Genomics 114 (2022) 110333

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Original Article

Bovine rumen epithelial miRNA-mRNA dynamics reveals post-transcriptional regulation of gene expression upon transition to high-grain feeding and phytogenic supplementation

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ARTICLE INFO

Keywords:
miRNAome
Dairy cattle
High-grain feeding
Differential expression
miRNA-mRNA interactions
Feed additive

ABSTRACT

The rumen epithelium has a pivotal role in nutrient uptake and host health. This study aimed to explore the role of microRNAs (miRNAs) in the epithelial transcriptome during diet transition from forage to high-grain feeding and the modulation through supplementation with a phytogenic feed additive. Rumen biopsies were collected from 9 ruminally-cannulated non-lactating Holstein cows fed a baseline forage diet (FD) and then transitioned to high-grain feeding (HG; 65% concentrate on a dry matter basis). Cows were randomly allocated into a control group (CON, $n = 5$) and a group supplemented with a phytogenic feed additive (PHY, $n = 4$). MiRNA and mRNA sequencing was performed in parallel and transcripts were analyzed for differential expression, pathway enrichment analysis, and miRNA-mRNA interaction networks. We identified 527 miRNAs shared by all samples of the rumen epithelium, from which, bta-miR-21-5p, bta-miR-143 and bta-miR-24-3p were the most expressed. Six miRNAs were differentially expressed between CON and PHY and 8 miRNAs between FD and HG feeding, which were mainly associated with fat metabolism. Transcriptome analysis identified 9481 differentially expressed genes (DEGs) between FD and HG, whereas PHY supplementation resulted in 5 DEGs. DEGs were mainly involved in epithelium development and morphogenesis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with tricarboxylic acid and short chain fatty acid (SCFA) metabolism were enriched in DEGs between diets. MiRNA target prediction and anti-correlation analysis was used to construct networks and identify DEGs targeted by DE miRNAs responsive to diet or PHY. This study allowed the identification of potential miRNA regulation mechanisms of gene expression during transition from FD to HG feeding and phytogenic supplementation, evidencing a direct role of miRNAs in host responses to nutrition.

1. Introduction

The stratified squamous epithelium of the bovine rumen plays a pivotal role in the uptake, metabolism and transport of short-chain fatty acids (SCFA) and other metabolic substrates for the host. Rumen epithelial cells are the first line of defense against hostile rumen conditions, in particular, acidic pH and harmful microbial-derived metabolites that accumulate in the rumen when cattle are fed grain-rich (HG) diets [1–6]. Research has shown that the rumen epithelium undergoes major structural alterations during HG feeding [2], which are reflected at the level of the gene expression, transcriptomic profile [3–5], and protein level [6]. Besides modulating genes involved in the epithelial metabolic regulation [3,7] and growth (i.e., insulin-like growth factor-binding proteins (*IGFBP*) [7]), feeding of HG diets downregulates the expression of genes involved in catalytic and binding activities [8], cellular protective functions [6] as well as upregulates the pro-inflammatory cascade genes [9] in the rumen epithelium.

MicroRNAs (miRNAs) are small (22–23 bp) non-coding nucleic acids that modulate gene expression primarily by acting on the 3′ untranslated region of mRNA in the cytoplasm, playing a crucial role in orchestrating

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https://doi.org/10.1016/j.ygeno.2022.110333
Received 3 November 2021; Received in revised form 17 February 2022; Accepted 6 March 2022
Available online 9 March 2022

post-transcriptional regulation by inhibiting target mRNA translation or promoting target mRNA degradation [10–12]. Their activity on gene regulation and their ubiquitous presence make them eligible as biomarkers for various perturbed biological processes and development of several diseases [13–15]. In fact, miRNAs have also been demonstrated to be fundamental for immunity response and also for the integrity of the gut epithelial barrier, by modulating relevant gene expression [16]. Several authors have demonstrated that different feeding regimes can generate pathophysiological changes that reflect into distinct miRNA expression patterns in cattle [17–19]. However, the miRNAome of rumen papillae and their role in downstream gene regulation remains largely underexplored in cattle. Few studies have focused on the role of ruminal miRNAs in the rumen tissue, and mainly studied miRNA regulation of rumen development of calves [20–23] or goats [24,25].

Phytogenic feed additives are rich in bioactive herbal compounds. Their supplementation can facilitate the transition from forage to HG feeding by enhancing the stability of the epimural microbiota, which in turn was reported to correlate with changes in the host epithelial gene expression [26]. In addition, phytogenic supplementation has been shown to increase ruminal pH and ruminal butyrate after 1 week of HG feeding, possibly due to an improvement in the rumen milieu through the increase in epithelial nutrient uptake and absorption capacity, as butyrate can act as a positive regulator of papillae development [27]. However, the implications of such feed additives on miRNA expression and their target genes have remained unexplored.

To gain insight into the regulation of miRNAs and genes involved in molecular mechanisms of transition from FD to HG, we have used small RNA in parallel with mRNA sequencing approaches to identify differences in the epithelium of non-lactating Holstein cows transitioned from 0% to 65% concentrate diets. Additionally, the effect of the supplementation with a phytogenic feed additive on papillae gene expression and miRNA content was investigated. We hypothesized that HG feeding will trigger changes at the level of the epithelial transcriptome and host miRNA regulation as a response to changes in the rumen milieu and accumulation of SCFA. In a previous study, high papillae lesion scores were diagnosed only in the first week of HG diet [2], and therefore, effects of HG feeding are expected to be more severe shortly after transition to the HG diet. Hence, we choose to perform the next generation sequencing of cow papillae samples within the first week of high-grain feeding. Furthermore, we hypothesized that phytogenic supplementation could positively improve the ruminal environmental conditions, and therefore modulate the adaptation of the ruminal papillae to the new dietary conditions compared to no supplementation.

2. Results

2.1. Characterization of miRNAs in epithelial tissue

Small RNA sequencing of 18 epimural biopsies yielded a total of 944,213,355 raw input reads, an average of 52,456,298 ± 2,980,805 raw reads per sample. After quality (PHRED >20) and size (min count 2, read length > 15) filtering approximately 81.4 ± 0.57% reads were retained for downstream analysis (Supplementary Table 1). Read length distribution analysis allowed the identification of a peak at 20–23 nucleotides, which is characteristic of mature miRNAs [28] (Fig. 1A). The reads were mapped first to the bovine genome (*Bos taurus* release 3.1 – UMD3.1) and secondly to the human genome in order to identify homologues. Reads belonging to other RNA categories (e.g., tRNA, rRNA, snoRNA, snRNA) were excluded from this analysis (Fig. 1B). From the 769,385,062 filtered reads, only 3.06 ± 0.40% did not map to the bovine genome.

