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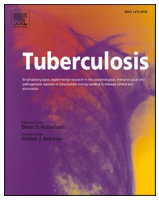

Characterization of the global bovine microRNAome of peripheral blood mononuclear cells isolated from *Mycobacterium bovis* exposed cattle

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ABSTRACT

Accurate diagnostics are urgently needed for bovine TB – an economically devastating disease posing a re-emerging threat to veterinary and public health worldwide. MicroRNAs, post-transcriptional gene regulators involved in a range of biological processes and immunological pathways, have emerged as promising diagnostic biomarkers for numerous diseases. In human TB, microRNAs have been widely studied, but not much is currently known about their role in bovine TB. This study aimed to investigate the diagnostic potential of microRNAs in bTB through comprehensive analysis of their expression profiles in disparate states of *M. bovis* exposure. We used RNA-sequencing to characterize the global microRNAome of peripheral blood mononuclear cells from cattle that were either unvaccinated, BCG-vaccinated, unprotected or protected. A total of 468 microRNAs were detected across all samples, none of which were uniquely expressed in any group. Significant differential expression was observed for bta-miR-6122–3p, bta-miR-3533 and bta-miR29b in various comparisons. Subsequent target analysis of bta-miR-29b, a candidate biomarker in human tuberculosis, revealed that several genes (*ACVR2A*, *PIK3R1*, *TBX21*, *TGFBR1* and *TGFBR2*) involved in a number of relevant T-cell and immune signaling pathways, were amongst the predicted targets. Overall, this study provides evidence that microRNAs could be promising novel biomarkers for bovine TB diagnostics.

1. Introduction

Bovine tuberculosis poses a re-emerging threat to veterinary and public health globally [1]. Despite decades of intensive efforts, adequate control of bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*), remains elusive. Historically, widespread pasteurization of milk and the use of the tuberculin skin test (TST) together with abattoir surveillance were pivotal in preventing transmission to humans and the control of bTB in cattle in the developed world [2,3]. Regardless, the global prevalence of bTB is estimated to be 9 % and rising, leading to huge economic losses of ±$3 billion annually due to condemnation of carcasses, production losses, and trade and export restrictions [2,3]. Furthermore, an estimated 140,000 cases and 11,400 deaths due to zoonotic TB in humans occur annually [4]; figures that are considered gross underestimations [5]. As such, bTB is recognized as an enormous threat to public and veterinary health [1].

Accurate diagnosis of bTB is hampered by limitations in the performance of available assays, and various logistical concerns [6,7]. Many countries, particularly those considered officially TB-free, rely on abattoir surveillance and contact-tracing alone, the sensitivity of which is low [7]. Additionally, the specificity of the TST and interferon-gamma release assays is impacted by cross-reactivity with closely related mycobacteria [6], including *M. avium* subsp. *Paratuberculosis* (MAP), which causes Johne's disease and occurs throughout Europe [8], and the

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M. bovis vaccine strain bacillus Calmette-Guérin ("BCG") which provides only partial and variable protection [9]. Control is further complicated by the presence of *M. bovis* in various non-bovine domestic species and wildlife reservoirs in the UK, Ireland, Spain and several countries in central-Western Europe [10,11]. These reservoirs provide a constant source of re-infection for cattle, while limited or no diagnostics are available for these species. It follows that there is an urgent need for the development of innovative diagnostic assays for bTB driven by novel diagnostic biomarker discovery.

Small non-coding RNAs (sncRNAs) play an essential role in gene expression through post-transcriptional regulation [12]. Recently, microRNAs (miRs) (a class of sncRNAs ~22 nt in size [13]) emerged as a highly promising novel class of diagnostic biomarkers [14], in both non-infectious diseases such as cancers [15], as well as in various infectious diseases, including TB [16]. The advantages of using miRs as biomarkers include detection early in infection, high stability, and accessibility in biofluids, rendering miRs highly suitable for point-of-care assays [14,15]. While great strides have been made studying miRs involved in human TB [16], very little is currently known about miRs in bovine TB [17–19]. Therefore, the purpose of this study was to characterize the global microRNAome of cattle with disparate exposures to *M. bovis* (BCG).

2. Materials & methods

2.1. Peripheral blood mononuclear cells

All samples used in this study were sourced from a previous BCG vaccination and *M. bovis* challenge study [20]. In that study, animals acquired from a TB-free herd in a non-endemic area were screened for bTB using a blood-based IFN-γ release assay and included when negative. Animals were either unvaccinated (controls) or vaccinated subcutaneously with 0.5 mL of 4.6×10^6 CFU *M. bovis* BCG Danish SSI 1331 (Statens Serum Institute) [20]. Nine weeks post-BCG vaccination, all animals were infected with 10^4 CFU *M. bovis* AF2122/97 using endobronchial administration [20]. All animals were sacrificed for post-mortem examinations (PM) at 20 weeks post-BCG (11 weeks post-challenge) [20]. For the present study, samples from six animals at two different time points were selected based on the presence (VL) or absence (NVL) of visible lesions at PM. For subsequent analysis, animals were grouped according to their immunological and infection status ("status") as an interpretation of vaccine outcomes observed in the original study [20]: controls (unvaccinated, prior to challenge), vaccinated (BCG-vaccinated, prior to challenge), unprotected (unvaccinated, post-challenge, VL) and protected (BCG-vaccinated, post-challenge, NVL) (Table 1). Samples consisted of peripheral blood mononuclear cells (PBMC) isolated from whole blood collected at weeks 8 and 17 post-BCG vaccination, cryopreserved in FCS + 10 % DMSO and stored in the vapor phase of liquid nitrogen.

