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# Elucidating genes and gene networks linked to individual susceptibility to milk fat depression in dairy goats

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Dietary supplementation with marine lipids modulates ruminant milk composition toward a healthier fatty acid profile for consumers, but it also causes milk fat depression (MFD). Because the dairy goat industry is mainly oriented toward cheese manufacturing, MFD can elicit economic losses. There is large individual variation in animal susceptibility with goats more (RESPO+) or less (RESPO-) responsive to diet-induced MFD. Thus, we used RNA-Seq to examine gene expression profiles in mammary cells to elucidate mechanisms underlying MFD in goats and individual variation in the extent of diet-induced MFD. Differentially expression analyses (DEA) and weighted gene co-expression network analysis (WGCNA) of RNA-Seq data were used to study milk somatic cell transcriptome changes in goats consuming a diet supplemented with marine lipids. There were 45 differentially expressed genes (DEGs) between control (no-MFD, before diet-induced MFD) and MFD, and 18 between RESPO+ and RESPO-. Biological processes and pathways such as "RNA transcription" and "Chromatin modifying enzymes" were downregulated in MFD compared with controls. Regarding susceptibility to diet-induced MFD, we identified the "Triglyceride Biosynthesis" pathway upregulated in RESPO- goats. The WGCNA approach identified 9 significant functional modules related to milk fat production and one module to the fat yield decrease in diet-induced MFD. The onset of MFD in dairy goats is influenced by the downregulation of *SREBF1*, other transcription factors and chromatin-modifying enzymes. A list of DEGs between RESPO+ and RESPO- goats (e.g., *DBI* and *GPD1*), and a co-related gene network linked to the decrease in milk fat (*ABCD3*, *FABP3*, and *PLIN2*) was uncovered. Results suggest that alterations in fatty acid transport may play an important role in determining individual variation. These candidate genes should be further investigated.

## KEYWORDS

goat, milk-fat depression, RNA-Seq, nutrigenomics, individual susceptibility, WGCNA

## Introduction

Several feeding strategies have proven efficient in modulating ruminant milk composition toward a healthier fatty acid (FA) profile for consumers (1, 2). This includes, for instance, reductions in atherogenic saturated FA and increases in CLA, vaccenic acid or long chain n-3 polyunsaturated FA (3, 4). However, some of these strategies (namely, diet supplementation with marine lipids) can induce milk fat depression (MFD) in dairy ruminants, although goats are less prone than cows to this syndrome (2, 5). Because most caprine milk is used for cheese manufacturing, MFD is always related to economic losses in this species.

Interestingly, a large individual variation in the extent of diet-induced MFD has been observed in dairy ruminants (6, 7), including dairy goats from the same experimental flock when consuming a diet supplemented with 2% fish oil (8). When trying to improve milk fatty acid composition, understanding the genetic mechanisms behind this variability, which remain uncertain, would help explain individual variation in fish oil-induced MFD severity and select for less-susceptible animals.

The introduction of high-throughput technologies to study the genome provides ruminant scientists with a valuable tool to better understand animals' responses to nutrition, and it has boosted research in the field of nutrigenomics [i.e., the study of genome-wide influences of nutrition (9, 10)]. Technologies such as RNA-sequencing (RNA-Seq) along with bioinformatics allow for examining the expression of the entire set of transcribed genes in a sample, offering key information on the fundamental effects of dietary components on physiological outcomes (10). This approach has been applied for elucidating genes involved in mammary transcriptome responses to dietary lipid supplementation in cows and sheep (11–13). In dairy goats, less information on the effects of lipid supplementation on lipogenic gene expression is available (14); we are only aware of one *in vivo* study applying RNA-Seq to examine the transcriptomic changes associated with MFD in the mammary gland of goats fed different levels of degradable starch (15). However, in this species, MFD associated with dietary starch is a much less usual condition than marine lipid-induced MFD (1, 2, 8). Thus, the characterization of the transcriptomic changes underlying marine lipid-induced MFD would provide useful information to determine the genetic mechanisms influencing this complex trait. Moreover, the identification of genes and variants involved in the individual susceptibility to MFD in goats and their potential use as biomarkers could be useful to improve the classification of the animals and select those less susceptible to diet-induced MFD.