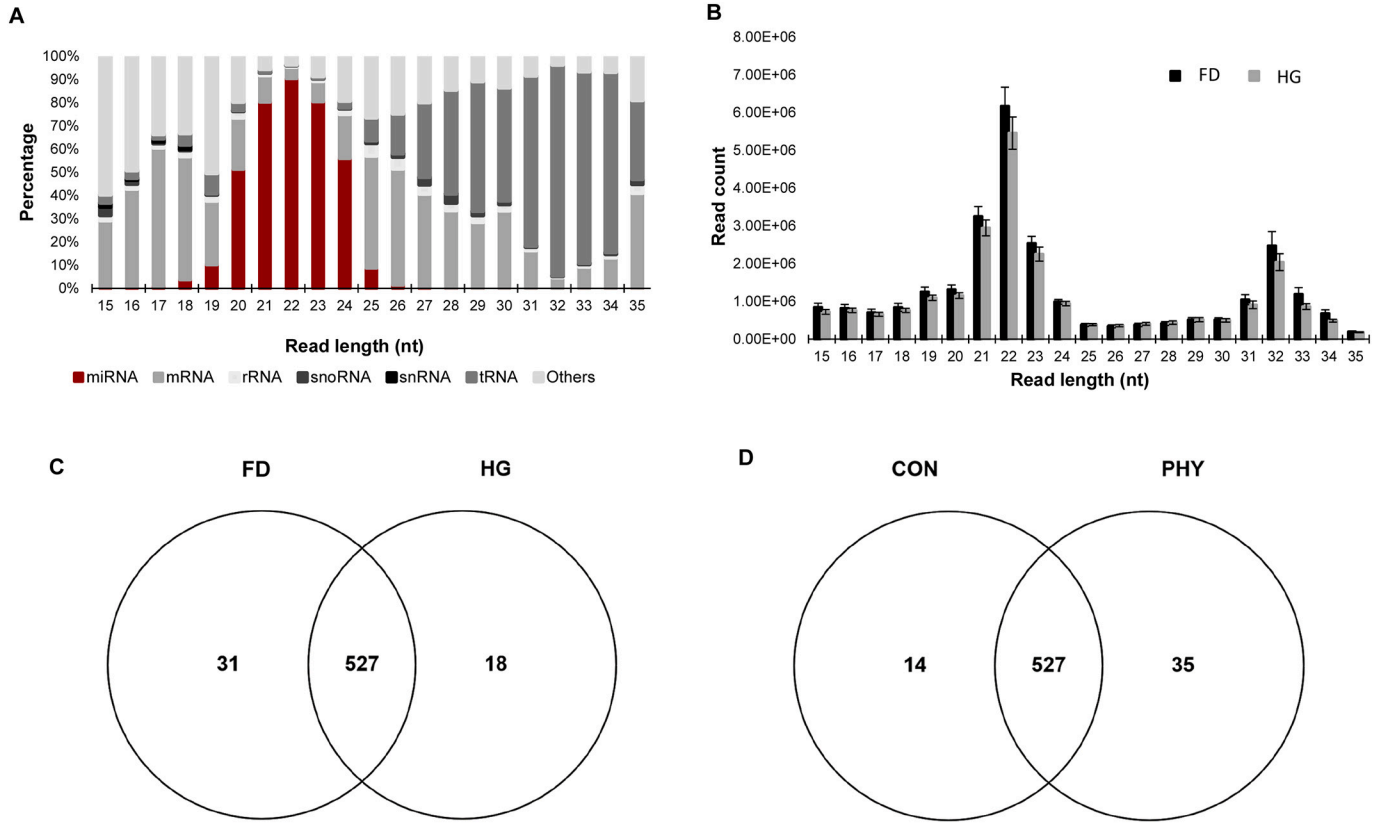

Fig. 1. Length distribution (mean ± SEM) of reads from bovine rumen epithelium (A). Percentage of reads assigned as miRNA, mRNA, snoRNA, snRNA, tRNA and others according to the read length distribution (B). Number of shared and unique miRNAs between forage and HG (C) and between CON and PHY (D).

2.2. Shared and unique miRNAs

Further filtering (duplicate removal, min read count 10 in at least one sample) was applied in order to identify shared and unique miRNAs in the papillae samples under different conditions. A total of 673 miRNAs were found in epithelial samples, from which 527 were shared by all samples. The 10 most abundant bovine miRNAs are depicted on Supplementary Table 2. Bta-miR-21-5p, bta-miR-143 and bta-miR-24-3p were three most expressed miRNAs, accounting for as much as 33.4% of the total reads. During forage feeding, 558 miRNAs were detected, while during HG feeding 545 miRNAs were found. When looking at unique miRNAs, 31 were found only during FD, while 18 were only found during HG feeding (Fig. 1C). Regarding treatment, 35 miRNAs were uniquely found in PHY samples, whereas 14 were exclusive to CON samples (Fig. 1D, Supplementary Table 3).

2.3. Differentially expressed (DE) miRNAs

Principal component analysis (PCA) of miRNA read counts adjusted to the multiple mappings in the bovine genome was performed in order to investigate the clustering of the samples. The first PC (PC1) explained 21% of the variance between samples, while the second PC (PC2) was responsible by 19% of the variation (Fig. 2A). No clear cluster was obtained by unsupervised analysis. However, a total of 8 and 6 miRNAs were differentially expressed (FDR $<$ 0.05) between forage and HG feeding and between CON and PHY, respectively (Fig. 2B, C). The miRNAs bta-miR-320a (hsa-miR-320a-3p), bta-miR-93, bta-miR-671, bta-miR-25 (hsa-miR-25-3p), hsa-miR-93-5p were up-regulated while bta-miR-143, bta-miR-146a and bta-miR-30c were down-regulated during HG feeding. The supplementation of the PHY compound elicited the up-regulation of hsa-miR-412-5p, bta-miR-454 (hsa-miR-454-3p), hsa-miR-409-3p, bta-miR-382 (hsa-miR-382-5p), and the down-regulation of bta-miR-190a (hsa-miR-190a-5p) and bta-miR-29a (hsa-miR-29a-3p).

2.4. Transcriptome analysis and DE genes

RNA sequencing yielded an average of 73.3 ± 3.42 million paired-end reads for 18 samples, ranging between 50.7 and 98.0 million reads per sample. From these, an average of 61.7 ± 3.25 million fragments were mapped to the bovine genome, resulting in the identification of 27,270 transcripts. A heatmap of the top 50 most expressed genes revealed different clusters of expression for the FD and the HG diet (Supplementary Fig. 1). Furthermore, a PCA was performed with the regularized log transformed read counts in DESeq2 method in order to investigate if samples show a cluster for the diet or the feed additive. The first two principal components (PCs) explained already 78% of the variation among the samples and both diets formed distinct clusters (Fig. 3A), whereas no clustering based on the treatment could be found. These results indicate a distinct transcriptomic profile of animals fed a forage-based diet upon switching to a 65% HG diet. Differences in gene expression would thus allow us to identify candidate genes explaining the differences between both diets.

