---|---|---|---|
| | Chicken embryonic lungs and DF-1 cells, | miR-19a | [139] |

### *2.3.1 MiRNA and Marek's disease virus infection*

Marek's disease, a highly contagious viral neoplastic disease caused by infection of *Gallid herpes virus* 2 (GaHV-2) or Marek's disease virus (MDV), has remained a major concern in the poultry industry owing to the continual emergence of new virulent strains [140]. Several *in vitro* approaches have been employed to unravel the roles played by host-encoded miRNAs in different scenarios of MDV infection [115,117-120,141-144]. Among the first studies, Lian et al [115] y the method of deep sequencing identified 28 downregulated and 11 upregulated miRNAs in MDV-infected chicken liver samples (tumorous spleen and MD lymphoma) as compared to control samples. Furthermore, the functions of target genes of many DE miRNAs were related to lymphomagenesis (Lian et al…). Tian et al. also identified 64 DE miRNAs (58 downregulated and 6 upregulated) between MDV-infected and non-infected chicken cells [116]. Using white leghorn chicken experimentally inoculated with the oncogenic RB-1B strain as a model to investigate the connection between chicken miRNA response and the oncogenic nature of MDV, Stik et al. [117] reported upregulation of miR-21 in chicken inoculated with RB-1B strain as compared to chicken vaccinated with a non-oncogenic strain CVI988. In a similar experiment, Li et al. [118] showed that miR-26a was downregulated in MDV-infected chicken spleens during different phases of tumor formation while Han *et al*. [119] indicated that miR-103 was downregulated in tumor samples from spleen and liver of infected chickens. Zhao *et al*. [120] observed that miR-219b promoted cell apoptosis via regulation of the expression of genes in the apoptosis pathways during MDV infection. Heidari *et al*. [121] identified 300 novel miRNAs and 54 DE miRNAs between bursal tissues from chickens infected with MDV compared to controls or between the infected resistant and susceptible chickens. Functional analysis further indicated that the target genes of DE miRNA were enriched for many reactome immune related pathways such as cytokine signaling, innate and adaptive immune system, Toll-like receptors and interleukins pathways [121].

### *2.3.2. MiRNA and Avian leukosis virus infection*

Avian leukosis virus (ALV) belongs to the genus *Alpharetrovirus* of the *Retroviridae* family. This virus can induce tumors in avian hosts, including B-cell lymphoma, hemangioma and myelocytoma [145]. In a study examining miRNA-mediated control of Avian leukosis virus subgroup J (ALV-J) infection, Li *et al*. [122] proposed that seven upregulated miRNAs (mir-221, mir-222, mir-1456, mir-1704, mir-1777, mir-1790, and mir-2127) identified in the liver of ALV-J-infected 10-week-old chickens might play a tumorigenic role, whereas five downregulated miRNAs (let-7b, let-7i, mir-125b, mir-375 and mir-458) might have associations with loss of tumor-suppressive functions. Li *et al*. [123] confirmed, through an *in vivo* experiment, that miR-375 was downregulated in the liver of ALV-J-infected chickens compared to non-infected chickens. The authors also indicated that overexpression of miRNA-375 led to significant inhibition of the proliferative capacity of DF-1 chicken fibroblasts cells, likely *via* targeting and repressing yes-associated protein 1 (*YAP1*), cyclin E (*CCNE1*) and *Drosophila* inhibitor of apoptosis protein 1 (*DIAP1*) genes [123]. Results of a microarray analysis of liver tumors from ALV-J-infected chickens identified miR-221, miR-193a, miR-193b and miR-125b as DE miRNAs [124]. Through an *in vitro* experiment, miR-221 was found to act as a tumorigenic agent by targeting B-cell lymphoma 2 (*BCL-2)* modifying factor, thus confirming its function [125]. In addition, Li et al. [126] indicated that increased replication of ALV-J was associated with the upregulation of miR-23 in the spleen of ALV-J-infected chickens. The authors showed that miR-23 could target and suppress *IRF1* (interferon regulatory factor 1), thus allowing enhanced virus replication. Li et al. [127] reported that mir-34b-5p can suppress melanoma differentiation-associated gene 5 (*MDA5*) signaling pathway to promote avian leukosis virus subgroup j (ALV-J)-infected cell proliferation and ALV-J replication. Moreover, Ji et al. [128] reported temporal changes of miRNA let-7b and let-7i expression in chickens challenged with subgroup ALV-J. Zhou et al. [129] studied the miRNA expression profiles in CEF cells infected with ALV-J (ALV-J treatment), reticuloendotheliosis virus (REV treatment) or both pathogens (synergistic infection) (both treatment) and identified 54 DE miRNAs (23 upregulated and 31 downregulated) when comparing ALV-J treatment with both treatment [129]. The authors revealed that some DE miRNAs (miR-184-3p, miR-146a-3p, miR-146a-5p, miR-3538 and miR-155) participated in virus-vector interaction, oxidative phosphorylation, energy metabolism, and cell