Differential gene expression analyses (DEA) have been successfully used to determine genes that are differentially expressed between experimental conditions (16, 17). However, the use of network approaches, such as the Weighted Gene Co-expression Network Analysis (WGCNA) method, has emerged

as a useful alternative to elucidate the genetic basis of complex traits. These approaches do not aim to identify individual differentially expressed transcripts but clusters (modules) of highly co-expressed genes that are up/down regulated under certain biological, chemical or environmental conditions (18).

Based on the hypothesis that MFD in goats is partly driven by changes in the mammary gland gene expression profile, the aim of the present study was to use RNA-Seq data from the milk somatic cells (MSC) to identify genes and gene co-expression networks that differentiate distinct responses to diet induced MFD in goats.

## Materials and methods

### Animals, diets, and management

A detailed description of procedures, including animals, diets, and sampling, is provided by Della Badia et al. (8). All experimental procedures were performed in accordance with European Union and Spanish legislations (Council Directive 2010/63/EU and R. D. 53/2013). The animal use protocol was approved by the Research Ethics Committees of the *Instituto de Ganadería de Montaña*, the Spanish National Research Council (CSIC), and the *Junta de Castilla y León* (Spain).

Briefly, 25 Murciano-Granadina goats (body weight:  $30.5 \pm 3.6$  kg) at a similar stage of their first lactation (days in milk:  $40.1 \pm 5.9$ ) were housed in individual tie stalls. During a 3 weeks adaptation period, goats were fed a TMR formulated with alfalfa hay (particle size > 4 cm) and concentrate (forage:concentrate ratio 50:50) without lipid supplementation [see Della Badia et al. (8) for details]. Subsequently, goats were supplemented with 20 g of fish oil (FO; Afampes 121 DHA, Afamsa)/kg of diet DM, which is known to cause MFD in dairy goats (8), for five additional weeks to induce a stable inhibition in milk fat synthesis. At the end of this second period, and on the basis of the magnitude of MFD, the five most-responsive goats (RESPO+; with a mean decrease in milk fat content of 26.0%) and the five least-responsive goats (RESPO-; with a mean decrease in milk fat content of 9.7%) were selected. Animals were milked once daily at ~08:30 h in a 10-stall milking parlor (DeLaval). They had *ad libitum* access to diets daily, and clean drinking water was available at all times.

### Sampling, RNA-extraction and RNA-sequencing

Milk samples for RNA extraction were collected at the end of the adaptation period (no-MFD, control samples) from all goats and at the end of the fish-oil (FO) supplementation period from goats selected as RESPO- and RESPO+. Sample collection and RNA extraction were performed according to

Toral et al. (19), with little modifications. Milk somatic cells from healthy udders have been proven to represent the lactating mammary gland transcriptome in ruminants and a valid proxy to more invasive approaches, with correlations of 0.98 with mammary gland biopsies (20, 21). Moreover, this source of RNA has already been successfully applied in nutrigenomic studies in sheep to determine genes and pathways involved in MFD associated with FO and conjugated linoleic acid supplementation (11, 12). Briefly, milk samples were collected 1 h after milking and 10 min after injection of 5 IU of oxytocin/animal (Facilpart, Laboratorios SYVA) to maximize milk somatic cell concentration. To prevent RNA degradation, udders were cleaned with soap and water and disinfected with povidone-iodine, and the nipples were also flushed with RNaseZap (Ambion). A sterile gauze was used to cover the collection tube to avoid contamination.

For RNA extraction, the MSCs from 50 ml of fresh milk were pelleted by centrifugation at  $650 \times g$  for 10 min at  $4^{\circ}\text{C}$  in the presence of a final concentration of 0.5 mM of EDTA. Then, the pellet was washed three times with 10, 2, and 1.5 ml of PBS (pH 7.2 and 0.5 mM of EDTA), followed by centrifugation at  $650 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The last pellet was kept in RNAlater (Sigma-Aldrich) and stored at  $-80^{\circ}\text{C}$  until RNA extraction using 500  $\mu\text{l}$  of TRIzol (Invitrogen). The RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). The mean RNA integrity number (RIN) of the samples was  $8.5 \pm 0.7$ .

A total of 15 samples were used for RNA sequencing: RESPO+ ( $n = 5$ ), RESPO- ( $n = 5$ ), and no-MFD (controls,  $n = 5$ ). Milk production records (content and yield) in controls, RESPO+ and RESPO- animals, after and before FO supplementation are summarized in [Supplementary Table S1](#); particularly, the milk fat percentage variation ranged between 21.74%–29.68% and 7.77%–10.81% in the RESPO+ and RESPO- groups, respectively. With the aim of maximizing transcriptomic differences between goats with and without MFD, the latter (i.e., the 5 no-MFD) corresponded to control samples collected from RESPO+ goats before offering them the diet that induced MFD.