We observed 9481 differentially expressed genes (DEGs) between FD and the HG group (FDR $<$ 0.05) (Fig. 3B). During high-grain feeding, 6542 genes were found to be up-regulated and 2939 down-regulated in comparison to baseline feeding (Supplementary Table 4). The PHY treatment resulted in five DEGs (Fig. 3C). Carbonic anhydrase 1 (*CA1*), rho family GTPase 3 (*RND3*) and ENSBTAG00000048514 were found to be down-regulated in the PHY group, while ENSBTAG00000053508 and zinc-alpha-2-glycoprotein (*AZGP1*) were up-regulated.

Gene ontology (GO) enrichment analysis was performed for DEGs

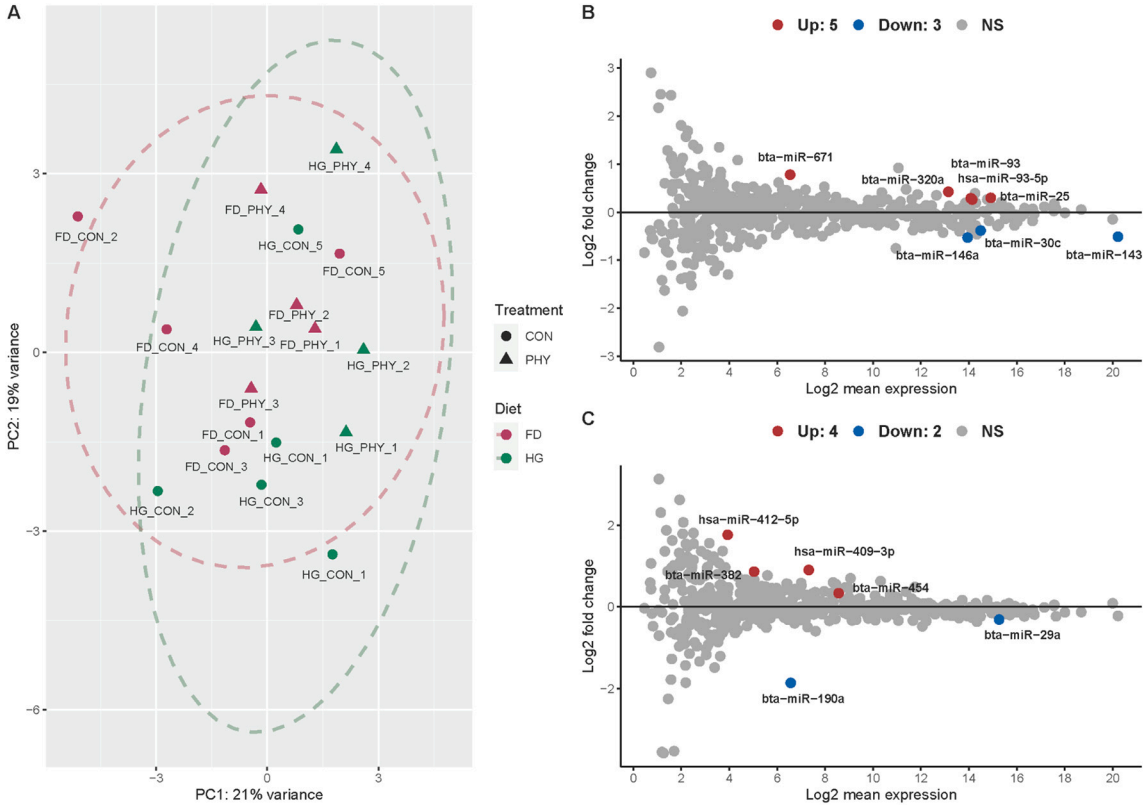

Fig. 2. Principal component analysis (PCA) of miRNA composition, showing 95% confidence ellipses for diet: pink; forage, green; HG (65% grain) (A). Scatter plot of log2 fold changes versus log2 mean expression of normalized counts obtained from DESEq2 in regards to diet (B) and phytogenic supplementation (C). In red, up-regulated miRNAs, and in blue, down-regulated miRNAs. Non-significant (NS) miRNAs are shown in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

