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RNA-sequencing studies suggest that microRNAs and alternative splicing of pre-mRNAs modulate immune and inflammatory responses in Holstein cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

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RNA-Sequencing (RNA-Seq) studies using bovine samples from *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-infected animals have identified a range of differentially expressed mRNAs with potential as biomarkers for MAP infection. As bioinformatic tools continue to develop, microRNAs (miRNAs) and alternative splicing (AS) have emerged as important contributors to host responses during infections. Although RNA-Seq enables AS analysis, most transcriptomic studies still emphasize gene-level expression due to the complexity of AS workflows, the need for deeper sequencing, and incomplete transcript annotations, even in humans. Nevertheless, interest in AS is growing, driven by its recognized role in tissue-specific functions and disease mechanisms. Recent findings have revealed distinct miRNAs and AS profiles in MAP-infected cattle compared to uninfected cows, indicating that post-transcriptional regulatory mechanisms are altered during MAP infection. In this comprehensive review, we examine critical aspects of post-transcriptional regulation in the context of MAP infection. We focus on recent studies investigating miRNAs and AS profiles, highlighting their roles in modulating immune responses and their potential as novel diagnostic biomarkers. Notably, overlapping mechanisms involving miRNAs and AS have been identified in paratuberculosis and in several human diseases, suggesting conserved pathways of host-pathogen interaction and immune regulation.

KEYWORDS

paratuberculosis, miRNAs, alternative splicing, gene regulation, biomarkers

1 Bovine paratuberculosis

PTB is an infectious chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which affects ruminants worldwide. Due to its socio-economic importance, notification to the World Organization for Animal Health (WOAH) is obligatory (1). PTB is distributed worldwide, with herd-level prevalences higher than 50% in the USA and Europe (2). Clinical signs of the disease include diarrhea, weight loss, decreased milk production, and premature culling (1). Economic losses due to PTB can be directly caused by the clinical signs of the disease, such as animal death or decrease in milk production, or by indirect consequences, such as the cost of the diagnostic testing of all the animals in the herd or the premature culling of animals. In a recent study, Rasmussen et al. estimated the 10-year average annual loss per cow due to PTB at 53.30 US\$ in the USA, 34.40 US\$ in Great Britain, 33.45 US\$ in Italy, 48.91 US\$ in Canada, 31.25 US\$ in Australia, 45.99 US\$ in Spain, 21.72 US\$ in Belgium, 8.31US\$ in Brazil, and 81.53US\$ in Japan (3). These losses represent between 0.72% and 1.41% of the annual milk revenue. Estimates of global annual losses due to PTB currently can reach up to 4–5 billion US \$ (4).

MAP also poses a potential risk to human health. There are similarities between the histopathological lesions and clinical signs that appear in PTB-infected cattle and human patients with gastrointestinal inflammatory diseases such as Crohn’s disease (CD) and inflammatory bowel disease (IBD) (5–7). Moreover, MAP has been identified in blood and tissue samples from patients with CD (8–10), and in the milk of pregnant women with CD (11). Additionally, anti-mycobacterial antibiotics can induce remission of pediatric CD (12). However, a causal association between MAP and CD has not been demonstrated (13). Recent studies have also shown an association between MAP and other human diseases such as type-1 diabetes (14), sarcoidosis (15), Alzheimer’s disease (16), rheumatoid arthritis (17), Hashimoto’s thyroiditis (18), Blau’s syndrome (19), multiple sclerosis (20) and cancer (21, 22). Colorectal cancer can appear as the result of complications of the two forms of idiopathic IBD: CD and ulcerous colitis (23).

2 RNA-Sequencing: a powerful tool to discover novel biomarkers

PTB control measures include the culling of MAP-infected animals and the enhancement of farm biosecurity measures. The current diagnostics tools for the direct detection of MAP are fecal real-time quantitative polymerase chain reaction (qPCR) and bacteriological culture, with the latter being considered the gold standard. However, both show limitations in detecting subclinical MAP-infected cattle with low bacterial load in feces and gut tissues. The lack of sensitivity of current diagnostic tests poses a limitation mainly for the identification of cattle with subclinical infection, which requires the performance of periodic tests and increases costs. The detection of subclinical infections remains a challenge,

and novel tools are needed to detect MAP-infected animals at the subclinical stages of the disease. RNA sequencing (RNA-Seq) is a high-throughput sequencing technique that provides data on the transcriptome of a cell or tissue. RNA-Seq can provide biological information on how an animal responds to an infection and is a source of novel biomarkers. RNA-Seq studies have used different samples from MAP-infected cattle, including those from the ileocecal valve (ICV) (24–26), salivary glands (27), whole blood (26, 28), Peyer’s patches (29), and jejunum and ileum (30). Other RNA-Seq studies used MAP-infected macrophages (31–36) and human monocytic cell lines differentiated to macrophages (37, 38). The mRNA of the ABCA13 transporter — previously identified in subclinical MAP-infected cattle — has been validated as a serum biomarker for detecting Holstein cows with focal PTB-associated lesions (26, 39). A summary of RNA-Seq studies using MAP-infected macrophages and bovine samples from MAP-infected animals is presented in Table 1.

Other potential biomarkers that can be used for MAP diagnosis include non-coding RNAs (ncRNAs) like microRNAs (miRNAs). miRNAs are post-transcriptional regulators of gene expression

TABLE 1 RNA-Seq studies that identified biomarkers associated with MAP infection.

Sample type	Group	N° of animals	Reference
Ileocecal valve	Control	5	(24)
	Subclinical infection	5	
	Clinical infection	5	
	Infected	5	(25)
	Uninfected	5	
	Without lesions	3	(26)
	With focal lesions	5	
	With diffuse lesions	5	
Bovine macrophages	Uninfected	7	(31)
	2h p.i.	7	
	6h p.i.	7	
	Uninfected	68	(32)
	2h p.i.	68	
	6h p.i.	68	
	24h p.i.	68	
	Uninfected	3	(33)
	6h p.i.	3	
	Uninfected	6	(34)
	Infected	6	
	Uninfected	6	(36)
	6h p.i.	6	

(Continued)

TABLE 1 Continued

Sample type	Group	N° of animals	Reference
Salivary glands	Infected at 1, 4, 8 and 24h p.i.	18	(27)
	Uninfected at 1, 4, 8 and 24h	6	
Whole blood	Infected	5	(28)
	Uninfected	5	
	Without lesions	3	(26)
	With focal lesions	5	
	With diffuse lesions	5	
Peyer's patches	Infected	10	(29)
	Uninfected	5	
Jejunum and ileum	Infected	4	(30)
	Uninfected	3	
Human macrophages	3h p.i.	THP-1 cells*	(37)
	Uninfected	THP-1 cells*	
	3h p.i.	2	(38)
	Uninfected	2	

*Monocyte cell line isolated from peripheral blood of an acute monocytic leukemia patient.

through their binding to their target mRNAs. Biomarkers associated with specific stages of MAP infection can also include transcript isoforms, caused by alternative splicing (AS). AS of pre-mRNA consists of the removal of introns and the assembly of exons contained in eukaryotic genes. AS events can influence transcript stability or structure with important physiological consequences. While RNA-Seq has enabled AS analysis, most transcriptomic studies still focus on gene-level expression rather than transcript isoform diversity. AS analysis requires more complex computational pipelines, greater sequencing depth, and careful interpretation. There is also the challenge of incomplete annotations of all transcript isoforms, even in well-studied organisms like humans. However, this area is gaining more attention, especially with the growing understanding of how crucial AS is to tissue-specific functions and disease mechanisms.