growth following GO annotation and KEGG pathway functional analysis of the miRNA target genes [129] .

### *2.3.3. MiRNA and other chicken diseases*

Bursal disease, caused by infectious bursal disease virus (IBDV), is a highly contagious disease that predominantly affects the bursa of Fabricius in birds [146,147]. IBDV targets the host immune system by destroying B lymphocytes, attracting T cells, and activating macrophages [147]. In poultry, vaccination has contributed to the overall reduction of disease burden [148]; however, a comprehensive understanding of the complexity of virus and host interaction is limited [147]. The roles of miRNAs in the regulation of IBDV infection have been the focus of many investigations. Initially, Shen *et al* [149] reported that recombinant avian adeno-associated virus (AAAV)-delivered VP1- and VP2-specific miRNAs can inhibit the replication of IBDV efficiently in transducing 8-day-old specific-pathogen-free chicken embryos. Two other studies reported the important roles of miR-9a and miR-2127 in IBDV infections. Ouyang *et al.* [130] found that miR-9 was induced 2, 4, 12 and 24 h after infection with IBDV and that miR-9 can promote IBDV replication by repressing the production of type I IFN. In a subsequent experiment, Ouyang *et al.* [131] provided evidence that miR-2127 function in IBDV infection is via downregulation of CHP53 mRNA translation and attenuation of CHP53-mediated antiviral innate immune response against IBDV. More recently, Fu *et al.* [132] reported that miR-130b suppreses IBDV replication via directly targeting the viral genome and cellular Suppressor Of Cytokine Signaling 5 (*SOCS5).*

Avian influenza, caused by avian influenza viruses (AIVs), is an important disease for many bird species. Wild waterfowls or aquatic birds are the natural reservoir hosts of all influenza A subtypes [150] except for two novel IAV subtypes, H17N10 and H18N11, in bats [151]. The first study on miRNA gene expression in AIV infected chickens using a deep sequencing approach was performed by Wang *et al.* [133], who reported 73 and 36 DE miRNAs between low pathogenic H5N3 infected and non-infected chicken lungs and tracheae, respectively, 4 dpi. Some of the DE miRNAs such as miR-146, miR-15 and miR-21 function in immune-related signal pathways in mammals [133]. In boiler chicken, Wang *et al.* [134] suggested miR-34a, miR-122-1, miR-122-2, miR-146a, miR-155, miR-206, miR-1719, miR-1594, miR-1599 and miR-451, as strong candidate miRNAs and *MX1, IL-8, IRF-7* and

*TNFRS19* as strong candidate genes involved in the regulation of host response to AIV infection. Examining avian influenza virus H9N2-infected and non-infected chicken embryo fibroblasts, Peng *et al.* [135] identified 48 DE miRNAs (e.g. miR-146c, miR-181a, miR-181b, miR-30b, miR-30c, miR-30e and miR-455), which were predicted to target immune response-related genes. Koenen *et al.* [152] reported more upregulated than downregulated miRNAs in broiler chickens, whereas a reversed trend was seen in layers, suggesting differential viral effects in chicken breeds. This might reflect differences in humoral immunity between the breeds since broiler chickens have weak short-term humoral immunity, whereas layers possess long-term humoral immune response and strong cellular immunity [152]. When performing miRNA expression profiling in spleen, thymus and bursa in chicken and duck, Li et al. [153] reported divergent changes in the miRNA expression upon H5N1 infection in both breeds and suggested that miRNAs can account for the level of susceptibility upon H5N1 infection. Recently, O'Dowd et al. [154] reported 67 upregulated miRNAs and 157 downregulated miRNAs across cellular and extracellular vesicle samples released from chicken tracheal cells stimulated with polyI:C and LPS from E.coli 026:B6 or infected with low pathogenic avian influenza virus H4N6. The authors indicated that miR-146a, miR-146b, miR-205a, miR205b and miR-449 can be used as miRNA-based antiviral agents or vaccine adjuvants in alternative strategies for the control of AIV in chickens [154].