The RNA sequencing was conducted at Centro Nacional de Análisis Genómico (CNAG, Barcelona, Spain), where the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) was used for library preparation. A NovaSeq™ 6000 Sequencing System (Illumina) was used to generate stranded paired-end reads of 51 bp.

## Alignment and quantification

RNA-Seq data files were aligned to the ARS1 goat reference genome (GCF\_001704415.1\_ARS1 from NCBI database) using STAR v. 2.7.6a (22). In addition to the default arguments for

the alignment, we added the options “–outFilterType BySJout” to reduce spurious junctions, “–outWigStrand Stranded” to indicate that our RNA-Seq data was stranded, and the option “–quantMode TranscriptomeSAM” to the necessary output for the quantification with RSEM software (23). For the quantification with RSEM, we used the option “–paired-end” to indicate our data were paired-end and “–no-bam-output” to indicate that no bam output should be created. In addition, we used the options “–estimate-rspd” to estimate the start position of the distribution, “–calc-ci” to calculate 95% credibility intervals and posterior mean estimates, “–seed 12345” to set the seed for the random number of generators used in calculating posterior mean estimates and credibility intervals, and “–p 8” to fix the parallel environment. RSEM software can deal with mapping uncertainty due to multi-mapped reads using the expectation maximization (EM) algorithm to estimate the maximum likelihood value of gene or transcript abundance (24).

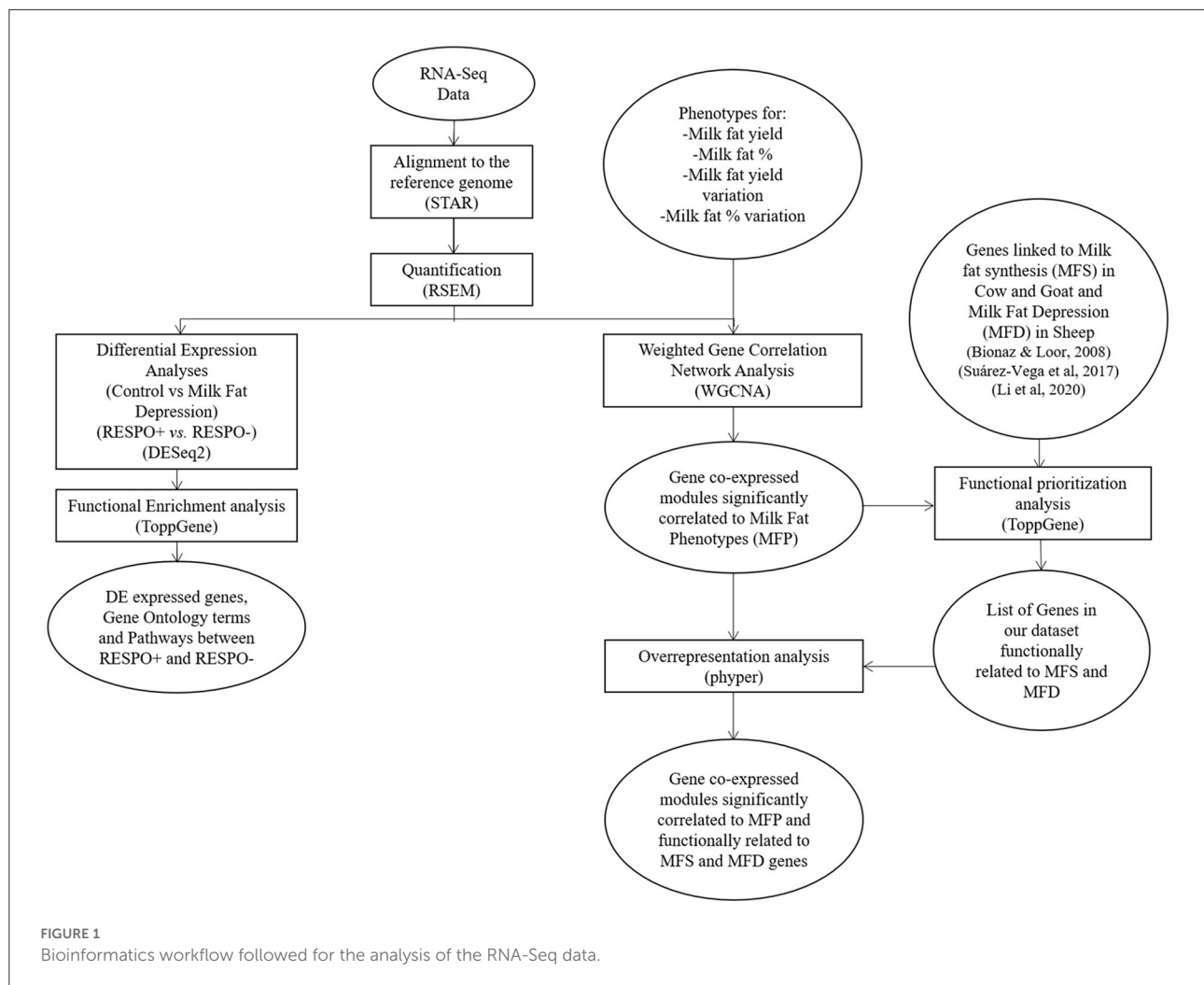
After alignment and quantification, we followed two different analytical approaches: a standard DEA and a WGCNA. The bioinformatics workflow applied to analyze the RNA-Seq data is summarized in [Figure 1](#).