3 MiRNAs

MiRNAs are highly conserved small ncRNAs (18 to 25 nucleotides) that regulate the expression of their target mRNAs. The first identified miRNA was detected in *Caenorhabditis elegans* by Lee et al. and was named lin-4 as it is a regulator of the LIN-14 protein (40). Years later, miRNAs were found in abundance in different invertebrate and vertebrate species with a high degree of conservation, which suggested that they were associated with important regulatory processes common between species.

3.1 Biogenesis of miRNAs

MiRNA biogenesis is classified into the canonical and non-canonical pathways (41) (Figure 1). In the canonical pathway, miRNA synthesis starts with a large primary transcript (pri-miRNA), an RNA stem-loop with a cap at 5', and a polyadenine at 3', usually transcribed by the RNA polymerase II (42). The 5' cap and 3' polyadenine of the pri-miRNA are cleaved in the cellular nucleus by a microprocessor complex formed by the type-III RNase Drosha and a double-stranded RNA binding protein, *DiGeorge syndrome critical region 8* (*DCGR8*) in mammals, to produce the pre-miRNA, which only contains the stem-loop structure (43). The pre-miRNA is translocated to the cytoplasm by the Ran/GTP/Exportin 5 complex, where it is processed by Dicer, another type-III RNase, which breaks the stem-loop and forms a miRNA-miRNA duplex. One of the strands is the mature miRNA, while the other is the "passenger" strand (44). The non-canonical pathway usually starts with an ncRNA, such as an intron or a short hairpin RNA (shRNA). The shRNA is initially cleaved by the microprocessor complex and exported to the cytoplasm via Ran/GTP/exportin5. The pre-miRNAs are further cleaved through the Argonaute-2 (AGO2), but Dicer-independent, cleavage (45). Mirtrons, which are produced from the introns of mRNAs during splicing, and 7-methylguanosine (m⁷G)-capped pre-miRNAs are dependent on Dicer to complete their cytoplasmic maturation, but they differ in their nucleocytoplasmic shuttling. Mirtrons are exported via Exportin5/Ran/GTP, while m⁷G-pre-miRNA are exported via Exportin1. The mature miRNA generated from the canonical or non-canonical pathways is integrated into the RNA-induced silencing complex (RISC), which guides the miRNA to its target mRNA. The binding of miRNAs to their target mRNAs induces gene silencing and/or mRNA degradation. The binding of miRNAs to the 5'-untranslated region (5'UTR) and other DNA elements could enhance translation and transcription (46). miRNAs tend to associate with proteins and/or travel in serum and plasma encapsulated within vesicles, which makes them more resistant to degradation than other RNAs and allows them to be measured with high sensitivity (47).

3.2 MiRNA profiling in MAP infection

The first study analyzing miRNA expression in MAP-infected animals did not show differential miRNA patterns between interferon-γ (IFN-γ) responders and unchallenged controls, probably due to limitations of the available miRNA annotation (48). However, the authors did identify differential expression of miR-205, miR-10b, miR-92b, miR-432, miR-27a, miR-127, miR-126, and miR-143 between controls at time point zero and controls at 6 months post-infection. These miRNAs are associated with proliferation and development roles in different mammal species, which suggests that miRNA expression also changes during development processes. Shaughnessy et al. did not detect differentially expressed (DE) miRNAs in serum at either the early (6 months) or late (43, 46, and 49 months) intervals between