Chronic respiratory diseases caused by *Mycoplasma gallisepticum* have severe consequences in the poultry industry. Zhao et al. [136] identified 45 and 68 DE miRNAs at 3 and 10 dpi with *Mycoplasma gallisepticum*, respectively, and suggested the mitogen-activated protein kinase pathway as a key regulatory route for the effect of miRNAs in the regulation of disease infection, as well as highlighted miR-8, miR-499 and miR-17 families as potentially important in *Mycoplasma gallisepticum* infection. In a follow-up study, Zhao et al. [137] identified miR-99a as a key player in *Mycoplasma gallisepticum* infection through the regulation of *SMARCA5*. Further important miRNAs for this pathogen include miR-101-3p [138] and miR-19a [139]. MiR-19a regulates the expression of the host *EZH2* (enhancer of zeste homolog 2) gene through binding to its 3' untranslated region [138]. MiR-19a might also suppress the expression of *ZMYND11* in *Mycoplasma gallisepticum* -infected chicken embryonic lungs and DF-1 cells [139]. MiR-19a can activate the NF--κB signaling pathway and promote pro-

inflammatory cytokines expression, cell cycle progression, and cell proliferation to defend against *Mycoplasma gallisepticum* infection [139].

### 2.4 Potential regulatory roles of miRNAs in small ruminant diseases

Small ruminants, including sheep and goats, are an important source of meat, milk, and wool throughout the world. MiRNA studies in small ruminants have focused on muscle [155-157], embryo/ovary [158-162], mammary gland development [163-165], milk-related phenotypes (yield and composition) [166-168] and hair/skin-related phenotypes [169-171]. An initial study on miRNA in small ruminants performed by Wenguang et al. [170] used microarray analysis to characterize the expression of 159 miRNAs in skin samples from body and ear of goats and sheep and identified 105 miRNAs conserved between the two species with significant roles in hair follicle differentiation. Subsequent studies using high-throughput sequencing techniques identified 106 precursors and 153 mature miRNAs in sheep and 267 precursors and 436 mature miRNAs in goat (Table 1). MiRNAs with important roles in sheep and goat diseases are shown in Table 5.

**Table 5. MiRNAs with important roles in small ruminant diseases**

| Species | Disease | Pathogen | Tissue | MIRNA | Citation |
|---|---|---|---|---|---|
| Sheep | Cystic echinococcosis | *Echinococcus granulosus* | Intestinal tissue | miR-21-3p, miR-542-5p, miR-671, miR-134-5p, miR-26b and miR-27a | [172] |
| Sheep and goat | Enzootic nasal adenocarcinoma | Enzootic nasal tumor virus | Tumor and para-carcinoma nasal tissues | miR-449b-3p, miR-449a-3p, miR-133a-3p, miR-449c, miR-133b, miR-9-5p, miR-148a-3p, miR-296-3p, miR-873-3p and miR-331-3p | [173] |
| Sheep | Bluetongue virus infection | Bluetongue virus | Testis | let-7d, let-7f, miR-106b, miR-10a, miR-10b, miR-136, miR-148a, miR-17-5p, miR-191, miR-194, miR-29a, miR-29b, miR-30a-3p, miR-30b, miR-362, miR-369-3p, miR-369-5p, miR-379-5p, miR-3958-3p, miR-409-3p, miR-412-3p, miR-432, miR-493-5p, miR-541-5p and miR-758-3p | [174] |

| | | | | | |
|---|---|---|---|---|---|
| Sheep | Peste des petits ruminants | Peste des petits ruminants virus | Spleen and lung | miR-21-3p, miR-1246, miR-27a-5p, miR-760-3p, miR-320a and miR-363 | [175] |
| Sheep | Prion diseases | Prion virus | Plasma | miR-342-3p, let-7b and miR-21-5p | [176] |
| Sheep | Peste des petits ruminant disease | Peste des petits ruminants virus | Peripheral blood lymphocyte | miR-150, miR-370-3p and miR-411b-3p | [177] |