## Differential gene expression analyses

We used the R package tximport (25) to import the results from the quantification step with RSEM software into R environment. The program DESeq2 v.1.34.0 (16) was used to perform the DEA. First, we used the function “collapseReplicates” in DESeq2 to collapse technical replicates. We computed the Euclidean distance among the samples using “dist” function in R and plotted the hierarchical clustering using “hclust” to check for outliers. Moreover, PCAtools (26) was used to perform a principal component analysis (PCA) to cluster samples based on gene expression data. Then, we performed differential expression analysis to identify differentially expressed genes (DEG) between control ( $n = 5$ ) and MFD samples [the latter including both RESPO+ ( $n = 5$ ) and RESPO- samples ( $n = 4$ ; after removing outliers)]. In addition, we performed a DEA to evaluate differences between RESPO+ ( $n = 5$ ) and RESPO- goats ( $n = 4$ ). For both analyses, we considered genes as differentially expressed at a  $P$ -adjusted value ( $P_{\text{adj}}$ )  $< 0.05$ , i.e., after the correction of  $P$ -values for multiple testing using the Benjamini–Hochberg’s approach.

## Functional enrichment analyses

Functional enrichment analyses were performed using the web-tool ToppFun in ToppGene Suite (27). Functional enrichment was performed for Gene Ontology (GO):Molecular function, GO:Biological Processes, GO:Cellular



Component, and Pathway databases. The following options were applied to perform the analyses: “*P*-value Method = probability density function,” “multiple test correction = false discovery rate (FDR),” “FDR cutoff < 0.05,” “Gene Limits  $1 \leq n \leq 2,000$ .” These analyses were performed for the different lists of DEGs obtained from the previous analyses, i.e., the upregulated or downregulated genes identified by the different comparisons between control and MFD samples and between RESPO+ and RESPO- samples.

## Weighted gene correlation network analyses

Expression values of genes were normalized using DESeq2 to obtain a matrix of counts to be used in the R package WGCNA (18), for weighted gene co-expression network construction.

Briefly, the similarity matrix between each pair of genes across all samples was constructed by calculating Pearson correlations. Then, the similarity matrix was transformed into an adjacency matrix (*A*) raised to a soft threshold power based on the free-scale topology criterion. We used the function “pickSoftThreshold” to calculate the soft-thresholding power. In this study, the soft threshold ( $\beta$  parameter) was 12, which corresponds to a free-scale topology of  $R^2 > 0.80$ . Subsequently, the topological overlap matrix (TOM) was used to define modules based on dissimilarity ( $1 - \text{TOM}$ ). Then, a hierarchical clustering from the dissimilarity matrix was generated with the “hclust” function. Modules of co-expression were then detected by using the dynamic tree cut (DTC) algorithm. The minimum size selected to construct the gene co-expression modules was 15.

Once the gene modules were detected, their associations with the studied milk fat traits (fat yield, fat percentage, variation in fat yield, and variation in fat percentage; [Supplementary Table S1](#)) were estimated using the correlation

between the module eigengene (ME) and phenotypic values for each trait and animal, allowing the identification of modules highly correlated with the interest traits. Genes of modules with significant module-trait associations ( $P$ -value  $\leq 0.05$ ), for at least one trait, were used for functional enrichment analysis.