Table 1
Immunological and infection status of study animals. The study animals were part of a previously described longitudinal study [20] of two groups (BCG and control) at two timepoints (week 8 and week 17). In the present study, animals were grouped based on their immunological and infection status (n = 3 per status).

Status	Timepoint	Group	Vaccination[a]	Challenge[b]	PM
Control	Week 8	Control	n/a	n/a	n/a
Vaccinated	Week 8	BCG	*M. bovis* BCG Danish SSI 1331	n/a	n/a
Unprotected	Week 17	Control	n/a	*M. bovis* AF2122/97	VL
Protected	Week 17	BCG	*M. bovis* BCG Danish SSI 1331	*M. bovis* AF2122/97	NVL

[a] Vaccinated animals received 0.5 mL 4.6×10^6 CFU *M. bovis* BCG Danish subcutaneously at week 0.

[b] Challenged animals were infected through the endobronchial route with 10^4 CFU *M. bovis* AF2122/97 at week 9. Samples were collected at week 8 and week 17. n/a = not applicable; VL = visible lesions; NVL = no visible lesions; PM = post-mortem.

2.2. RNA-sequencing

Total RNA extraction, library construction and RNA-sequencing were carried out at Novogene (Cambridge, UK). Briefly, PBMCs were thawed and total RNA extracted and analyzed for quantity, integrity and purity on the Agilent 2100 bioanalyzer. All samples passed QC: an average of 456 ng/μL (range 273–680 ng/μL) RNA was extracted, with an average RNA integrity number (RIN) of 8.1 (range 7.5–8.5). A double-stranded cDNA sequencing library was constructed using the NEB Next® Multiplex Small RNA Library Prep Set for Illumina® (Set 1) (Cat No. E7300) according to the manufacturer's protocol (New England Biolabs, Hitchin, UK). Single-end sequencing of 50 bp reads was performed on the NovaSeq platform (Illumina, Cambridge (UK).

2.3. Bioinformatic analyses

Quality control of raw reads was carried out using FastQC (v 0.11.9) [21] with default settings, and adapters and low-quality reads removed using TrimGalore (v 0.6.4) [22] (parameters: -quality 30 –illumina –length 17 –max_length 28 –fastqc). miRDeep2 (v 0.1.3) [23] was used for processing and mapping of reads, identification of miRs and quantification or expression profiling. First, reads were processed and mapped against an index of the reference genome, bosTau9 (ARS-UCD1.2) [24], built with bowtie [25], using the miRDeep2 mapper module [23] (parameters: e -h -j -k AGATCGGAAGAGCACACGTCT -l 17 -m). Next, using miRbase v22 [26] and the miRDeep2 module [23], known and novel miRs present in the reads were identified (parameters: t Cow). The criteria for inclusion of novel miRs consisted of the following points: i) a high (≥90 %) predicted probability of being a true positive according to miRDeep2; ii) a significant (≤0.05) randfold *p*-value of the predicted hairpin structure; and iii) expression of the mature sequence in ≥10 samples at a minimum of 100 reads per million (RPM). Finally, quantification of known miRs was performed with the quantifier module [23], using the default settings and miRbase v22 [26]. Based on earlier work [27], miRs which did not meet the threshold of ≥50 counts across samples were filtered out. Differential expression analysis was subsequently carried out with DESeq2 (design ~ timepoint + group + group: timepoint) (v 1.36.0) [28], using R (v 4.2.0) [29] in R Studio (v 2023.03.1) [30], on the remaining miRs in the dataset. microRNAs with a Benjamini-Hochberg adjusted *p*-value ≤0.05 were considered significantly differentially expressed (DE). TargetScanHuman (v 7.2) [31] was used to predict the mRNA targets of the identified miRs of interest (MOIs). Finally, miRNA target filter of the Qiagen IPA (Ingenuity Pathway Analysis) tool [32] was used to decipher the target mRNAs of the miRs of interest using the following filters: i) confidence – experimentally observed; ii) species – human; iii) disease – immunological disease, infectious disease, inflammatory disease, inflammatory response and respiratory disease; and iv) pathways – Th1 and Th2 activation pathway.

2.4. Ethics statement

The original animal study [20] was reviewed and approved by the Animal and Plant Health Agency Animal Welfare and Ethical Review Body committee. All animal experiments were conducted within the limits of a United Kingdom Home office license under the Animal (Scientific Procedures) Act 1986.

2.5. Data availability statement

Raw and processed data are available through GEO accession number GSE285119.