The functions of miRNAs have been reported for several small ruminant diseases such as *Cystic echinococcosis* infection [172], an epithelial tumor induced in goats and sheep by enzootic nasal tumor virus (ENTV) [173], vascular endothelial inflammation in placental dysfunction (Yuan et al. 2017), bluetongue virus [174], Peste des petits ruminants (PPR) infection [175] and prion disease [176]. Small ruminants are highly susceptible to *Cystic echinococcosis*, a chronic zoonotic infection caused by infection with the larval stage of the cestode, *Echinococcus granulosus*. Jiang et al. [172] profiled the miRNA expression in intestinal tissues of sheep with resistant and non-resistant Major Histocompatibility Complex (MHC) haplotypes after peroral infection with *E. granulosus* eggs and identified 83 (75 up- and eight downregulated) miRNAs as DE between the groups. The authors identified some highly DE miRNAs as NF--κB pathway-responsive miRNAs (miR-21-3p, miR-542-5p, miR-671, miR-134-5p, miR-26b and miR-27a) due to their involvement in the inflammation process in response to *E. granulosus* infection. MiRNAs also play important roles in Enzootic nasal adenocarcinoma, an epithelial tumor induced in goats and sheep by enzootic nasal tumor virus [173]. Wang *et al.* [173] identified 116 DE miRNAs in the tumor and para-carcinoma nasal tissues of Nanjing yellow goats with Enzootic nasal adenocarcinoma and showed involvement of the predicted target genes in cell proliferation, signal transduction, and other processes associated with cancer.

In an effort to explore the mechanisms of Bluetongue virus infection, Du *et al.* [174] identified 25 known and 240 novel DE miRNAs candidates in Bluetongue virus infected and uninfected primary sheep testicular cells as well as significant enrichment of target genes in MAPK, PI3K-Akt, endocytosis, Hippo, NF—κB viral carcinogenesis, FoxO and JAK-STAT signaling pathways [174]. Peste des petits ruminants (PPR) is a highly contagious viral disease characterized by fever, sore mouth, conjunctivitis, gastroenteritis and pneumonia, and primarily affects goats and sheep.

Recently, Pandey *et al.* [175] examined the function of miRNAs in PPR virus (PPRV) infected lung and spleen tissues of sheep and goats and reported 67 and 37 DE miRNAs (20 in common) in the lung and 50 and 56 DE miRNAs (11 in common) in the spleen of goats and sheep. Downstream analyses of DE miRNAs suggested that PPRV-induced miR-21-3p, miR-320a and miR-363 might act cooperatively to enhance viral pathogenesis in the lung and spleen of sheep by downregulating several immune response genes [175]. To identify potential miRNA biomarkers for small ruminant prion diseases, Sanz Rubio *et al.* [176] analyzed ten potential candidate miRNAs from circulating blood plasma of naturally infected scrapie sheep by quantitative reverse transcription PCR and identified miR-342-3p and miR-21-5p as circulating biomarkers of prion disease [176]. Yang *et al.* [177] studied the miRNA expression profile in peripheral blood lymphocyte (PBMC) and primary testicular (ST) cells from sheep inoculated with PPR vaccine virus *in vivo* and *in vitro* and identified a high number of DE miRNAs between different time points; such as 373 DE miRNAs at 3 dpi compared with 0 dpi or 115 miRNAs (12 upregulated and 103 downregulated) at 5 dpi compared with 0 dpi [177]. In ST cells, 271 miRNAs were DE when comparing 3 dpi with 0 dpi or 102 miRNAs were DE when comparing 5 dpi with 0 dpi [177]. Three DE miRNAs, miR-150, miR-370-3p and miR-411b-3p were common to PBMC cells and ST cells [177].