## Functional enrichment analysis for milk fat genes

To determine if the gene co-expressed modules significantly correlated to the phenotypic traits were functionally enriched in genes involved in milk fat synthesis or MFD, we developed the following functional enrichment analysis. First, we performed a functional prioritization analysis on ToppGene (27) using the genes from the gene modules significantly correlated to our traits as the test list. The training set was a list of 432 known genes linked to milk fat synthesis in cattle and goats, and to MFD in sheep (Supplementary Table S2) (11, 28, 29). The functional enrichment analysis for the test set was performed for the Gene Ontology (GO) (GO:Biological Process, GO:Molecular Function, and GO:Cellular Component), and the Pathway and Gene family databases, using as  $P$ -value method the probability density function and FDR  $< 0.05$ . Genes in the test set were considered functionally prioritized when the overall  $P$ -value for the analysis was  $< 0.05$ .

Once we had our list of genes functionally associated to milk fat synthesis and/or MFD, composed by the training gene set and the functionally prioritized genes (milk fat gene list, 850 genes; Supplementary Table S3), we performed an overrepresentation analysis to check which of the gene co-expressed modules was enriched for the genes in this list. A hypergeometric test was performed for each significantly correlated module of genes using the phyper package (30) in R. For each evaluated module, we computed the probability that genes overlapping (specific for each gene co-expressed module) with the milk fat gene list was higher than expected by chance (i.e., overrepresented) using as background the 11,624 genes used to create the weighted-gene correlation networks. The  $P$ -value obtained was corrected for multiple testing using FDR and Bonferroni methods with the  $P$ .adjust function in R.

## Results

### Sequencing and alignment of the goat MSCs transcriptome

The statistics regarding RNA-Seq are summarized in Supplementary Table S4. The average of reads per sample was

37,335,520 (SD = 4,929,042.77), with a mode of technical replicates per sample of 2. The mapping rate to the goat reference genome *Capra hircus* assembly ARS1 was 93.86% (SD = 0.01), with a percentage of uniquely mapped reads of 74.87% (SD = 0.05) and 19% of multi-mapping reads (SD = 0.04).