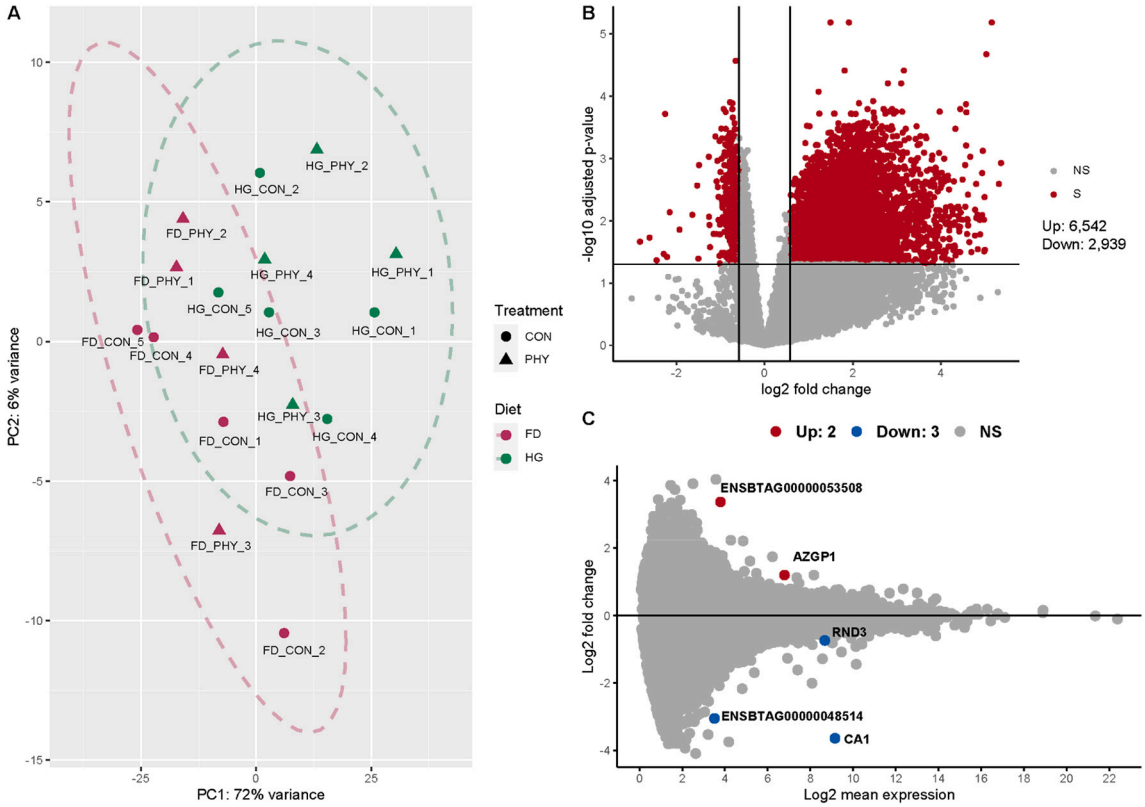

Fig. 3. Principal component analysis (PCA) of bovine transcriptome, showing 95% confidence ellipses in regards to diet: dark pink; forage, dark green; HG (65% grain) (A). Volcano plot of DEGs obtained from DESEq2 in regards to diet (B). Scatter plot of log2 fold changes versus log2 mean expression of normalized counts obtained from DESEq2 in regards to phytogenic supplementation. In red, over-expressed genes, and in blue, down-regulated genes. Non-significant (NS) genes are shown in grey (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

according to the diet, using DAVID with *B. taurus* as a reference. From the 9481 DEGs, 6412 were successfully assigned to the reference, while 3069 were marked as unknown. The most enriched GO terms for "Biological process" (GOTERM_BP_FAT), "Cellular component" (GOTERM_CC_FAT), "Molecular function" (GOTERM_MF_FAT) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs are given in Supplementary Table 5. A total of 67 biological processes, 62 cellular components and 58 molecular function significantly enriched GO terms were identified (FDR < 0.05). In addition, 65 KEGG pathways were significantly enriched (FDR < 0.05). No significantly enriched GO terms were found for DEGs in regards to the treatment.

2.5. Target gene prediction of DE miRNAs

The eight DE miRNAs between FD and HG were shown to target 690 mRNAs based on a cumulative weighted context++ score of < -0.4, while the six DE miRNAs in regards to the treatment group were found to target 333 genes. Among these genes, 627 and 181, were present in our bovine rumen epithelial transcriptome dataset of the same animals for diet and treatment, respectively. In order to further explore the function of these putative miRNA gene targets, GO enrichment analysis was performed, as above. When considering diet, 33 GO terms associated with "Biological process" (GOTERM_BP_FAT) (Fig. 4) were enriched (FDR < 0.05). The pathways with a higher fold enrichment (> 2) were associated with morphogenesis of a branching structure (GO:0001763), morphogenesis of a branching epithelium (GO:0061138), tube development (GO:0035295), morphogenesis of an epithelium (GO:0002009), tissue morphogenesis (GO:0048729), and negative regulation of cell proliferation (GO:0008285). No GO terms associated with "Cellular component" (GOTERM_CC_FAT) were found to be significantly enriched, while for "Molecular function" (GOTERM_MF_FAT), protein kinase inhibitor activity (GO:0004860) and kinase inhibitor activity (GO:0019210) were found to be significantly enriched (FDR < 0.02). Additionally, the KEGG pathway associated with axon guidance (bta04360) was also found to be enriched (FDR $= 0.01$). When considering the GO term enrichment analysis in regards to treatment group, 48 GO terms associated with "Biological process" (GOTERM_BP_FAT) were significantly enriched (FDR < 0.05). The top 25 are illustrated in Supplementary Fig. 2. No GO terms associated with "Cellular component" (GOTERM_CC_FAT) were found to be significantly enriched, while for "Molecular function" (GOTERM_MF_FAT) 24 GO terms were significantly enriched (FDR < 0.05). KEGG pathways analysis revealed that the phosphorylated mechanistic target of rapamycin (mTOR) signaling pathway (bta04150), forkhead box O (FoxO) signaling pathway (bta04068) and oocyte meiosis (bta04114) were significantly enriched (FDR < 0.05).

2.6. miRNA-mRNA interactions

After filtering of significant negative correlations between DEGs and DE miRNAs (Spearman $r < -0.7$, p-value <0.05) in regards to diet and treatment, 52 and 8 interaction pairs were given as an input and visualized as an interaction network (Fig. 5). FYVE and coiled-coil domain autophagy adaptor 1 (*FYCO1*) gene was initially identified as a target gene of miR-93/93-5p, to which it was moderately correlated to, but it was also found to be highly correlated with miR-320a, miR-671 and miR-25. Genes RNA exonuclease 1 homolog (*REXO1*) and MAPK interacting serine/threonine kinase 2 (*MKNK2*) were also found to be highly correlated with miR-671 and miR-25. *REXO1* was a predicted target of miR-25, while *MKNK2* was a predicted target of miR-93. Interestingly, both *REXO1* and *MKNK2* were also moderately correlated to miR-93/93-