3. Results and discussion

3.1. Characterization of the microRNAome of M. bovis exposed bovine cells

To study the potential of miRs to be used as biomarkers of BCG vaccination or disease following *M. bovis* infection, the global expression of miRs in PBMCs of cattle either vaccinated with *M. bovis* BCG, challenged with virulent *M. bovis*, or both, was investigated using RNA-sequencing. Raw sequencing reads were generated using the NovaSeq platform. A summary of general statistics of the reads is provided in Table 2. The sequencing data comprised an average of 13.4M (range 12.2–15.4M) raw reads per sample with a read length of 50 bp. Overall, on average 90.8 % (range 86.2 %–96.8 %) of raw reads remained after trimming of low-quality reads and adapters, resulting in an average of 12.1M (range 10.7–14.2M) clean reads of 17–28 bp in length, with a Phred score of 37.

To evaluate the presence of known and novel miRs in our data, we used miRDeep2 for mapping and quantification. The percentage of reads that mapped to the bosTau9 (ARS-UCD1.2) reference genome is shown in Fig. 1A. As expected, the mapping of reads was similar in each group. On average, the vast majority of reads (96.2 %) mapped to the reference genome, with only a low percentage remaining unmapped (3.8 %). We quantified the number of known and novel miRs that were detected in our data. After filtering for miRs with ≥50 counts across samples, to increase robustness, the dataset comprised 468 known miRs. As all 468 miRs were detected in each of the groups, none of the miRs were uniquely expressed. The identity and normalized counts of the known miRs detected in each sample are given in Supplementary File 1. Principle component analysis of the variance-stabilized transformed count data showed there was considerable overlap between the groups of animals, with PC1 and PC2 describing 23 % and 18 % of the variance in the dataset, respectively (Fig. 1B). The within group variance was greatest in the control group, and smallest in the protected group, the samples of which cluster closer together (Fig. 1B). Interestingly, the protected group falls completely within the unprotected group in PC1 and PC2 (Fig. 1B). The close similarity of the expression profiles of the groups is further demonstrated by analysis of the top 50 most expressed known miRs in each group (Fig. 1C). The VENN diagram shows that only 54 genes comprised the top 50 of all individual groups, and 47 (87 %) of these were expressed in each group (Fig. 1C). Only three genes were uniquely present in the top 50 of any group: bta-miR-223 in controls; and bta-miR-374b & bta-miR-32 in protected animals (Fig. 1C). A further four miRs were common between only a subset of either 2 or 3 groups: bta-miR-532 (Control & Vaccinated), bta-miR-423–3p (Vaccinated & Unprotected), bta-miR-106b (Unprotected & Protected), and bta-miR-339a (Control, Vaccinated & Unprotected) (Fig. 1C). The comparative levels of expression of the top 50 most expressed miRs in each group is shown as a heatmap in (Fig. 1D). This figure shows that the expression of the majority of these miRs is highest in the protected group, and lowest in the control group (Fig. 1D). Similarly, the expression is higher in the unprotected group than the vaccinated group (Fig. 1D). Taken together, this suggests that exposure to *M. bovis* may alter the expression of these miRs, and that challenge with virulent *M. bovis* affects this expression more so than the attenuated vaccine strain BCG.

Table 2
General statistics of the sequencing data. Numbers of reads represent averages per group (n = 3).

Group	Raw reads	Clean reads	% trimmed	Length of reads*	Phred score^{+}
Control	12,458,767	11,373,400	91.3	17–28	37
Vaccinated	13,916,856	12,524,057	89.9	17–28	37
Unprotected	13,324,896	11,952,494	89.6	17–28	37
Protected	13,790,317	12,732,488	92.2	17–28	37

The *length of reads and $^{+}$Phred score are those obtained post-trimming.

3.2. Detection of novel miRs

The expression of potential novel miRs was also evaluated using miRDeep2. In total, 3 predicted novel miRs had an estimated probability of 90 %, a significant randfold *p*-value and had an expression level ≥100 RPM, but their expression was only detected in 1 sample (Supplementary File 2). No novel miRs were submitted to miRbase.

3.3. Exploring the potential of miRs to differentiate animals of disparate M. bovis exposure statuses

In order to further investigate whether the expression of miRs could differentiate between animals of disparate statuses of exposure to *M. bovis* (BCG), a differential expression analysis was performed. In this analysis, the expression of miRs in each group was compared to the control group (unprotected vs. control, protected vs. control and vaccinated vs. control) and also to each other (unprotected vs. protected, unprotected vs. vaccinated and vaccinated vs. protected). The results of the DE analysis are shown in Supplementary File 3 and Fig. 2. Overall, the number of significantly differentially expressed miRs was low in all comparisons. In the comparisons of vaccinated vs. control and unprotected vs. protected groups, no miRs were found to be differentially expressed. Three miRs stood out as being differentially expressed in various other comparisons: bta-miR-6122–3p, bta-miR-3533 and bta-miR-29b (Figs. 2 and 3).