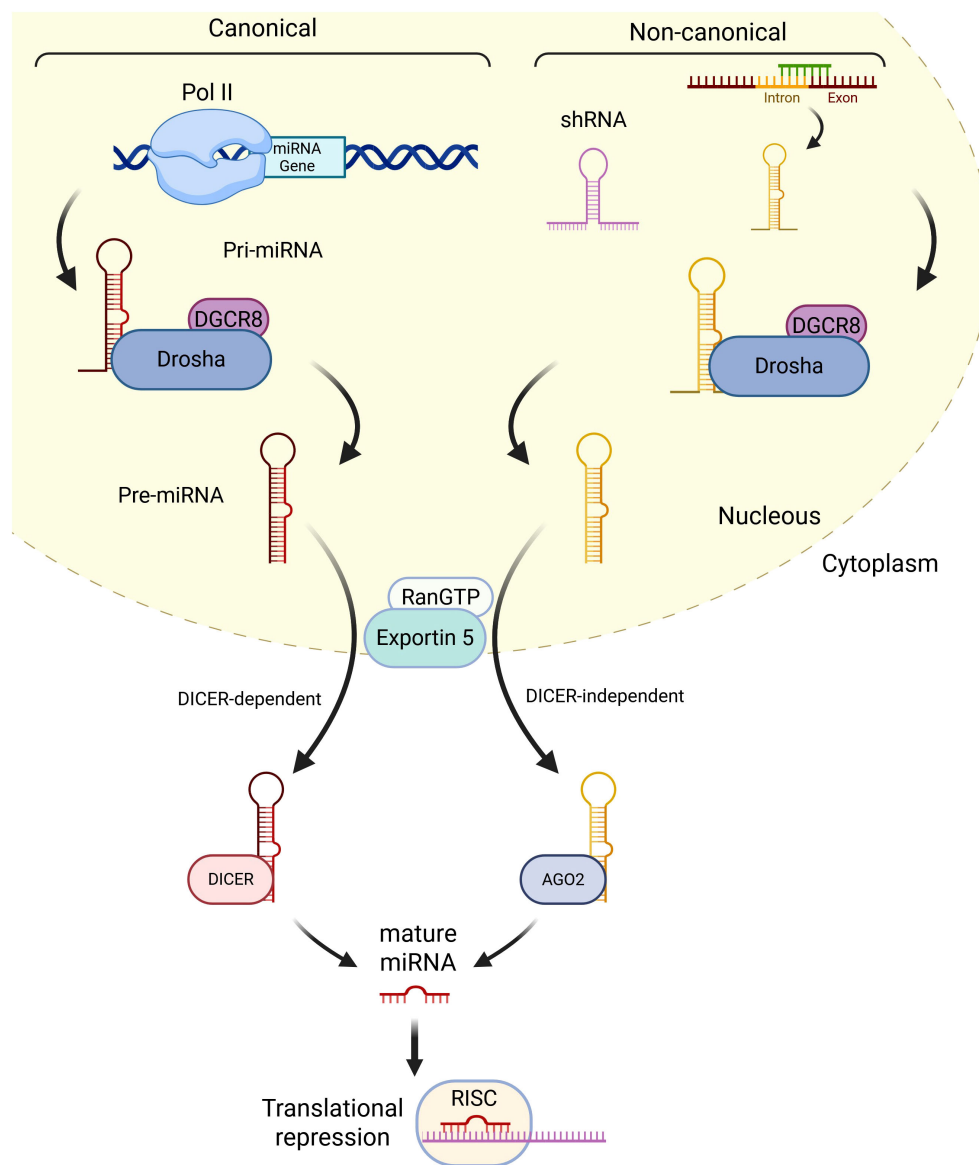


FIGURE 1

Summary of miRNA biogenesis. In the canonical pathway, miRNAs are usually transcribed by RNA polymerase II and generate pri-miRNAs, which are cleaved by DGCR8 and DROSHA into a stem-loop structure called pre-miRNA. RanGTP/Exportin 5 complex translocates the pre-miRNA to the cytoplasm, where it is processed by DICER into a miRNA duplex containing the mature miRNA and the passenger miRNA. After the duplex is undone, the mature miRNA is incorporated into the RISC complex and can induce gene silencing and/or mRNA degradation. In the non-canonical pathway, shRNAs are initially cleaved by the microprocessor complex and exported to the cytoplasm via RanGTP/Exportin5. They are further processed via AGO2-dependent, but DICER-independent, cleavage. All pathways ultimately lead to a functional miRISC complex. In most cases, miRISC binds to target mRNAs to induce translational inhibition (Created in Biorender.com).

seropositive and seronegative animals. However, some miRNAs, such as bta-miR-92b, bta-miR-29a, bta-miR-205, and bta-miR-345-3p, were DE in seropositive animals at time point 0 and time points 6, 43, 46, and 49 months. These miRNAs are associated with immune functions, suggesting that they could be associated with immune maturation.

A summary of the miRNAs with differential expression between MAP-infected and uninfected cattle is shown in Table 2. Wang et al. identified DE miRNAs in monocyte-derived macrophages (MDMs) infected with MAP 6 hours after infection (49). These DE miRNAs were associated with modulation of the MAPK pathway (miR-12023

and miR-1343-3p), the NF- κ B pathway (miR-214, bta-miR-133a, and bta-miR-1246), and apoptosis (bta-miR-1246, bta-miR-1306, bta-miR-454, and bta-miR-150). Using BoMac cells and MDMs, Wright et al. showed that MAP can modulate miR-19a, miR-129, miR-24, and miR-24-3p expression to induce macrophage polarization (50). MiR-19a and miR-129 are associated with an M1 macrophage phenotype and regulate lipid efflux and inflammation. MiR-24-3p, however, inhibits phagocytosis and promotes the M2 macrophage phenotype. These findings suggest that MAP can interfere with inflammation and macrophage function by altering miRNA expression. Additional studies in MAP-infected bovine macrophages found that bta-miR-

TABLE 2 MiRNAs that are dysregulated upon MAP infection.

Sample type	miRNAs	Up/ downregulation	Role of the miRNAs	Reference	
Macrophages	bta-miR-92b, bta-miR-29d-5p, bta-miR-454, bta-miR-1306, bta-miR-1343-3p, bta-miR-2443, and bta-miR-12023	Downregulated	MAPK, NF-κβ, and apoptosis	(49)	
	bta-miR-132, PC-3p-7159_279, bta-miR-1246, bta-miR-2484, bta-miR-150, bta-miR-451, bta-miR-214, bta-miR-677, bta-miR-380-3p, bta-miR-122, bta-miR-212, bta-miR-1434-5p, bta-miR-369-3p, and bta-miR-133a	Upregulated			
	miR-129-5p	Downregulated	Macrophage polarization, lipid efflux, and inflammation, phagocytosis	(50)	
	miR-129-5p, miR-19b-3p, and miR-24-3p	Upregulated			
	bta-miR-133b, bta-miR-92a, and bta-miR-184	Upregulated	Interleukin signaling	(51)	
Whole blood	bta-mir-19b, bta-mir-19b-2, bta-mir-1271, bta-mir-100, bta-mir-301a, bta-mir-32, 14_7917 (Novel), and 27_25982(Novel)	Downregulated	Phagocytosis, macrophage function, and cytokine production	(52)	
	bta-mir-6517, bta-mir-7857, bta-mir-24-1, bta-mir-24-2, and bta-mir-378c	Upregulated			
Ileal segments	bta-miR-105a, bta-miR-433, bta-miR-2400, bta-miR-137, bta-miR-424-3p, bta-miR-138, and bta-novel-53(Novel)	Downregulated	TLR signaling, lymphocyte activation, inflammatory response, and phagocytosis	(25)	
	bta-miR-146b, bta-miR-196b, bta-miR-2483-5p, bta-miR-133b, bta-miR-1247-5p, bta-miR-184, and bta-miR-202	Upregulated			
Feces	miR-501-5p, miR-658	Downregulated	ND	(53)	
	miR-92a-3p	Upregulated			
Sera	bta-miR-147, bta-miR-196a, bta-miR-346, bta-miR-655, and bta-miR-2426	Downregulated	Ras and Wnt pathways	(54)	
	bta-miR-363, bta-miR-374b, bta-miR-2887,	Upregulated			
	and miR-92a-3p				
Whole blood diffuse lesions vs. no lesions	bta-miR-144, bta-miR-19a, and bta-miR-101	Downregulated	Not identified	(56)	
	bta-miR-2425-3p, and bta-miR-139	Upregulated			
Whole blood diffuse vs. focal lesions	bta-miR-144, bta-miR-19a, and bta-miR-32	Downregulated	Not identified		
Ileocecal valve focal lesions vs. no lesions	bta-miR-23b-3p and bta-miR-23a	Downregulated	RNA polymerase		
	bta-miR-2478 and bta-miR-150	Upregulated			
Ileocecal valve diffuse lesions vs. no lesions	bta-miR-135b and bta-miR-433	Downregulated	MAPK signaling		
Ileocecal valve diffuse vs. focal lesions	bta-miR-204, bta-miR-99a-5p, bta-miR-10174-3p, bta-miR-154c, bta-miR-433, bta-miR-23b-3p, bta-let-7e, bta-miR-214, bta-miR-23a, bta-miR-382, bta-miR-145, bta-let-7c, bta-miR-30a-5p, bta-miR-27b, and bta-miR-411a	Downregulated	MAPK, P53, lysosome, PI3K/ Akt, JAK-STAT, and regulation of immune system		
	bta-miR-146a, bta-miR-146b, and bta-miR-10167-3p	Upregulated			

133b, bta-miR-92a, and bta-miR-184 were downregulated upon MAP infection (51). Target gene function analysis suggested that miR-92a was involved in interleukin signaling, hence regulating the immune response against MAP.