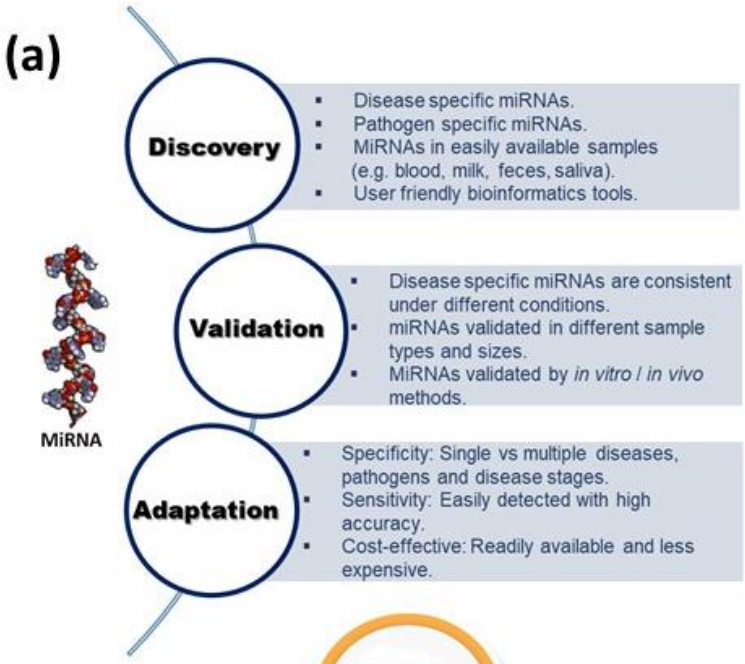
## 3. MiRNA biomarker development and potential therapeutic applications

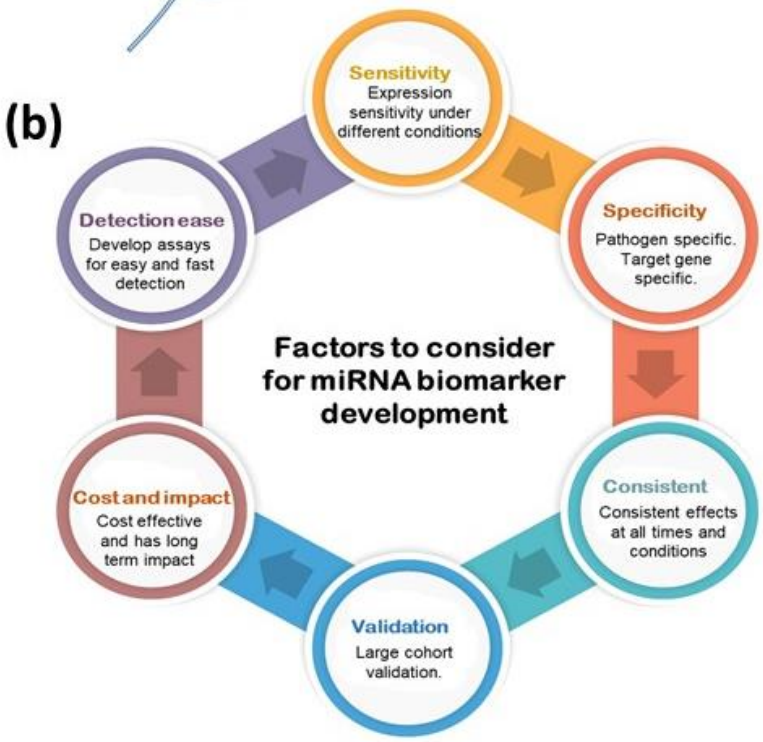
As discussed in sections above, miRNAs impact animal health and offer possibilities for its exploitation for enhanced health management. The possibility and the practical aspects of using miRNAs as biomarkers have been intensively reviewed in many human diseases such as cancers [178-180], rheumatic diseases [181] and diabetes mellitus [182,183]. In farm animals, discovering biomarkers will be crucial for the management of disease [184].

Initial biomarkers used in human disease management were protein biomarkers but detecting new and enhanced protein biomarkers has proven to be an expensive venture and time consuming mainly due to the low availability of clinically relevant proteins, their complex nature and the lack of accurate and repeatable detection methods [180]. Taylor [185] recently summarized the common qualifying factors of a biomarker as: (1) a biomarker should be easily accessible (i.e. discovered and measured easily and using minimal invasive procedures); (2) be specific to the condition under investigation

(specificity); (3) have high sensitivity (be easily and accurately detected, ideally before the appearance of clinical symptoms and potentially vary according to disease stages or response to therapy) and (4) be translatable from research/development to application. As compared to other nucleic acids, miRNAs are ideal biomarker candidates as they are very stable under a wide range of conditions and can be extracted from a variety of liquid biospeciments (e.g. blood, urine, milk, feces, etc.) and tissue samples. MiRNAs are also highly specific to tissues and cell types and its ability to delineate disease stages has been successful used to differentiate cancer stages and to monitor the responsiveness to therapy[186,187].