First, bta-miR-6122–3p was significantly downregulated in unprotected vs. control (L2FC −1.80; adjusted *p*-value 0.001) and protected vs. control (L2FC −1.55; adjusted *p*-value 0.014) (Figs. 2 and 3A). These results suggest that exposure to virulent *M. bovis* downregulates bta-miR-6122–3p expression. This miR has only been described in cattle and thus far appears to be species-specific [26]. It was first discovered as a novel miR in bovine retinal microvascular endothelial cells by Guduric-Fuchs et al. (2012) [36]. Because this miR is species-specific, the QIAGEN IPA tool could not determine the associated pathways or up/down-stream molecules. However, the predicted targets according to TargetScanHuman are listed in Supplementary File 4A.

Next, bta-miR-3533 was significantly downregulated in unprotected vs. control (L2FC −2.31; adjusted *p*-value 0.028), protected vs. control (L2FC −2.19; adjusted *p*-value 0.047) and unprotected vs. vaccinated (L2FC −2.43; adjusted *p*-value 0.010), whereas it was significantly upregulated in vaccinated vs. protected (L2FC 2.31; adjusted *p*-value 0.018) (Fig. 2). Expression of this miR was similar in vaccinated and control animals, but exposure to virulent *M. bovis* appeared to downregulate its expression, as can be seen by the low expression in both unprotected and protected groups (Fig. 3B). This miR has thus far only been described in alveolar macrophages of cattle [37], epithelial cells of lungs and tracheas of chickens [38], and two species of marine lophotrochozoans [39]. However, no functional data is currently available. Although bta-miR-3533 has not been described in humans, it belongs to the same family of miRs as hsa-miR-573 and hsa-miR-3616–5p according to TargetScanHuman, suggesting they may have similar physiological functions. While no functional data is available for hsa-miR-3616–5p, several studies have previously reported on hsa-miR-573. Recently, hsa-miR-573 has been found to suppress the proinflammatory response in Dengue virus 2 infections, through direct interaction with the 3 'UTR of the Toll-like receptor (TLR) - 2 gene, leading to a downregulation of its expression [40]. Given that immunity to tuberculosis is mediated by Th1 CD4^{+} T-cell responses characterized

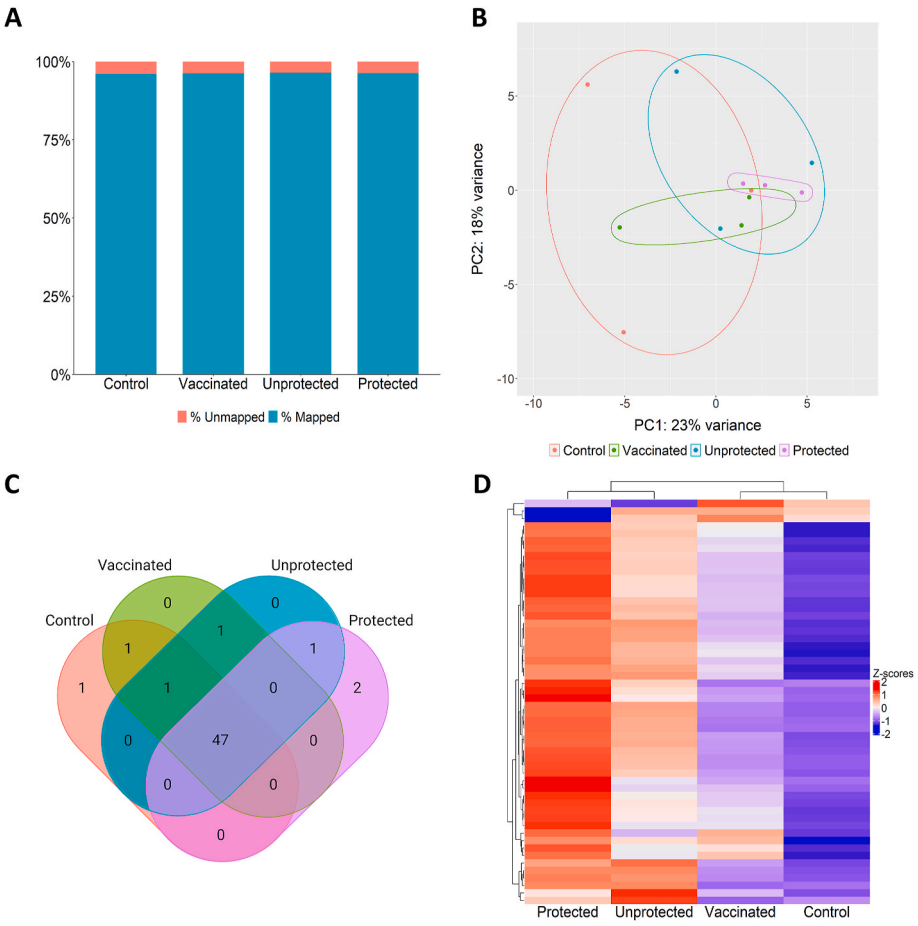

Fig. 1. RNA sequencing and differential expression analysis results. A) Mapping of reads. Percentages of reads that were either mapped to the reference genome bosTau9 (ARS-UCD1.2), or unmapped. Percentages shown reflect averages per group (n=3). B) Principle component analysis plot. The variance described by the first two principle components (PC1 and PC2), using variance-stabilized transformed count data of the 468 miRs present in the final dataset. The ellipses do not reflect confidence intervals, they visualize the clustering of samples in each group. C) Disparity in the top 50 most expressed miRs in each group. Lists of expressed miRs were sorted according to counts and the top 50 most expressed miRs in each group used to create a VENN diagram. Created using the Venn diagram tool of the Bioinformatics & Systems Biology department of VIB/UGent [33]. D) Expression of the top 50 most expressed miRs in each group. Normalized counts of miRs were averaged per group and transformed into z-scores representing the expression distribution over the groups. Created using ggplot2 (v 3.4.3) [34] and ComplexHeatmap (v 2.12.1) [35] in R studio (v 2023.03.1) [30] and Graphpad Prism (v 10.1.1).