An analysis of whole-blood samples collected from MAP-infected cows and uninfected cows from a MAP-free herd based on ELISA results and absence of clinical cases revealed differential expression of miRNAs with roles in the regulation of the immune

response and inflammation upon bacterial or viral infection (52). In the infected cows, downregulation of bta-miR-100 and bta-miR-301a and upregulation of bta-miR-32 were observed. The targets of these miRNAs associated with phagocytosis, macrophage function and cytokine production were the *Tweety Family Member 3* (*TTYH3*), *HIC ZBTB Transcriptional Repressor 1* (*HIC1*), *Inosine Monophosphate Dehydrogenase 1* (*IMPDH1*), *ARF Like GTPase 2* (*ARL2*), *Adaptor Related Protein Complex 2 Subunit Alpha 1* (*AP2A1*), and *Zinc Finger and BTB Domain Containing 4* (*ZBTB4*). Other differentially expressed miRNAs were bta-miR-19b, bta-miR-19b-2, bta-miR-1271, and a novel miRNA (Novel:14_7917). The authors hypothesize that the identified miRNAs may be involved in regulating early immune responses.

MAP infection can also result in miRNA upregulation in the ileal segments of newborn calves, such as miR-146b, which inhibits proteins in the TLR signaling pathway such as interleukin-1 receptor-associated kinase 1 (*IRAK1*), interleukin-1 receptor-associated kinase 2 (*IRAK2*), and tumor necrosis associated factor 6 (*TRAF6*) (25). Therefore, MAP could be interfering with TLR signaling and downstream inflammatory responses. This study also found an upregulation of bta-miR-202, bta-miR-184, bta-miR-196b, and bta-miR-1247, associated with lymphocyte activation, muscle and epithelium development, inflammatory response, the proliferation of endothelial cells, phagocytosis, and epigenetic regulation. Downregulation of bta-miR-133b, bta-miR-433, bta-miR-105a, and bta-miR-137, associated with lymphocyte activation, the proliferation of endothelial cells, muscle and epithelium development, and vesicle transport, was also detected.

Fecal samples have also been used to identify DE miRNAs between cows with clinical signs of PTB and with a bacteriological culture and ELISA-positive result vs. healthy cows (53). The results showed downregulation of miR-501-5p and miR-658, and upregulation of miR-92a-3p. When comparing miRNA-Seq data obtained from serum samples of cows in different stages of PTB based on ELISA and PCR results and clinical signs vs. control cows, Choi et al. detected differential expression of the bta-miR-363, bta-miR-196a, bta-miR-147, bta-miR-655, bta-miR-346, and bta-miR-2426, associated with the Ras and Wnt pathways (54). Two miRNAs (bta-miR-374b and bta-miR-2887) were identified in cows with PCR-positive and ELISA-negative results and with key molecules such as interleukin-10 (*IL-10*) and transforming growth factor beta (*TGF-β1*).

Previous studies have compared the miRNA profiles of cattle with positive and negative ELISA and fecal PCR results (52–55). In cattle, however, focal lesions in gut tissues can be detected before fecal shedding and ELISA. Therefore, examining the miRNA profiles of MAP-infected animals selected according to the presence of focal histopathological lesions facilitates the identification of miRNAs in animals in the subclinical stage of the infection when the amount of MAP and antibodies cannot be detected with current pre-mortem diagnostic methods. Our research team recently used miRNA-Seq to quantify and compare the expression of miRNAs in ICV and whole-blood samples collected from cows with focal or diffuse PTB-associated lesions in gut tissues vs. control cows without lesions (56). To our knowledge, this was the first study that used sRNA-Seq to

compare miRNA expression in blood and gut tissues of cattle classified according to histopathological data. None of the identified miRNAs were differentially expressed in both the PB and ICV samples, and, therefore, our results showed PB and ICV-specific miRNAs expression. Specifically, in blood samples, we identified eight miRNAs in the comparison of cows with diffuse lesions vs. controls (bta-miR-2425-3p, bta-miR-144, bta-miR-19a, bta-miR-32, bta-miR-139, bta-miR-101, bta-miR-27a-5p, and bta-miR-181a) and three in the comparison of cows with diffuse vs. focal lesions (bta-miR-19a, bta-miR-144, and bta-miR-32). Among the eight miRNAs differentially expressed in blood samples from cows with diffuse lesions vs. controls, three (bta-miR-19a, bta-miR-144, and bta-miR-32) were also found downregulated in PB samples of cows with diffuse vs. focal lesions. Interestingly, bta-miR-32 was also downregulated in a previous study where the miRNA profiles of Holstein cattle positive and negative for MAP antibodies were compared (52). In the ICV samples, we identified 4, 5, and 18 miRNAs that were differentially expressed in cows with focal lesions vs. controls, diffuse lesions vs. controls, and diffuse vs. focal lesions, respectively. This suggests that the miRNA expression in ICV changes more as PTB-associated lesions become more severe. In the comparison of cows with focal lesions vs. controls, two upregulated (bta-miR-2478 and bta-miR-150) and two downregulated (bta-miR-23a and bta-miR-23b-3p) miRNAs were identified. DE miRNAs in the comparison of cows with diffuse lesions vs. controls (bta-miR-433, bta-miR-146a, and bta-miR-99a-5p) were also dysregulated in the comparison of cows with diffuse vs. focal lesions. Interestingly, the human ortholog of bta-miR-146a is an inhibitor of the pro-inflammatory immune response (57). We, therefore, speculated that the upregulation of miR-146a would negatively regulate TRAF6 and control the pro-inflammatory immune response (58). Two miRNAs (bta-miR-23a and bta-miR-23b-3p) were downregulated in both comparisons, i.e., in cows with focal lesions vs. controls and diffuse vs. focal lesions. Some of the miRNAs identified in our study had been previously identified in studies describing miRNA expression in response to mycobacterial infections, including bta-miR-27a-5p, bta-miR-32, bta-miR-23a, bta-miR-135b, bta-miR-146a, bta-miR-146b, bta-miR-433, bta-let-7e, and bta-let-7c (25, 52, 59, 60).

4 Alternative splicing

Splicing is the process by which the pre-mRNA's introns are removed, and the exons are rearranged.