Therefore, miRNA can be used as biomarkers, capable of serving diagnostic, prognostic, or therapeutic purposes, for the management of livestock disease (Figure 1). Still, it is essential to consider different features of biomarker development such as sensitivity, specificity, stability, spectrum and cost [188,189]. The development of a biomarker generally requires some major steps: (i) discovery: to identify the potential candidate markers for specific conditions, (ii) confirmation and validation: to test the identified markers in other populations or other samples (iii) adaptation: development of suitable arrays or tests for the identified markers (Figure 1a). Up till now, most miRNA studies in animal diseases have focused on profiling miRNA changes (discovery stage). Although the functions of some miRNAs have been validated as summarized in sections above, those experiments were mostly *in vitro* and may not reflect the actual underlying complex biological regulatory mechanisms. To the best of our knowledge, there are no available commercial miRNA biomarkers for use in livestock disease management. Many factors must be considered for the development of miRNAs as biomarkers in livestock species, including- specificity, expression sensitivity, validation in a large cohort to ensure its effectiveness, consistency of results, and impact on both cost (cost-effective) and time (long-term) (Figure 1b). Ideally, a validated miRNA biomarker



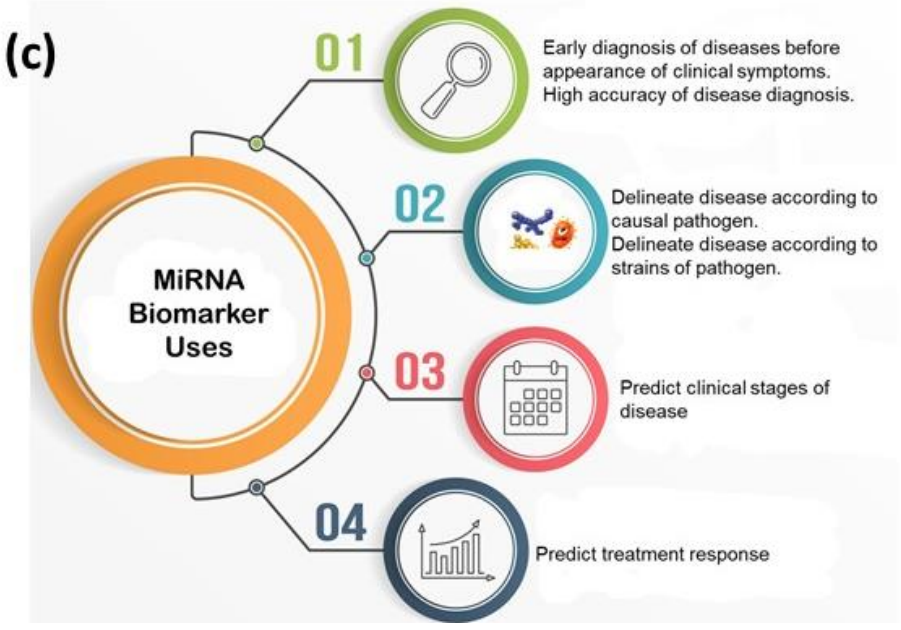



Figure 1: An oveiw of steps involved in the development of miRNA biomarkers for livestock disease management. (a): Steps/stages to follow in miRNA biomarker development, (b): Factors to consider in miRNA biomarker development and (c): Potential application of miRNA biomarkers.

can be used for early diagnosis, prognosis and for predicting the response to therapy (Figure 1c). For instance, different groups of miRNA biomarkers can be used for the management of JD in livestock as follows: (i) for early diagnosis of JD infection, (ii) for classifying JD due to different strains of MAP, (iii) for predicting the stage of JD and (iv) as effective treatment (therapeutic) for JD. The development of miRNAs as biomarkers for livestock diseases requires collective action and different stakeholders' involvement. Researchers, veterinarians and farmers have the prerogative to identify the issues and the need for biomarkers for specific purposes. The experimental design and data analysis require both the statisticians and bioinformaticians to derive the best analytical tools to deliver robust results in the discovery and validation phases. A crucial link is required between academia (researchers) and industry to develop cost-effective and reliable tests and to select the best option for developing biomarkers. Finally, validated and reliable miRNA-based diagnostic kits and biomarkers for breeding for resistance will support livestock improved productivity. Therefore, successfully developed miRNA biomarkers might serve as new tools that could enhance current methods or lead to the development of new methods or therapies for managing farm animal diseases.