by IFN-γ production, it would be of interest to determine whether bta-miR-3533 could have a similar function in bTB to that of hsa-miR-573 in Dengue infections. Additionally, hsa-miR-573 has been implicated in various cancers [41–44], where it may be involved in regulation of cell proliferation, migration and invasion. Because bta-miR-3533 has not been described in humans, the QIAGEN IPA tool could not determine the associated pathways or up/down-stream molecules. However, the predicted targets according to TargetScanHuman are listed in Supplementary File 4B.

Finally, bta-miR-29b was upregulated in protected vs. control (L2FC 0.98; adjusted *p*-value 0.016) (Fig. 2B). Here, a clear upward trend in expression of this miR was seen with increasing experimental exposure to both *M. bovis* BCG and virulent *M. bovis* (Fig. 3C), with expression in protected animals being the highest and the only group in which the expression was significantly different to the control group. bta-miR-29b is part of the miR-29 family which consists of miR-29a, miR-29b and miR-29c, and is recognized to be broadly conserved with known orthologs in various species (including humans, non-human primates, mice, dogs, chickens and several others) [31]. The miR-29 family and miR-29b, specifically, have been widely studied and have been implicated in various processes and diseases such as cancer [45], metabolism & metabolic disease [46], (immune) regulation pathways [47,48], and shown to have antifibrotic and proapoptotic properties [49,50]. Of particular interest are previous findings of the effects of miR-29b on T-cell regulation and differentiation [51–53]. Steiner et al. [51] demonstrated that miR-29b decreases the number of IFN-γ producing T-cells and overall IFN-γ production through targeting of T-bet and Eomes – two well-known T-cell transcription factors [54,55]. Knowing the importance of IFN-γ producing $CD4^+$ T-cells in tuberculosis, the potential interference herewith by this miR could be of consequence in disease progression vs. resolution. In fact, Ma et al. [56] showed that miR-29 suppresses immunity to *M. tuberculosis* (*Mtb*) by directly targeting the 3′ UTR of IFN-γ (Supplementary Fig. 1A), thereby lowering its expression. They further showed that miR-29 sponge-transgenic mice produced more IFN-γ and were more resistant to infection with *M. bovis* BCG and *Mtb* HR37v [56]. We compared the sequence of the 3′UTR of the bovine *IFNG* gene to the sequence of bta-miR-29b and found a nucleotide difference within the seed region, while human and mouse sequences showed perfect pairing (nucleotides 2–8; Supplementary Fig. 1A). Although it is known that imperfect pairing in the seed region can sometimes be rescued by complementary pairing in the 3′ region of the miR [57], in this case only 3 further, non-consecutive nucleotides are a match for Watson-Crick pairing (Supplementary Fig. 1A). While complete degradation of IFN-γ mRNA is therefore unlikely, translation could still be repressed [58]. As a result, the effect of bta-miR-29b on IFN-γ mRNA in bovines is presently unclear, and this would have to be studied