4.1 AS mechanism and types

This process is mediated by the spliceosome, a complex formed by highly dynamic small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4, U5, and U6) and accessory proteins (61–63) (Figure 2A). The same mRNA can be spliced in different ways to produce different proteins. Splicing events can be categorized depending on whether they are always (constitutive) or only sometimes (alternative) recognized by

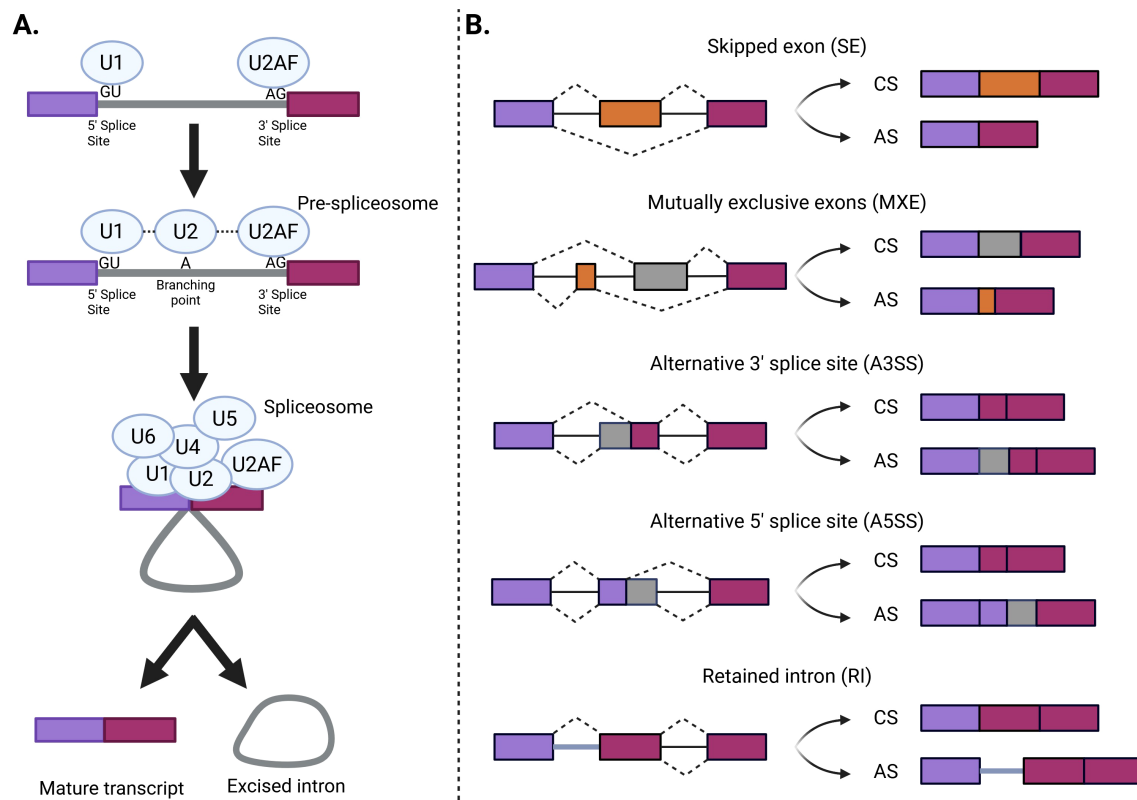


FIGURE 2

The splicing process and AS events. **(A)** First, a recognition of the 5' and 3' splice sites by the U1 and U2 small nuclear ribonucleoprotein complexes (snRNPs) occurs. Then, the U4, U5, and U6 snRNPs are recruited into the active spliceosome. Finally, the functional spliceosome excises the intron and binds the two exons. **(B)** Different types of alternative splicing events. These classes include skipped exon (SE), in which a single exon is included or excluded from the final transcript; mutually exclusive exons (MXE), in which the final transcript only contains one of the two affected exons; alternative 3' splice site (A3SS), in which the acceptor site (at 3') is changed; alternative 5' splice site (A5SS), in which the donor site (at 5') is changed; and retained intron (RI), in which an intron gets included in the final transcript. Violet and magenta bars represent the constitutive sequence that always forms part of the mature mRNA, while yellow and grey represent the alternative sequences that can be either included or excluded in the mature mRNA. Exons and introns are represented by boxes and solid lines, respectively (Created in [Biorender.com](https://www.biorender.com)). AS, alternative splicing; CS, constitutive splicing.

the spliceosome and spliced in the mature mRNA. The most frequently used splice sites contain highly conserved sequences easily recognized by the spliceosome and lead to constitutive splicing (CS), whereas less frequently used splice sites lead to AS and are only used when specific splicing regulatory elements are activated. Depending on the splice site locations, AS events can be classified into different classes (Figure 2B). AS events can have multiple detrimental consequences in the mature transcript, such as a decrease in mRNA stability, a change in protein function or target, a change in final protein location, and the generation of premature termination codons, which are natural targets of the nonsense-mediated decay pathway and can cause changes in gene expression (64, 65). Changes in the frequency of AS events can be the cause of specific diseases, modify their severity, or be associated with their susceptibility (66).

4.2 AS profiling in MAP infection

Genes and pathways associated with AS event changes in MAP-infected cattle have been identified and are summarized in Table 3.

Using surgically isolated intestinal segments, significant changes in the frequency of two AS events between MAP-infected and non-infected tissues were detected 1 month post-infection (25). There were 16 genes that displayed significantly different AS events between the control and infected compartments of control and infected animals. RT-qPCR was performed to verify the differences between MAP-infected and control tissues in alternative spliced forms of the *adenosine deaminase (ADA)* and *monocyte-to-macrophage differentiation-associated (MMD)* genes. The identified AS events affected *ADA* and *MMD* mRNAs and appeared more frequently in MAP-infected intestinal segments. The event affecting *ADA* is a retained intron (RI) event, in which the intron 4 was retained significantly more frequently in MAP-infected segments. The event affecting *MMD* consisted of a mutually exclusive exon (MXE) event, in which the alternative first exon was detected more frequently in infected segments. *ADA* is involved in purine metabolism, and its deficiency results in the accumulation of adenosine in the lysosome, as well as lysosome enlargement, alkalinization, and dysfunction (67). *MMD* is expressed in mature macrophages and participates in macrophage activation through

TABLE 3 Pathways affected by AS events associated with MAP infection.