Moreover, the important functions of miRNAs in livestock diseases makes them an attractive option for the development of therapeutic approaches for livestock disease management. MiRNA-based therapeutic approaches can be through miRNA inhibition (diminish the expression of disease-induced miRNAs) or miRNA replacement (re-establish the expression of disease repressed miRNAs )[190]. Several approaches have been used for miRNA inhibition such as antisense anti-miR oligonucleotides, locked nucleic acid anti-miRs, antagomiRs and miRNA sponges[191]. Similarly, several small molecules, synthetic miRNA mimics, and DNA plasmids have been used in miRNA restoration therapy [192,193]. The success of miRNA therapies majorly depends on suitable, effective and specific delivery systems[194,195]. Some high efficient and specific miRNA delivery methods have been via exosome and nanoparticles [196,197]. MiRNA therapies to cure diseases in livestock could be an attractive option to complement or replace current disease-management methods.

However, understanding the specific functions and mechanisms of interaction between miRNA and other biomolecules in disease pathogenesis is the first step in the development of miRNA therapeutics for livestock diseases.

**4. Challenges and opportunities for understanding miRNA biological roles**

Up to now, it is well known that miRNAs play important roles in many biological processes related to disease development in farm animals. Therefore, the application of miRNAs to improve disease resistance in farm animals is very promising. MiRNAs can be used as direct biomarkers or indirectly through other technologies. As direct biomarkers, such as circulating biomarkers, miRNA in biological fluids like blood, milk, saliva, urine, etc., can facilitate rapid detection of disease infection status. Indirectly, miRNA can find use in other technologies such as RNA interference or genome editing. Genome editing using CRISPR/Cas9 technology can robustly, specifically, and stably modify miRNA expression by editing either the seed sequence of miRNAs or the three prime untranslated regions of their target genes [198]. The success of the application of this technology in miRNA-mediated therapy has been proven in diseases in animal models [199-201]. Before adopting miRNAs as biomarkers, it is crucial to understand their roles in disease pathogenesis. Although affordable 'OMICS'-based technologies have enabled faster identification of miRNAs, the identification and validation of miRNA functions is still hindered by low sample size and poor reproducibility. Besides, a holistic approach for exploring and validating miRNA functions, given the complexity of livestock diseases is lacking. Since many livestock diseases are chronic in nature, miRNA functional studies should consider the different disease stages. Some diseases are also caused by multiple pathogens such as mastitis or impact numerous tissues or organs such as JD; therefore, the spatiotemporal-specific manner of the regulatory function of miRNAs needs to be considered. The lack of sensitive and reliable tools for detecting lowly expressed miRNAs might ignore some potentially important miRNAs with essential functions. Additionally, a miRNA can target hundreds of genes, thus making it difficult, costly, and labor-intensive to validate each miRNA gene target functionally. Lastly, the limited attention to *in vivo* experiments for miRNA validation is also a significant challenge for understanding their roles in livestock diseases.

Nevertheless, the lower cost of sequencing may lead to increase in sample sizes in miRNA studies and allow the inclusion of technical errors in experimental designs. Moreover, the present downward trend in the cost of sequencing may provide opportunities to sequence multiple types of molecules (miRNAs, lncRNAs, mRNAs, etc.) simultaneously, thereby enhancing the possibility of integrative analyses for further exploration of miRNA roles in interaction networks. Furthermore, the collaboration by different research groups can significantly improve the power of detection and validation of miRNA functions. Other technologies, such as single-cell sequencing will further understanding of disease pathogenesis [187] and miRNA functions, while genome editing [202] and RNA interfering technologies [203] could facilitate identification of the exact target genes and downstream impact of miRNAs on disease pathogenesis. Machine learning and deep learning methods could improve the ability to classify disease pathogens [189] and predict the roles of miRNAs in disease progression [204].

## 5. Conclusion

It is without doubt that miRNAs play significant regulatory roles in livestock disease pathogenesis and have substantial potential as biomarkers for the management of livestock diseases. However, the application of miRNAs in disease management is hindered by many factors such as inadequate diagnostic tools, lack of assessment for the accuracy, sensitivity, specificity of miRNAs, and potentially high-cost of developing miRNA biomarkers. Besides, an incomplete understanding of miRNA roles in disease pathogenesis is also a contributing factor limiting the use of miRNAs in livestock disease management. Nevertheless, given the pressing need to control livestock diseases, a significant increase in miRNA research has been observed in recent times. Deeper disease phenotyping methods such as sensor technology, lower cost of sequencing or miRNA genotyping, and more powerful computing resources and statistical methods are important assets for miRNA studies. Therefore, we believe that miRNA biomarkers will eventually be developed and employed as powerful tools to manage livestock diseases.