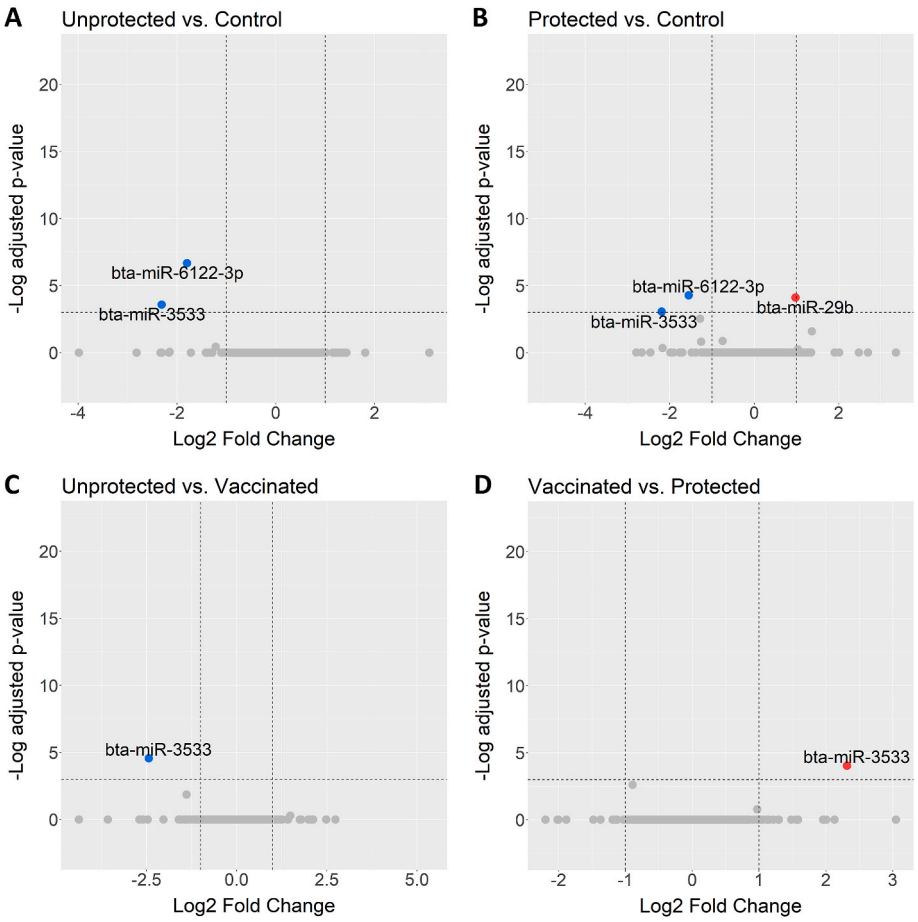

Fig. 2. Volcano plots of the differential expression analysis of miRs between groups with disparate *M. bovis* exposure status. Only comparisons which yielded differentially expressed miRs between groups (n=3) are shown. A) unprotected vs. control; B) protected vs. control; C) unprotected vs. vaccinated; D) vaccinated vs. protected. Blue = downregulated; red = upregulated; grey = non-significant miR. The horizontal line represents an adjusted *p*-value of 0.05; the vertical lines represent a log2 fold chance of -1 and 1, respectively. Created using DESeq2 (v 1.36.0) [28] in R studio (v 2023.03.1) [30].

further to be fully understood. Other predicted targets according to TargetScanHuman are listed in Supplementary File 4C. To further investigate the molecular activity of miR-29b, we used the QIAGEN IPA tool to contextualize the pathways and up-/down-stream regulators it is associated with. We found the following genes to be direct targets of miR-29b in humans: activin A receptor type 2A (*ACVR2A)*, phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*), T-box transcription factor 21 (*TBX21*), transforming growth factor beta receptor 1 (*TGFBR1*) and *TGFBR2* (Fig. 4) (Supplementary File 5). As can be seen from Fig. 4, each of these targets are predicted to be inhibited by the overexpression of miR-29b, and all are involved in various aspects of immunity, particularly processes encompassing T-cell pathways and signaling. ACVR2A, is a receptor for activins, which are part of the transforming growth factor-beta (TGF-β) superfamily [59]. This family also comprises *TGFBR1* and *TGFBR2*, which together form the receptor for the multifunctional cytokine TGF-β [59], which among other functions is critical for T-cell immunity. *TBX21* encodes the transcription factor T-bet, which as discussed previously has been shown to be a target of miR-29b in mice [51] and is a crucial regulator of IFN-γ expression and T-cell differentiation towards Th1 [54,59]. Finally, PIK3R1 belongs to a family of lipid kinases involved in cell proliferation and survival, and PIK3R1 specifically is associated with the metabolic activity of insulin [59]. Given that our analysis in IPA was based on human miRs, we investigated the binding potential of bta-miR-29b to the predicted bovine targets and the same mRNA in human and mouse. Of the predicted targets, only the 3′UTRs of *TBX21* and *PIK3R1* showed perfect pairing to the seed region of miR-29b in all three species (Fig. 5), while comparison of the 3′UTRs of *ACVR2A*, *TGFBR1* and *TGFBR2* showed multiple mismatches (Supplementary Figs. 1B–D). Therefore, it is likely that *TBX21* and *PIK3R1* are true targets for miR-29b in cattle, but this should be confirmed through additional studies.

Within the context of the present study, the suggested direct targeting of T-bet by bta-miR-29b in protected animals is of particular interest. Previously, T-bet has been shown to be pivotal in the control of *Mtb* infections and T-bet$^{-/-}$ mice show increased susceptibility [61], and more recently Grant et al. [62,63] showed that T-bet^{+} T-cells contribute to a reduction in bacterial burden within the tubercle. However, an excess of T-bet expression appears to be detrimental in TB immunity, due to a reduced capability of T-cells to migrate to the active site of infection (the lung) [64]. Protective immunity against TB is complex and depends on the right balance and timing. This is not dissimilar to what is known about IFN-γ, which on the one hand is a critical mediator of TB immunity and crucial for survival [65–67]; but on the other hand, has been correlated to an increase in bacterial burden when found in excess post-challenge [68–70]. Therefore, the precise effect of potential miR-29b regulated inhibition of T-bet in bovine tuberculosis remains to be determined.