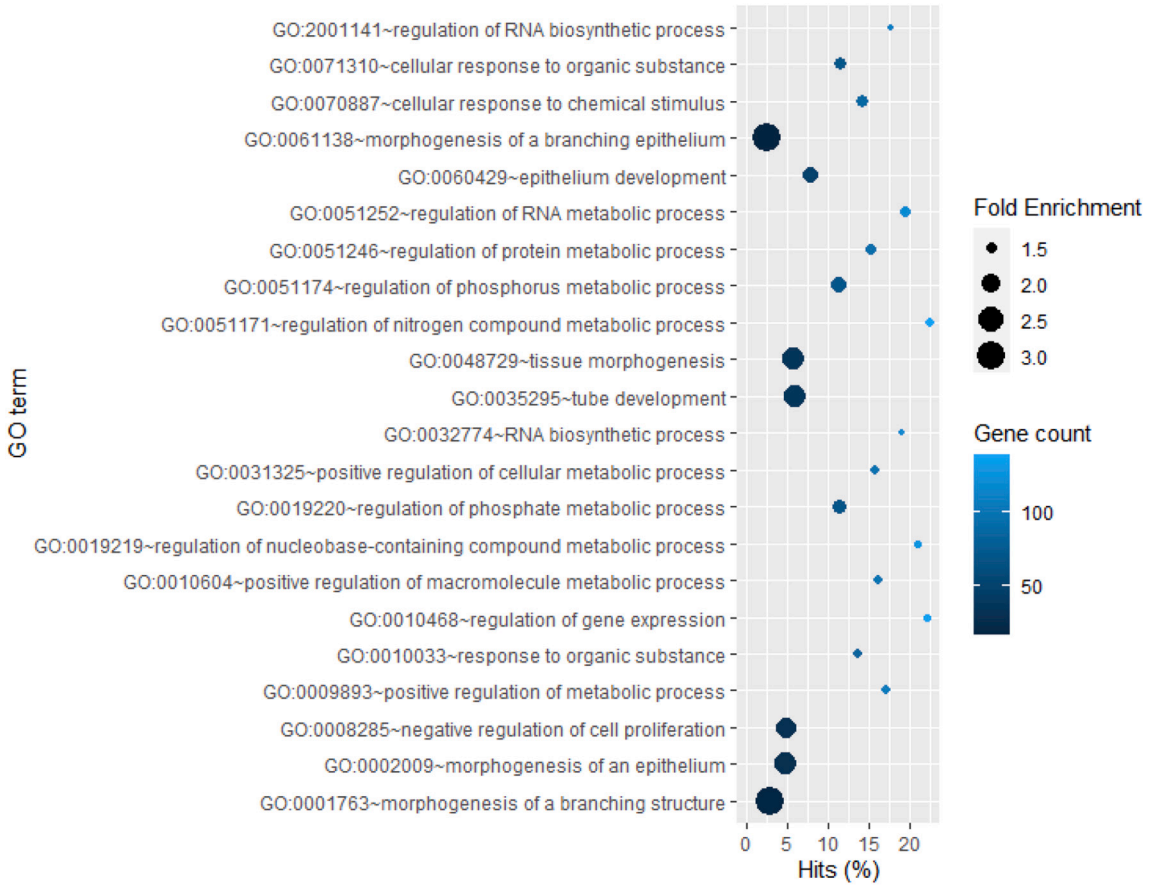

Fig. 4. Top 25 gene ontology (GO) associated with "Biological process" (GOTERM_BP_FAT) significantly enriched in the list of target genes from miRNAs DE between forage and HG.

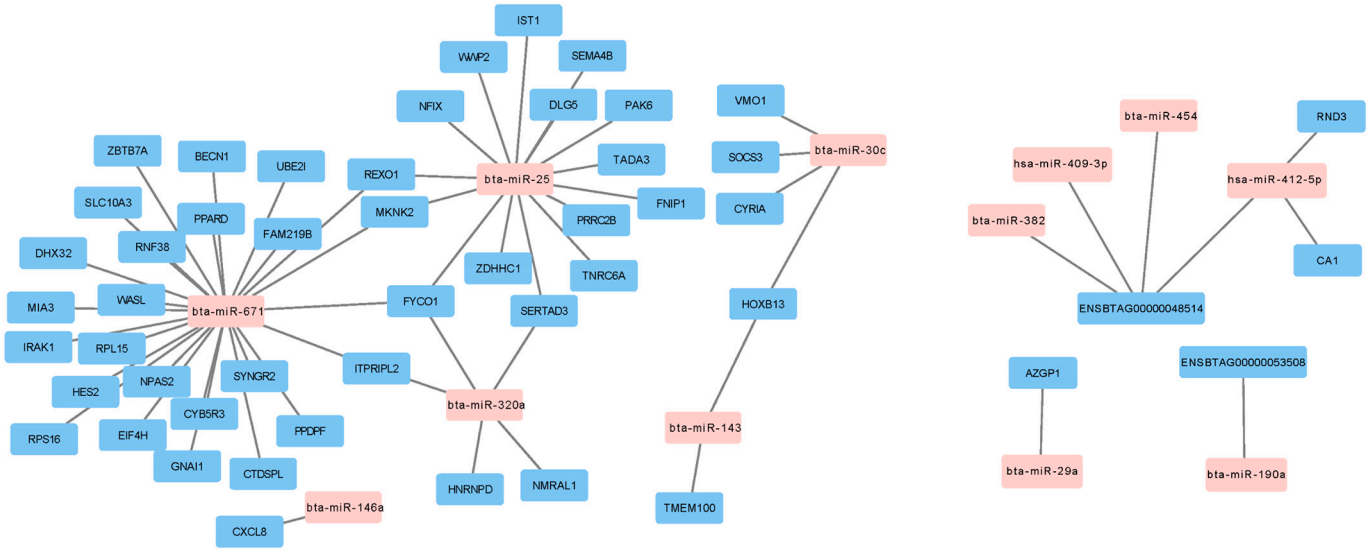

Fig. 5. Strongest ($r < -0.7$) miRNA-mRNA anti-correlations, allowing the identification of plausible miRNA-mRNA interaction pairs in regards to diet and phytogenic supplementation.

5p and miR-320a. Inositol 1,4,5-trisphosphate receptor interacting protein-like 2 (*ITPRIPL2)* was highly correlated to miR-671 and miR-320a and moderately correlated to 93/93-5p and miR-25. SERTA domain containing 3 (*SERTAD3*) was highly correlated with miR-25 and miR-320, while homeobox B13 (*HOXB13)* was highly correlated with miR-143 and miR-30c. When taking a look at miRNAs-mRNAs impacted by PHY supplementation, miR-382, hsa-miR-409-3p, miR-454 and hsa-miR-412-5p were all found to be highly correlated with the down-regulated gene ENSBTAG00000048514.