Sample type	Genes that displayed significantly different AS events between control and infected animals	Pathways affected by AS events	Reference
Ileal segments	<i>ADA</i> ¹ and <i>MMD</i> ¹	Macrophage maturation and lysosome function	(25)
Jejunum	<i>COPG2</i> , <i>KIF2C</i> ² , <i>EXOC7</i> ² , <i>RABEPK</i> , <i>DNASE1</i> , and <i>EEA1</i>	vesicle-mediated transport, regulation of acute inflammatory response, and tuberculosis	(69)
Peripheral blood			
Salivary gland			
Peripheral blood	<i>BOLA</i> , <i>CYTH1</i> , <i>LOC509006</i> , <i>DNM2</i> , <i>CLTA</i> , <i>JSP.1</i> , <i>RAB11FIP5</i> , <i>AP2M1</i> , <i>PRKCI</i> , <i>ARAP1</i> , <i>CD53</i> , <i>CLEC12A</i> , <i>CPNE1</i> , <i>ITGA2B</i> , <i>NCF1</i> , <i>SGSH</i> , <i>SIRPA</i> , <i>SLC11A1</i> , <i>TARM1</i> , <i>TMC6</i> , <i>CLTA</i> , <i>DNM1</i> , <i>DNM2</i> , <i>EPS15L1</i> , <i>FCGR2B</i> , <i>FCHO1</i> , <i>PICALM</i> , <i>NCOA7</i> , <i>DMXL1</i> , <i>SLC11A1</i> , <i>MAN2B1</i> , <i>LAPTM4A</i> , <i>AP3B1</i> , <i>NAGA</i> , <i>CTSW</i> , <i>ACTN1</i> , <i>LOC512286</i> , <i>LOC782367</i> , <i>LOC788175</i> , <i>TGFB2</i> , <i>ITGB2</i> , and <i>C8G</i>	Endocytosis, clathrin-mediated endocytosis, neutrophil degranulation, platelet activation, lysosome, and amoebiasis	(70)
Ileocecal valve ³	<i>BOLA</i> , <i>BOLA-NC1</i> , <i>LOC616942</i> , <i>GRK3</i> , <i>IL2RG</i> , <i>C2</i> , <i>C4A</i> , <i>PSMB10</i> , <i>NR1H3</i> , and <i>PSAP</i> , <i>ASAH1</i>	Endocytosis, antigen processing and presentation, complement activation, humoral immune response mediated by Igs, immune system processes, IgG-mediated immune response, B-cell mediated immunity, and the lysosome	

¹Validated by RT-qPCR. ²Differential AS events in these genes significantly changed protein structure. ³Genes displaying changes in both AS and gene expression.

TNF- α and nitric oxide production (68). MAP infection may cause these AS events to disrupt macrophage maturation and lysosome function. The retention of intron 4 in *ADA* could decrease transcript stability, gene expression, and impair macrophage lysosome function. An abnormal first exon of *MMD*, however, could disrupt protein synthesis or function and interfere with macrophage maturation and MAP clearance during the early stage of the infection.

RNA-Seq data from Holstein cows classified as PTB-positive or negative according to ELISA and fecal PCR results allowed the identification of 119 (90 genes), 150 (89 genes), and 68 (45 genes) differential AS events between MAP-infected and healthy Holstein cows in whole-blood, jejunum, and salivary gland samples, respectively (69). Furthermore, 14 genes (*CD3G*, *SCARB1*, *MYO5A*, *RABEPK*, *KIF2C*, *EXOC7*, *COPG2*, *REPS1*, *NFYC*, *SYK*, *EEA1*, *CARD9*, *DNASE1*, *TAC1*, and *RHBDD3*) were significantly enriched for immune-related pathways, including vesicle-mediated transport, regulation of acute inflammatory response, and tuberculosis. Two of the identified AS events may alter protein structure, with an event in *exocyst complex component 7* (*EXOC7*), which skipped exon 8 and appeared less frequently in MAP-infected animals, and an event in *kinesin family member 2C* (*KIF2C*), which skipped exon 10 and appeared more frequently in MAP-infected animals. *EXOC7* plays an important role in vesicular trafficking and the secretory pathway, and it has been reported that a lack of *EXOC7* causes a decrease in the number of phagosomes, reducing the efficiency of cytokine transport. *KIF2C* encodes for a protein associated with mitotic chromosome segregation and with immune cell migration and infiltration in humans. Thus, the skipping of exon 10 may affect the immune response against MAP by decreasing immune cell migration.

Our research team has recently used RNA-Seq to identify differential AS profiles between whole blood and ICV samples

collected from Holstein cattle with focal and diffuse PTB-associated lesions in gut tissues vs cows without lesions in gut tissues (70). The role of AS in the regulation of the immune response was studied while keeping in mind its potential application in the diagnosis of subclinical MAP infection. To the best of our knowledge, our study is the first genome-wide profiling of AS events in whole-blood and ICV samples from Holstein cows with PTB-associated lesions of distinct severity vs. uninfected cattle without lesions in gut tissues. In the PB of cows with focal lesions vs. controls, differential AS was identified in genes involved in endocytosis (*bta041144*) including *BOLA*, *LOC509006*, *JSP.1*, *Dynamin-2* (*DNM2*), *CLTA*, *CLTA Adaptor complex AP2, Mu subunit* (*AP2M1*), *Cytohesin 1* (*CYTH1*) *RAB11 Family Interacting Protein 5* (*RAB11FIP5*), the apoptosis regulator (*PRKCI*), *ArfGAP With RhoGAP Domain*, and *Ankyrin Repeat And PH Domain 1* (*ARAP1*). *CLTA* is the main structural component of the lattice-type cytoplasmic face of coated pits and vesicles generated during receptor-mediated endocytosis. *DNM2* participates in the release of *CLTA*-coated vesicles from the plasma membrane, and *AP2M1* is required for the activity of a vacuolar ATPase, which pumps protons into endosomes and lysosomes to acidify them. *CYTH1* regulates the adhesiveness of integrins at the plasma membrane and is involved in membrane trafficking, and *PRKCI* negatively regulates apoptosis. After entry into macrophages by endocytosis, MAP can survive within phagosomes by inhibiting apoptosis, phagosome acidification, and antigen presentation (71). In this context, our findings suggested that the AS of genes involved in endocytosis may contribute to phagosome maturation arrest and MAP persistence within the macrophages of cows with focal lesions in gut tissues.

In the comparison of PB samples from cows with focal lesions vs. controls, AS events in genes associated with the pathogenesis of herpesviruses that infect humans at an early age but remain

asymptomatic during for long time, such as the Kaposi sarcoma-associated herpesvirus infection (bta05167) and Epstein-Barr virus infection (bta05169), were identified. These genes affected by AS events include the *BOLA*, *LOC509006*, *Mitogen-Activated Protein 2 Kinase 7* (*MAP2K7*), *TNF Receptor Associated Factor 3* (*TRAF3*), *Interferon regulatory factor 7* (*IRF7*), *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta* (*PIK3CD*), and *JSP.1*. *IRF7* plays an important role in the regulation of Epstein-Barr virus latency. *TRAF3* can interact with Epstein-Barr virus-encoded latent infection membrane protein-1 (LMP1) along with other members of the TRAF family, acting as a negative NF- κ B regulator and inhibiting the inflammatory response (72). Our findings suggest that the AS of the *BOLA*, *LOC509006*, *MAP2K7*, *TRAF3*, *IRF7*, *PIK3CD*, and *JSP.1* genes may contribute to the development of focalized PTB-associated lesions and establishment of the latent stage of MAP infection, as it has been observed in Epstein-Barr virus and Kaposi sarcoma-associated herpesvirus latency.