3.4. microRNA expression in TB diagnostics

A recent review on the diagnostic value of microRNAs in human tuberculosis [71] analyzed 83 papers reporting more than 100 miRNAs described as differentially expressed across a variety of samples. Of these DE miRs, however, only 25 were consistently reported in a minimum of

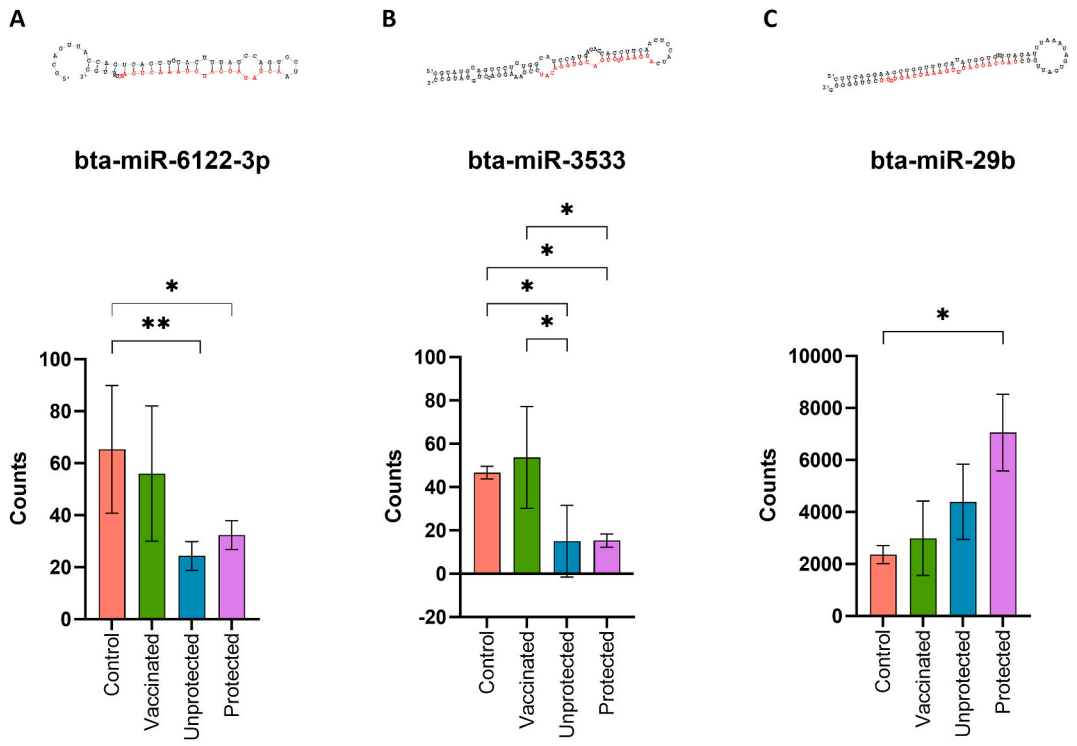

Fig. 3. Stemloop structure and expression levels of differentially expressed miRs. A) bta-miR-6122-3p; B) bta-miR3533; C) bta-miR-29b. Expression levels depicted as normalized counts. * = p-value ≤ 0.05; ** = p-value ≤ 0.01. Created using mirDeep2 (v 0.1.3) [23] in R studio (v 2023.03.1) [30] and Graphpad Prism (v 10.1.1).

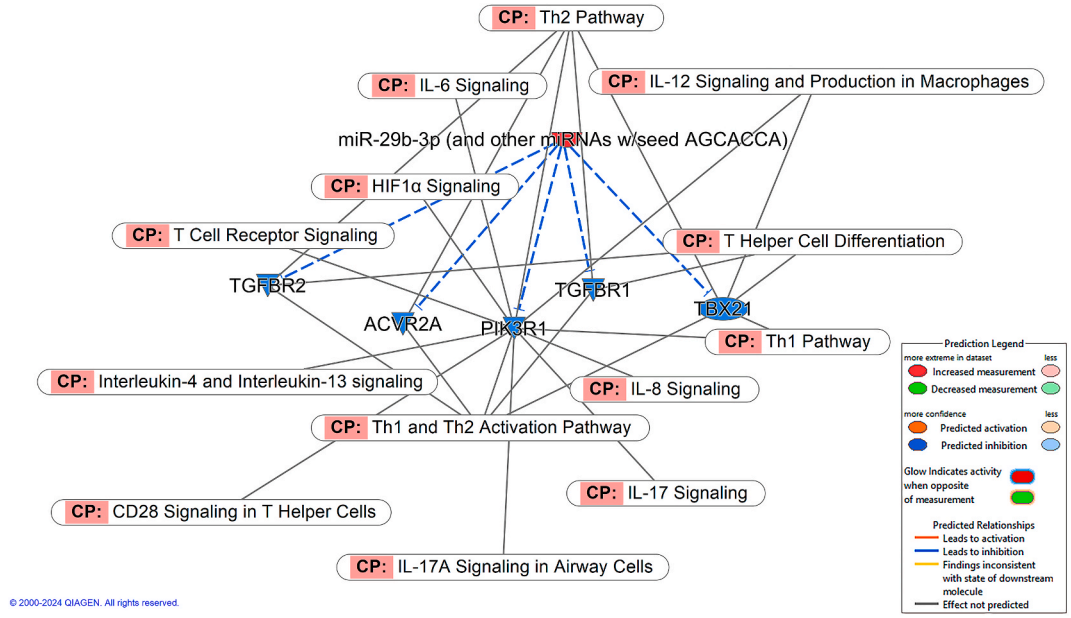

Fig. 4. Targets of miR-29b and their associated networks and pathways. Target genes (blue) of miR-29b (red) are visualized in the context of their associated networks and pathways. Created using the Qiagen IPA tool [32].