3. Discussion

To our knowledge, this is the first study attempting to characterize the rumen tissue miRNAome and its function with regards to diet transition or supplementation of a phytogenic feed additive in dairy cows. From the top 10 most expressed miRNAs in rumen papillae of our cows, miR-21-5p, mir-27b, let-7a-5p, let-7f and miR-205 were also previously reported in the blood of cows [28]. These highly abundant miRNAs have been previously reported to be associated with immunity and sensing of environmental stress in cattle [14], bovine skeletal muscle development [29,30], differentiation of connective tissue cells [23], regulation of glucose and lipid metabolism in mice [31] and regulation of macrophage and T-cell-mediated immunity in humans [32,33], showing the biological relevance in tissue development and immunity of the discovered miRNAs. The rumen epithelium is essential for nutrient absorption, transportation and SCFA metabolism. When HG diets are fed and SCFA concentrations are rapidly increased, the size of the rumen papillae must adapt in order to maximize the surface area for SCFA absorption, and provide buffering for the rumen environment. This is in accordance with the observations reported in our companion paper, as there was an increase in SCFA and lactate accompanied by a drop in ruminal pH during transition from FD to HG [34]. The dendrogram based on the hierarchical clustering of the samples also shows a clear separation of miRNA expression between samples taken during FD and those taken after 1 week on a HG diet (Fig. 6). Interestingly, the trait heatmap for pH, lactate and SCFAs corresponded to the miRNA expression dendrogram, which strengthens the hypothesis that miRNAs are heavily involved in transcriptional regulations as a response of feed-induced stress. One major aspect of high-grain feeding to non-lactating dairy cows is the massive over-supply of energy. In fact, we found miRNAs bta-miR-320a, bta-miR-93/hsa-miR-93-5p, bta-miR-671, and bta-miR-25, which are all in some aspects associated to the regulation of lipid metabolism and adipogenesis, to be up-regulated during HG feeding. In detail, miR-320a has been previously reported to affect lipid metabolism and fat deposition in beef cattle [35]. MiR-320 family promotes adipogenesis by blocking mesenchymal stem cells differentiation pathways [36] and has been found to regulate insulin resistance [37] as well as modulate glucose and fatty acid metabolism [38]. MiR-93/93-5p was found to impact the proliferation and differentiation of bovine adipocytes, by incrementing mitotic activity of adipocytes [39]. MiR-671 was found to be differentially expressed between postpartum dairy cattle with mild or severe negative energy balance [15]. MiR-25 is known to repress triacylglycerol synthesis and lipid accumulation in the mammary epithelium of goats [40], playing a role in lipid metabolism and synthesis of milk components [41]. These previous findings combined with the up-regulation found in the current study suggest a crucial role of the described miRNAs within the regulation of lipid metabolism and adipogenesis during HG feeding. Our results suggest a coordinated action of miRNAs to repress triacylglycerol synthesis and regulate insulin sensitivity and boost adipogenesis to cope with the excessive energy surplus. As adipogenesis does not occur at the level of the epithelium, these miRNAs might be secreted from the luminal epithelial cells towards the internal layers of the epithelium potentially as exosomes and reach the bloodstream, possibly triggering a signaling cascade for systemic adipogenesis or even reach the liver or the adipose tissue directly to exert an effect there. A growing body of evidence has also suggested that miRNAs may be used as a tool for cell-cell communication and that adipocytes can actually use miRNAs for both local and systemic communication, as a way to stimulate lipid storage [42,43]. Since these cows were not lactating, it is assumed that the energy surplus will be stored, potentially in the form of fat tissue. In fact, when the HG diet was fed for a period of 4 weeks, these cows gained an average 65 kg

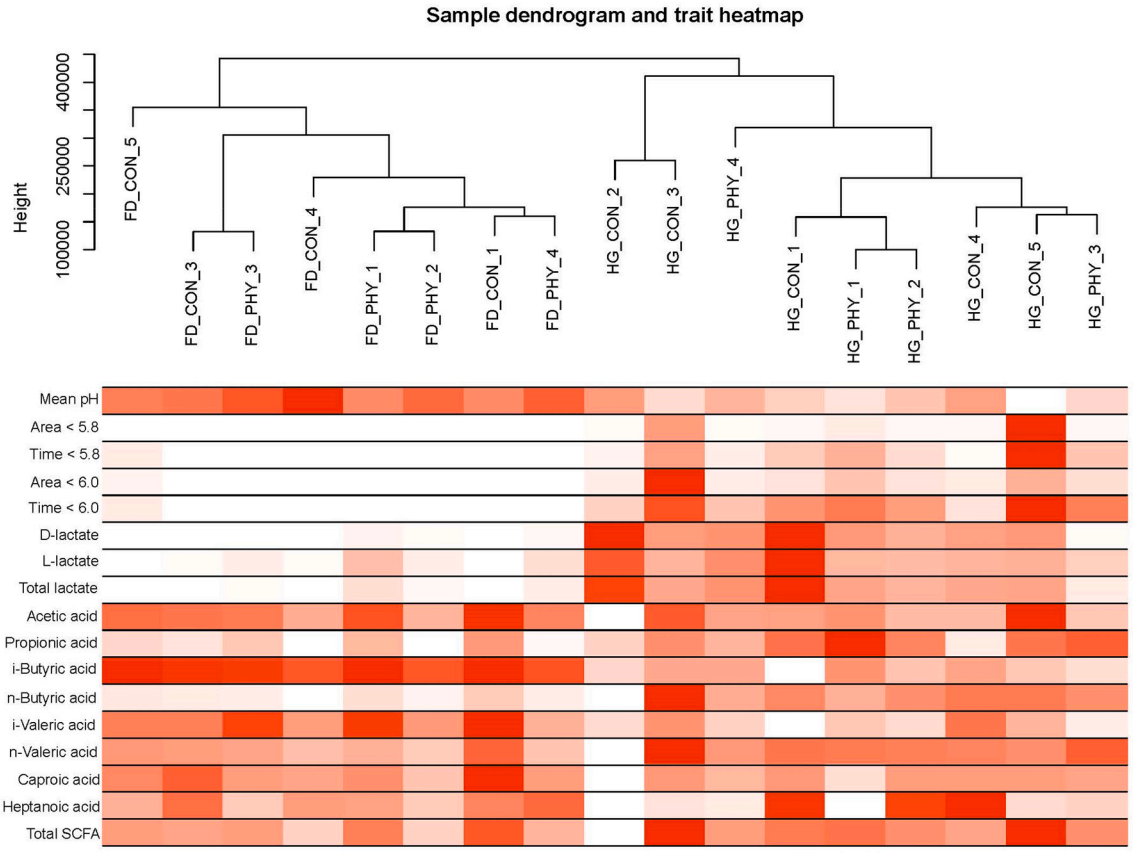

Fig. 6. Hierarchical clustering of miRNAs and phenotypic data. Outlier detection resulted in the exclusion of sample FD_CON_1. Colour scale is presented between white (low) and red (high). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of live body weight [34].

In addition to the effects of a high-grain diet on miRNA and mRNA expression, we were also interested in the effects of a phytogenic feed additive, since it was reported that the supplementation of a blend of menthol, thymol and eugenol during HG feeding could improve rumination, shorten the duration of pH <6.0 in the rumen, as well as reduce ruminal concentrations of LPS and biogenic amines in plasma [44,45]. However, the effect of such bioactive substances at the level of the ruminal epithelium remains deeply uncharacterized. In this study, supplementation with a phytogenic feed additive elicited the up-regulation of hsa-miR-412-5p, bta-miR-454, hsa-miR-409-3p, bta-miR-382 and the down-regulation of bta-miR-190a and bta-miR-29a. GO term enrichment analysis showed a fold enrichment of KEGG pathways associated with mTOR and FoxO family of transcription factors signaling pathways considering the predicted target genes of these six DE miRNAs. In a previous study, a phytogenic product containing thymol has been reported to regulate both mTOR and FoxO signaling pathways in the ileum and jejunum of broiler chickens [46]. This pathway comprises a serine/threonine kinase, which senses various environmental and intracellular changes such as nutrient availability and energy status and coordinates several cellular processes including cell growth differentiation, and survival. It is also involved in regulation of adipogenesis [47] and seems to be correlated with feed efficiency in beef cattle [48]. Given the results of our study and previous studies showing a regulation of mTOR and FoxO signaling pathways after supplementing diets with phytogenic feed additives, further studies are essential in order to elucidate the mechanisms of action of these phytogenic compounds.