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EMIA.; funding acquisition, EMIA. All authors have read and agreed to the published version of the manuscript.

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**Abbreviations**

| | |
|---|---|
| AAAV | Avian Adeno-Associated Virus |
| AIVs | Avian Influenza Viruses |
| ALV | Avian Leukosis Virus |
| ALV-J | Avian Leukosis Virus Subgroup J |
| ASF | African Swine Fever |
| *BCL-2* | B-Cell Lymphoma 2 |
| BMECs | bovine mammary epithelial cells |
| BVD | Bovine Viral Diarrhea |
| BVD | Bovine viral diarrhea |
| BVDV | BVD virus |
| CCNE1 | Cyclin E |
| CLCF1 | Cardiotrophin Like Cytokine Factor 1 |
| CMT | California mastitis test |
| DE | differentially expressed |
| DIAP1 | *Drosophila* Inhibitor Of Apoptosis Protein 1 |
| *DLG5* | Discs Large MAGUK Scaffold Protein 5 |
| DOAJ | Directory Of Open Access Journals |
| ELK1 | ETS-Like Gene 1 |
| ENTV | Enzootic Nasal Tumor Virus |
| FMD | foot and mouth disease |
| FMDV | FMD virus |
| GaHV-2 | *Gallid Herpesvirus* 2 |
| hpi | hours post-infection |
| Hsp70 | Heat Shock Protein Family A |
| IBDV | Infectious Bursal Disease Virus |
| IC | ileum control |
| IFN-$\alpha$ | type I interferon |
| IFN-$\beta$ | beta interferon |
| IL7R | Interleukin 7 Receptor |
| IR | ileum resistant |
| IRAK1 | interleukin-1 receptor-associated kinase 1 |
| IRAK2 | Interleukin-1 Receptor-Associated Kinase 2 |

| | |
|---|---|
| *IRF1* | Interferon Regulatory Factor 1 |
| IS | ileum susceptible |
| JAK1 | Janus kinase 1 |
| JD | Johne's disease |
| *KLF4* | Krüppel-like factor 4 |
| LD | Linear Dichroism |
| LS | low Salmonella shedding |
| MAP | *M. avium* subsp. *paratuberculosis* |
| MAPK | Mitogen-Activated Protein Kinase |
| *MDA5* | Melanoma Differentiation-Associated Gene 5 |
| MDPI | Multidisciplinary Digital Publishing Institute |
| MDV | Marek's Disease Virus |
| MHC | Major Histocompatibility Complex |
| miRNA-Seq | miRNA sequencing |
| ncRNAs | Non-Coding Rnas |
| *NFAT5* | Nuclear Factor Of Activated T-Cells 5 |
| NFATC4 | Nuclear Factor Of Activated T Cells 4 |
| NFIA | nuclear factor IA |
| NF-kappaB | nuclear factor-kappa B |
| NGS | Next-Generation Sequencing |
| *NPEPPS* | Aminopeptidase Puromycin Sensitive |
| *ORF4* | open reading frame 4 |
| P.I. | Post-Infection |
| PBMC | Peripheral Blood Lymphocyte |
| PCR | Polymerase Chain Reaction |
| PCV2 | Porcine circovirus type 2 |
| *PDCD4* | Programmed Cell Death 4 Gene |
| PID | Post-Infection Day |
| PPR | Peste Des Petits Ruminants |
| PPRV | PPR Virus |
| PRRS | Porcine Reproductive and Respiratory Syndrome |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PS | persistent Salmonella shedding |
| REV | Reticuloendotheliosis Virus |
| RXRB | retinoid X receptor β |
| *SOCS5* | Suppressor Of Cytokine Signaling 5 |
| SRP14 | signal recognition particle 14 |
| ST | *Salmonella enterica serovar Typhimurium* |
| ST | Primary Sheep Testicular |
| TGS | Third-Generation Sequencing |
| TLA | Three-Letter Acronym |

| | |
|---|---|
| VP1 | Virus Protein |
| YAP1 | Targeting and Repressing Yes-Associated Protein 1 |

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