In the comparison of the PB samples from cows with diffuse lesions vs. controls, genes involved in the CLTA-mediated endocytosis, neutrophil degranulation, and platelet activation pathways were affected by differential AS. More specifically, AS of genes belonging to the neutrophil degranulation and platelet activation pathways, including the *Cluster of differentiation 53* (*CD53*), *CLEC12A C-type lectin domain family 12 member A* (*CLEC12A*), *Solute Carrier Family 11 Member 1* (*SLC11A1*), and *T Cell-Interacting, Activating Receptor on Myeloid Cells 1* (*TARM1*) genes was observed. *CD53* contributes to transduction signals in T cells and natural killer cells, and *TARM1* increases the production of pro-inflammatory cytokines in macrophages and neutrophils via the toll-like receptor. Neutrophils phagocytose *Mycobacteria* from dying infected macrophages within the granuloma (73, 74). *CLEC12A* and *SLC11A1* are both associated with innate host resistance. *SLC11A1* is a membrane transporter involved in iron metabolism expressed in the late endosomal/lysosomal compartments of macrophages. We hypothesize that the AS of genes involved in neutrophil degranulation and platelet activation pathways may, at least in part, contribute to granuloma formation and pro-inflammatory immune response in MAP-infected cattle with diffuse lesions.

In PB samples from cows with diffuse lesions vs. focal lesions, several genes (*ACTN1*, *TGF β 2*, *ITGB2*, and *C8G*) with AS events associated with the lysosome (bta:04142) and amoebiasis (bta:05146) were identified. Amoebiasis is caused by *Entamoeba histolytica*, a human extracellular protozoan parasite that, like MAP, invades the intestinal epithelium and over time disrupts the intestinal mucus layer, followed by apoptosis of host epithelial cells (75). MAP infection also impacts the intestinal mucosa and, over time, causes serious damage to the ileum and jejunum, diarrhea, progressive wasting, and the eventual death of the infected animal in the more advanced stages of clinical PTB (76). The AS of several genes associated with the activation of the lysosomes and amoebiasis may also cause disruption of the intestinal mucus layer, apoptosis, and MAP dissemination to other organs and tissues, as seen in humans infected with *Entamoeba histolytica*.

In the ICV samples from the infected cows, genes that encode proteins with RNA-binding domains and coiled-coil domains and those involved in AS showed changes in AS. As RNA splicing is regulated by cis-regulatory elements in pre-mRNA and trans-regulatory elements, mainly RNA-binding proteins, AS of RNA-binding proteins may in turn affect the splicing of other pre-mRNAs and contribute to the various onsets of PTB.

5 Associations between miRNAs and AS profiles and gene expression regulation

Our previous studies advanced the understanding of gene expression regulation in response to MAP infection, specifically highlighting the roles of miRNAs and AS events (56, 70). Given that both miRNAs and AS contribute to the modulation of gene expression, we integrated the analysis of AS-regulated pathways with the regulatory influence of miRNAs to provide a comprehensive view of the post-transcriptional regulation of gene expression in MAP-infected cattle. In Figure 3, a graphical summary of the alternatively spliced genes, miRNAs, and key molecular mechanisms identified is presented. In cows with focal lesions vs. control cows (Figure 3A), which could be interpreted as the initial stage of MAP infection, both AS events and miRNAs contributed to the inhibition of the innate immune response, apoptosis, and antibacterial response. The response against MAP DNA mediated by Type I IFN, IL-6, and TNF was inhibited by the upregulation of bta-miRNA-2478, which inhibits RNA polymerase III G (*POLR3G*) expression. *POLR3G* downregulation may be responsible for the blockade of the NF- κ B-mediated innate immune responses that allow for MAP persistence within infected macrophages. The innate immune response was also inhibited via the AS of genes associated with endocytosis, such as *CLTA*, *CYTH1*, *JSP.1*, *RAB11FIP15*, *DNM2*, *AP2M1*, *PRKC1*, and *ARAP1*, and antigen presentation and recognition, such as *BOLA* and *LOC50906*.

In the comparison of cows with diffuse lesions vs. control cows (Figure 3B), which could be interpreted as the early clinical stage of the infection, upregulation of bta-miRNA-215 and bta-miRNA-146a inhibits the pro-inflammatory immune response via inhibition of BMX and TRAF6, respectively, which allows MAP to persist and grow inside granulomas. In this comparison, AS-affected genes associated with neutrophil degranulation and platelet activation, such as *CD53*, *CLEC12A*, *SLC11A1*, and *TARM1*, may stimulate the development of granulomas and the induction of a pro-inflammatory immune response. In the comparison of cows with diffuse lesions vs. focal lesions (Figure 3C), which could be interpreted as the advanced clinical stage of the infection, the downregulation of bta-miRNA-214, bta-miRNA-23a, bta-miRNA-99a-5p, bta-miRNA-204, bta-miRNA-let7c, and bta-miRNA-let7e promotes the expression of type I IFN and proinflammatory cytokines, causing the activation of a strong pro-inflammatory response. In the same way, AS events differentially expressed in this comparison were associated with endocytosis, antigen presentation, humoral IgG and B cell immune response, and complement activation. Aberrant inflammation and induction of