To gain more insights on regulated pathways, we additionally obtained the transcriptome of the same samples used for miRNA analyses from our cows. Functional enrichment analysis of 690 target genes of eight DE miRNAs between FD and HG diet indicated an increased fold-enrichment of pathways associated with epithelium morphogenesis and tissue development. The rumen epithelium is known to play a role in both the metabolism and transfer of digested nutrients using the portal venous system to peripheral tissues. A study analyzing the proteome of sheep highlighted the high prevalence of proteins associated with the metabolism of lipids and proteins in the epithelium [49]. As rumen papillae undergo rapid cell turnover and are vital for the SCFA uptake and nutrient exportation into the bloodstream, a higher degree of lipid metabolism during HG feeding might be associated with the metabolism of SCFA that are taken up by the epithelium from the lumen. In fact, an increment in total SCFA of approximately 20 mM was observed between FD and the first week of HG in our companion paper [34]. This is further supported by the GO term enrichment of 9418 DEGs between FD and HG feeding. Several KEGG pathways associated with TCA cycle, SCFA metabolism, glycolysis, bile secretion, fatty acid degradation and regulation of lipolysis in adipocytes were significantly enriched. This is in agreement with a previous report in steers, where transition from forage to concentrate mainly led to the differential expression of genes associated with tissue growth and structure, and fatty acid metabolism [50]. In comparison to 9418 DEG between diets, only five genes were significantly DE between the CON group and PHY-supplemented animals. The expression of *AZGP1*, an adipokine involved in the regulation of mobilization of body reserves in adipose tissue as well as the utilization of excess fatty acids released from enhanced lipolysis [51], was upregulated in the PHY group compared to CON cows. The *ENSBTAG00000048514* gene was downregulated within the PHY group. This uncharacterized protein has been previously associated with LPS stimulation of monocyte-derived macrophages in Holstein and Gir cattle [52], potentially playing a role in immunity. The *RND3* gene was also downregulated in the PHY group. The encoded protein acts as a negative regulator of cytoskeletal organization leading to loss of adhesion. It was found to be DE during feeding of high-concentrate in Charolais steers, playing a role in cholecystokinin/gastrin mediated signaling [53]. Furthermore we found the *CA1* gene, downregulated in the PHY group, which is involved in various biological processes, such as respiration, acid-base balance, saliva, and gastric acid formation [54]. The encoded CA1 enzyme is vital for absorption of SCFA in the gastrointestinal tract, polysaccharides degradation, NaCl resorption, and alkalinization of gut contents [55,56]. A subsequent up-regulation of *CA1* in the CON group might be associated with an attempt of buffering ruminal milieu, as the PHY supplementation seems to have modulated rumen fermentation and reduced the risk of subacute ruminal acidosis [34]. Although only a low number of genes were DEG between PHY and CON, these genes seem to have an important role within the metabolic regulation in our feeding model. However, studies at the level of the epithelium are lacking and a comprehensive analysis of the mechanisms of action of these bioactive compounds needs to be conducted.

Combining our findings from miRNAome and transcriptome, we performed network analysis based on target predictions and anti-correlated expression. Gene expression regulation by miRNAs can lead to complex regulatory networks. A given miRNA can regulate several target genes, while, simultaneously, one gene can be targeted by several miRNAs [57]. Within the network of DE miRNAs and DEGs between diets, *FYCO1* was negatively correlated to bta-miR-25, bta-miR 671 and bta-miR 320a. These miRNAs were found to be up-regulated during HG feeding. *FYCO1* was down-regulated in HG, and is associated with microtubule plus end–directed transport of autophagic vesicles [58]. *MKNK2* was simultaneously targeted by miR-671 and miR-25. High-fat fed knockout mice showed less weight gain and lowered adipocyte inflammation, highlighting the role of *MKNK2* gene in adipogenesis/lipogenesis [59]. Differentially expressed miRNAs were shown to be highly correlated with genes with key functions on the maintenance of both cellular and organismal homeostasis, highlighting the essential effect of diet in the disruption of epithelial signaling and downstream regulation. Furthermore, these types of analyses hint towards a regulation of fat metabolism through miRNAs. Network analyses of the PHY effect considering the 5 DEG and 6 DE miRNAs, showed that ENSBTAG00000048514, down-regulated in PHY group, was highly correlated with up-regulation of the miR-382, hsa-miR-409-3p, miR-454 and hsa-miR-412-5p, making this gene a very interesting candidate to further understand miRNA-mRNA dynamics during phytogenic feed additive supplementation and the genetic basics of active compound modulation in the rumen epithelium. Further validation of these potential miRNA gene targets is necessary.

4. Conclusions

Taken all our findings together, we could demonstrate that miRNAs are involved in the regulation of transcription upon transition to a high-grain diet, as well as in transcriptional alterations after feeding a phytogenic feed additive to the cows. For the PHY effect, we found even more differentially expressed miRNAs (6), than genes (5). For the diet effect we detected massive changes within the transcriptome (9481 DEGs) and 8 DE miRNAs. A post-transcriptional fine-tuning of gene expression and consequently protein production seems to be closely related to fat metabolism in non-lactating cows.

5. Materials and methods

5.1. Ethics statement

The feeding trial was approved by the Institutional Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to the law for animal experiments (protocol number: BMNWF- 68.205/0003-V/3b/2019).

5.2. Animals, diets, and experimental setup

For this research, we used a subset of samples collected in a previous experiment. The research included 9 non-lactating ruminally-cannulated Holstein cows (age = 10.5 ± 0.73 years, body weight = 887.7 ±

65.5 kg) housed at the Dairy Research Farm in Kremesberg of the University of Veterinary Medicine Vienna, Austria. From the 9 cows, 4 cows were randomly allocated to a treatment group, supplemented with a phytogenic mixture including menthol, thymol and eugenol (PHY; 0.04% of DM, Digestarom®, BIOMIN Holding GmbH; $n = 4$), and the other cows remained as a control group (CON; $n = 5$) using a neutral carrier. The experiment consisted in transitioning the 9 cows from an only forage diet (FD) to a high-grain diet (HG) using a step-wise adaptation over 1 week (10% daily to reach a 65% grain content on day 7). The composition of the FD, on dry matter basis, was 75% grass silage, 15% corn silage, and 10% hay (17.2% crude protein, 50.4% neutral detergent fiber and 4.2% starch).The HG diet formulated on dry matter basis included 26.3% grass silage, 8.7% corn silage and 65% concentrate to provide 19.5% crude protein, 31.0% neutral detergent fiber and 28.5% starch (Supplementary Table 6). During the FD feeding, the phytogenic mixture or control was added directly in the rumen through the cannula, while during HG feeding the treatment was included in the concentrate. The animals were fed once daily, at 7:00 am, with free access to their diets via computer-regulated individual feeders, mineral blocks and water throughout the day. Cows were housed in a free-stall barn with individual deep cubicles (2.6 × 1.25 m) with straw bedding.