3 publications, with the miR-29 family by far the most frequently reported (in 15 studies) [71]. Subsequent analysis of diagnostic parameters of miR-29 in active pulmonary tuberculosis (ATB) revealed a sensitivity and specificity of 80 %, an area under the curve (AUC) of 0.86, and a diagnostic odds ratio (DOR) of 16, concluding that miR-29 is a suitable biomarker in TB diagnostics in humans [71]. The finding that miR-29b was also amongst the markers identified in the present study validates the scientific approach used and strengthens the suggestion that miRs could also be useful biomarkers for bovine TB.

3.5. *Limitations of the study*

As the main aim of this study was to ascertain whether disparate vaccination and disease states of bTB would exhibit variation in their microRNAomes, samples from a previous study were selected on the basis of their vaccination status and the subsequent outcome of

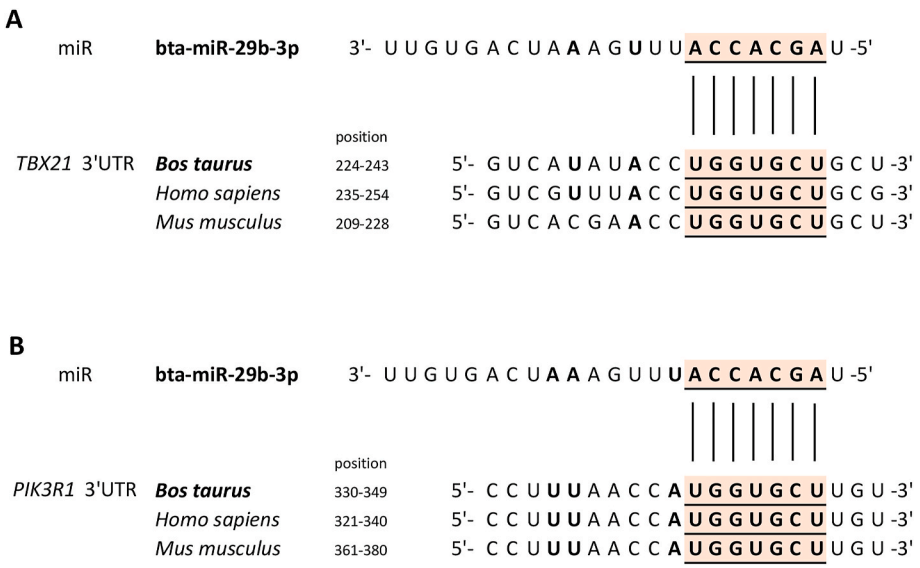

Fig. 5. Sequences of the 3'UTR of predicted targets of miR-29b that are complete matches. The seed region of the miR is in bold, underlined and shaded in orange. Nucleotides in the 3' region of the miR or the 5' region of the mRNA sequence in bold indicate complementary matches outside of the seed region. A) TBX21; and B) PIK3R1. Sequences were downloaded from Ensembl [60].

challenge. This was done in order to maximize the distinction between diseased and healthy animals, unlocking the greatest potential for divergent phenotypes. A limitation of this decision is the inherent selection bias introduced in this manner. In subsequent studies, this should be avoided in order to better capture natural variation. However, the authors felt that for the purpose of this study, this choice is justified as it allowed for more clearly defined status discrimination to address the research question.

For clarity, the terms control, vaccinated, unprotected and protected were introduced as an interpretation of the status of the animals under study, based on earlier work [20]. It is important to note however, that the analysis in the present study was done on the raw variables of group and timepoint, and their interaction. To address the paired nature of the samples, animal ID would ordinarily be included in the model as a random effect, but DESeq2 does not allow for this. While it is important to take note of this limitation, the authors believe this does not take away from the main finding and message of this paper – that miRs can be useful in the discrimination of various bTB vaccination and disease states.

4. Conclusion

This pilot study presents the first comprehensive characterization of the microRNAome of peripheral blood cells of cattle with disparate exposures to *M. bovis* and/or BCG. The data clearly demonstrate that, like in human TB, miRs can differentiate between distinct disease states of bovine TB, and therefore substantiate the feasibility and diagnostic potential of miRs in this context. Additional studies are warranted to more precisely map the determinants of these disparate vaccination and disease outcomes. Subsequent improved understanding of their miR expression dynamics can inform novel vaccination and diagnosis strategies (DIVA) as well as therapeutics towards better control of bTB.

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CRediT authorship contribution statement

Anna E. Karagianni: Writing – review & editing, Investigation, Formal analysis. **Lindert Benedictus:** Writing – review & editing, Resources. **Sabine Steinbach:** Writing – review & editing, Resources. **Femke Broere:** Writing – review & editing, Supervision. **Elisabeth M.D. L. van der Heijden:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interests

The authors declare no competing interests.

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Appendix. ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2025.102639.

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