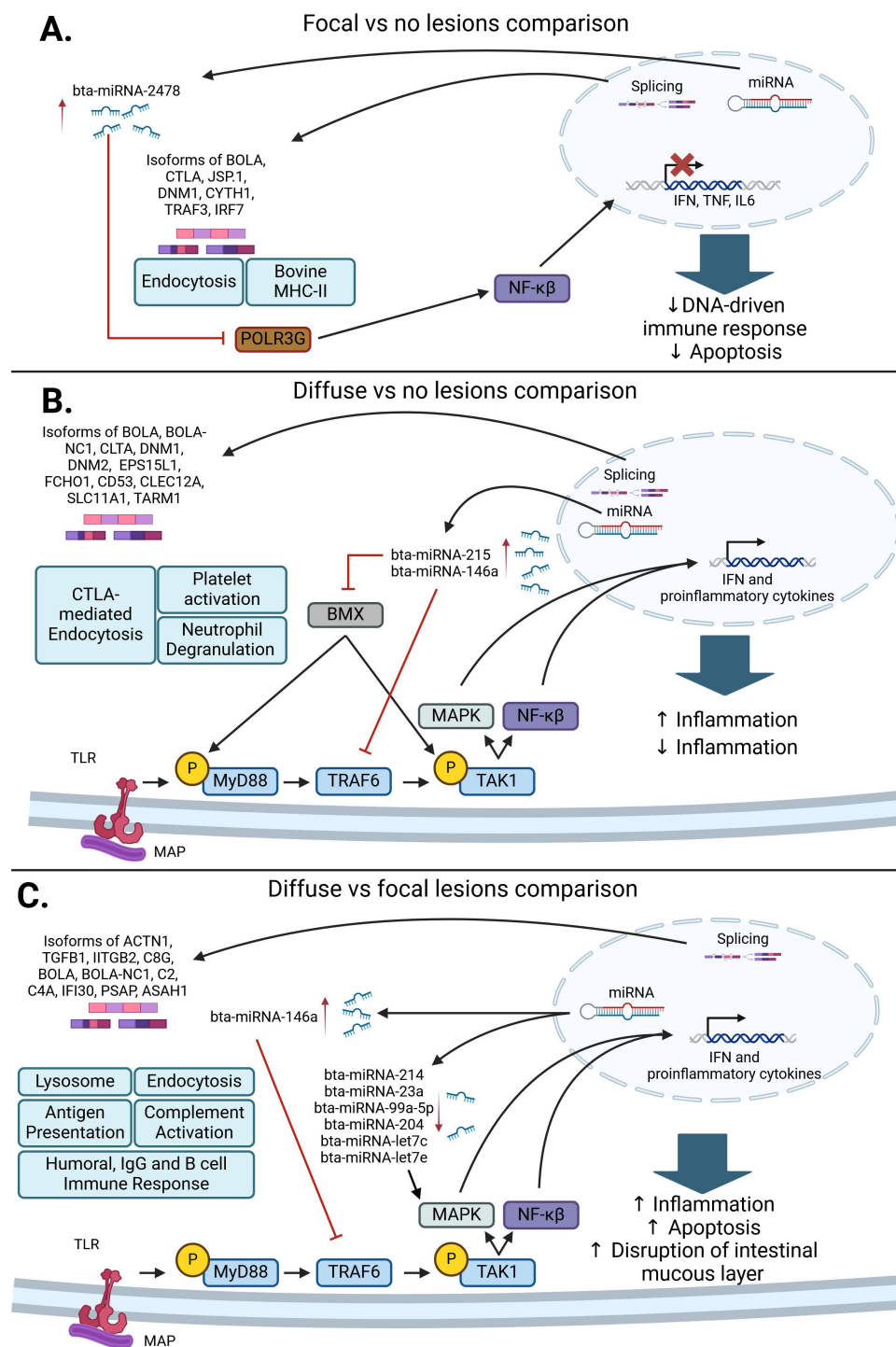


FIGURE 3

Post-transcriptional regulatory pathways associated with a response to MAP infection. **(A)** In the comparison of cows with focal lesions vs. control cows, bta-miRNA-2478 and AS in genes associated with endocytosis and antigen presentation and processing can interfere with NF- κ B and type-I IFN signaling, which would promote MAP survival. **(B)** In the comparison of cows with diffuse vs. no lesions, the differentially expressed miRNAs bta-miRNA-215 and bta-miRNA-146a inhibit BMX and TRAF6, respectively. The inhibition of these genes causes an inhibition of the MAPK and NF- κ B pathways and a decrease in the production of proinflammatory cytokines. Subsequently, the AS of genes affecting CTLA-mediated endocytosis, platelet activation, and neutrophil degranulation contributes to granuloma formation and the pro-inflammatory response observed in MAP-infected cattle with diffuse lesions. **(C)** In the comparison of cows with diffuse vs. focal lesions, a decrease in bta-miRNA-214, bta-miRNA-23a, bta-miRNA-99a-5p, bta-miRNA-204, bta-miRNA-let7c, and bta-miRNA-let7e expression, which target different components of the MAPK pathway, causes an increase in pro-inflammatory cytokine expression. AS events affecting genes associated with endocytosis, the complement system, antigen presentation, the humoral immune response, and the lysosome contribute to an excessive stimulation of the pro-inflammatory response and disruption of the intestinal mucus layer, which results in the development of severe lesions in gut tissues (56, 70) (Created in [BioRender.com](#)).

humoral immune response are usually observed in animals with advanced clinical infection. In addition, the AS of genes involved in the lysis of MAP-containing phagosomes was observed in this comparison, which may lead to apoptosis and disruption of the intestinal mucus layer.

6 Challenges in RNA-Seq and future directions

RNA-Seq has become a very useful tool for transcriptome profiling. This technology holds significant potential, as it allows for in-depth studies of disease pathogenesis and can be used to identify biomarkers for the development of novel diagnostic tools, drugs, and vaccines. We previously identified host mRNAs, miRNAs, and AS events that were DE in samples from MAP-infected animals with distinct PTB-associated lesions in gut tissues *vs.* controls (26, 56, 70). However, several challenges remain in data interpretation. Technical difficulties arising from RNA library preparation and sequencing depth can introduce data variability, especially in studies with small sample sizes or high biological variability. Accurately quantifying low-abundance transcripts, distinguishing closely related isoforms, and detecting AS events require high sequencing depth and robust bioinformatic pipelines, which are often limited by annotation quality and computational resources.

Looking ahead, future directions in RNA-Seq include the integration of long-read sequencing technologies (e.g., PacBio and Oxford Nanopore) to capture full-length transcripts. Additionally, multi-omics approaches that combine RNA-Seq with genomics (77), proteomics, epigenomics, and spatial transcriptomics have the potential to generate a more holistic understanding of gene regulation. Continued improvement in data integration tools, machine learning-based analysis, and publicly available annotation databases will be critical to fully realizing the potential of RNA-Seq in systems biology and host-pathogen interactions.

Besides the utility of miRNAs and AS events as biomarkers for the detection of subclinical MAP infection, modifying or targeting the expression of the identified miRNAs or AS events may open novel opportunities for the development of miRNAs and/or splicing-based PTB therapies. Novel splicing and miRNA-based therapies are being developed for human inflammatory and autoimmune diseases, but they are still rare in the veterinary context.

7 Conclusions

The current diagnostic tools for the direct detection of MAP are fecal real-time qPCR and bacteriological culture. However, both show limitations in detecting subclinical MAP-infected cattle. This review article highlights the contribution of RNA-Seq-based approaches to the discovery of biomarkers that may support both the early diagnosis of MAP infection and an understanding of the regulatory mechanisms involved in the host immune response. It provides a comprehensive review of RNA-Seq studies that explored

changes in miRNA expression and AS events in cattle infected with MAP, highlighting key findings in gene expression regulation during MAP infection and offering mechanistic insights into host immune regulation, which will be valuable for researchers in comparative immunology and veterinary science. The review also aimed to compile recent evidence indicating the importance of these regulatory elements in the pathogenesis of PTB, promoting an integrated view of transcriptomic data and its impact on the biology of the bovine immune system. MiRNAs and AS can modulate host responses against MAP infection, allowing MAP survival during the early stage of the infection and promoting granuloma formation, tissue damage, and humoral immune responses in the clinical stages of MAP infection.

Author contributions

GB-B: Investigation, Visualization, Methodology, Data curation, Formal analysis, Writing – original draft. MA-H: Investigation, Funding acquisition, Resources, Writing – review & editing, Methodology, Validation, Project administration, Supervision, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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