5.3. Sample collection

Epithelial sampling for miRNA and mRNA sequencing was performed during the week of FD and after 1 week, during the HG feeding. To collect papillae samples, the rumen was manually emptied, and digesta was stored in insulated pre-warmed plastic bins. The ventral rumen wall was inverted and the sampling area (circa 5 × 5 cm) was rinsed with phosphate-buffered saline (PBS). Rumen papillae were cut with aseptic scissors and tweezers through the ruminal cannula (approximately 20 cm below the opening of the cannula), as close as possible to the base of the papillae. The epithelial biopsies were immediately washed in PBS and snap frozen in liquid nitrogen. The tissue samples were collected in duplicates and stored at −80 °C. Immediately after sampling, the rumen wall was re-inverted and the rumen content was re-filled through the cannula.

5.4. Total RNA extraction

Total RNA was extracted using NucleoSpin miRNA kit (Macherey-Nagel, Germany). Briefly, 30 mg of papillae sample were disrupted and homogenized in 300 μl of ml buffer with ceramic beads (Roth Lactan) of 0.7 and 2.0 mm size using a FastPrep-24 instrument (MP Biomedicals). The obtained lysate was loaded on a filter column and centrifuged at 11,000 x*g* for 1 min. Further extraction was done following manufacturer's instructions for the isolation of small and large RNA molecules, adjusting the centrifugation steps for 1 min. DNA digestion was done on-column. RNA was eluted using 40 μl of RNase-free water, incubating the column at room temperature for 1 min, and centrifuging it for 1 min at 11,000 x g. Samples were frozen and stored at −80 °C. Quantity was assessed with a spectrometer (DS-11 FX+, DeNovix Inc., US). Quality control was performed using the 2100 Bioanalyzer System (Agilent, California, US). The chip for small RNAs and the RNA 6000 Nano chip (Agilent, California, US) were used for assessing the RNA integrity for small and for large RNAs, respectively.

5.5. RNA sequencing

Samples were sent to an external laboratory for sequencing (CeGaT GmbH, Tübingen, Germany). For mRNA, libraries were prepared from 10 ng total RNA using SMART Seq Stranded Total RNA-Seq Kit (Takara) and sequencing was performed on a NovaSeq 6000 (Illumina) platform using a 2 × 100 bp paired-end approach. For miRNAs, 200 ng of total RNA were used. Library preparation was conducted using the NEXTflex Small RNA-Seq Kit (Bioo Scientific). Sequencing was performed using a 50-base single-end approach on a NovaSeq 6000 (Illumina) platform.

The raw fastq files of the sequence data were submitted to the NCBI Gene Expression Omnibus (GEO) repository under the accession numbers GSE184575 and GSE184578.

5.6. Bioinformatics and pre-filtering

Sequencing reads were demultiplexed with Illumina bcl2fastq (version 2.20) and adapters were trimmed with Skewer (version 0.2.2) [60].

For miRNA, raw sequence data was checked for quality using FastQC v0.11.5-cegat [61]. Demultiplexed reads were analyzed using sRNAbench [62]. Reads with an average PHRED score below 20 were discarded. The analysis was run in genome mode using the cow genome (UMD3_1_mp) and miRBase 22 as a reference in order to identify bovine (bta) miRNAs and human (hsa) miRNA homologues. Before mapping, reads below 15 nucleotides in length were removed and not used for subsequent analyses. Bowtie was used to map sequence reads against the miRNA library with a maximum of two nucleotide mismatches allowed. The output files were filtered based on the read counts using package tidyverse (version 1.3.1) [63] in R [64]. Only human homologues for which a corresponding bovine miRNA was not identified were included in downstream analyses. The final dataset contained only miRNAs with >10 read counts in at least one sample. Visualization and lists of unique and shared miRNAs were obtained through Venny 2.1.0 [65].

For transcriptome data, the first three nucleotides of the second sequencing read derived from the Pico v2 SMART Adapter were trimmed. Trimmed raw reads were aligned to *B. taurus* ARS-UCD1.2 (bosTau9) using STAR (version 2.5.2b) [66].

5.7. Differential expression analysis

DE analysis of miRNA and mRNA data was calculated with DESeq2 (v1.30.0) [67] in R [64]. Genes with less than 2 reads over all samples were removed from the analysis. Read counts were median ratio normalized and a negative binomial generalized linear model including the diet, phytogenic treatment and their interaction was used (Y = diet + group + diet:group). The DE miRNAs and genes were identified using the Wald test and by computing the appropriate contrasts. Differences in expression were considered significant at a Benjamini and Hochberg [68] corrected p-value <0.05. The relationship between miRNA and transcriptome samples was visualized using principal component analysis (PCA) and hierarchical clustering on the rlog transformed data.

Clustering of samples based on normalized counts of miRNAs from DESeq2 was conducted with WGCNA (v1.69) [25] in R [21]. A sample dendrogram and associated phenotypic data, such as SCFA, lactate and pH data was visualized as a heatmap (Fig. 6).

5.8. miRNA target prediction and functional analysis

Target mRNAs of DE miRNAs were predicted using TargetScan (release 7.2, March 2018, http://www.targetscan.org/) [69]. Predicted targets with a cumulative weighted context++ score of < −0.4 were selected for further analysis [70]. In order to validate these results, miRNA target genes were enriched for GO terms and KEGG pathways using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 [28,29]. The GOTERM_BP_FAT, GOTERM_CC_FAT and GOTERM_MF_FAT parameters were used to analyze the 'Biological process', 'Cellular component' and 'Molecular function' terms, respectively.

5.9. Identification of miRNA-mRNA interactions

Putative target mRNAs were selected for Spearman correlation analysis using the package Hmisc [71] in R, if present in the transcriptome data. Negative correlated mRNAs ($r < -0.7$) that were found

to be differentially expressed, and with a significant p-value <0.05 were further selected and visualized as an interaction network in Cytoscape [72].

Declaration of competing interest

Nicole Reisinger is employed by Biomin GmbH, a company that manufactures and trades feed additives. This fact did not impact the analysis nor the interpretation of the results.

Acknowledgments

We thank the staff of the dairy farm of Vetmeduni Vienna for their help. We are also thankful to Thomas Enzinger, Parisa Kheirandish, Claudia Lang, Arife Sener-Aydemir and Veronika Strasser for their help in sampling. This research has been supported by Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development. BIOMIN Holding GmbH supports financially the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110333.

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