### Gene expression levels and sample distribution

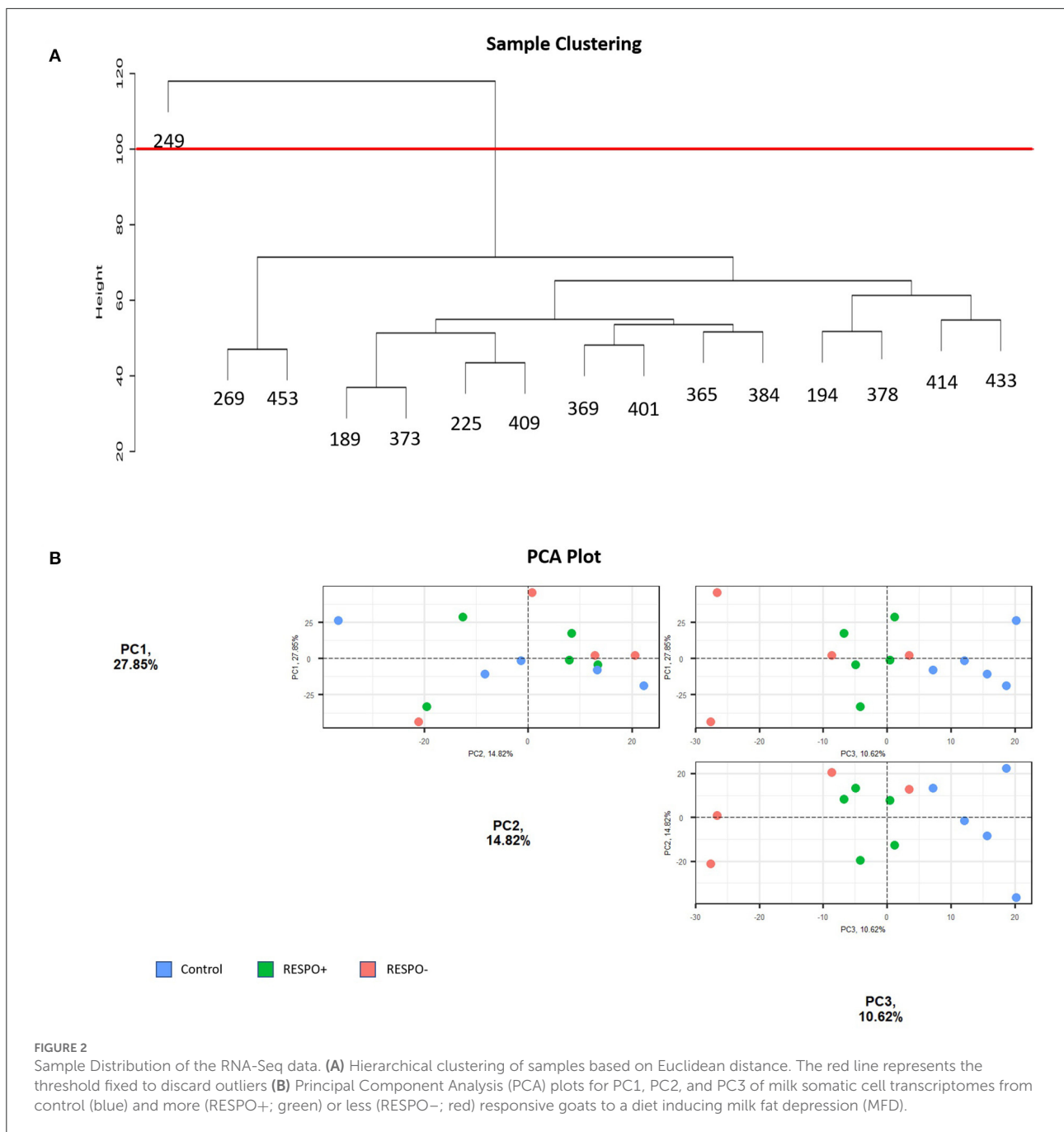
Gene counts normalized using the Fragments per Kilobase per Million Mapped Reads (FPKM) method were used to evaluate gene expression levels. According to their expression, genes were classified into four different categories: genes with an expression lower than 0.1 FPKMs, which were not used in subsequent analyses, low expressed ( $< 10$  FPKMs), middle expressed ( $> 10$ – $500$  FPKMs), and highly expressed ( $> 500$  FPKMs) genes (Supplementary Figure S1). The majority of genes ranged between 10 and 500 FPKM. Among 66 most expressed genes ( $> 500$  FPKMs) in goats, the top expressed genes were caseins (*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*) and whey (*PAEP* and *LALBA*) proteins.

Based on the Euclidean distance, we removed one outlier (ID = RESPO2\_249 sample) from the RNA-Seq dataset (Figure 2A). Regarding the PCA, Figure 2B shows the three principal components accounting for more than 10% of the variance. Altogether, PC1, PC2, and PC3 explained 53.29% of the variance. It was expected that lipid supplementation would not explain a high percentage of the variance in the PCA analysis when evaluating the whole transcriptome because of the animal variability and the fact that the number of genes expected to have a high impact on the complex quantitative traits is low. The first principal component (PC1), accounting for 27.85% of the variance, and the PC2 (accounting for 14.82%) were not able to group the samples by the conditions (Figure 2B). Nevertheless, the PC3, accounting for 10.62% of the variance, better discriminates the different conditions evaluated.

### Differentially expressed genes between control and MFD

A total of 45 genes were differentially expressed ( $P_{adj} < 0.05$ ) between control and MFD goats (Supplementary Table S5). Thirty-three of these were upregulated in goats with MFD and 12 in the control. The top three differentially expressed genes were a ncRNA *LOC102181690* ( $P_{adj} = 1.16E-14$ ,  $\log_2$ FoldChange =  $-2.16$ ) and the gene coding for 40S ribosomal protein S27 (*LOC102171808*,  $P_{adj} = 0.0004$ ,  $\log_2$ FoldChange =  $-2$ ), both upregulated in MFD samples, and the *SREBF1-like*





(*LOC108635517*,  $P_{\text{adj}} = 3.88\text{E}-05$ ,  $\log_2\text{FoldChange} = 1.11$ ), upregulated in control samples.

After functional enrichment analyses, genes upregulated in the goats with MFD were significantly clustered [False Discovery Rate Benjamini and Hochberg (FDR B&H) <0.05] in seven GO terms for the Molecular function category (GO:Molecular function), 50 terms in the GO:Biological Process category, 15 terms in the GO:Cellular Component category, and 37 in the pathway databases (Supplementary Table S6).

The highest enriched GO terms for each category were “structural constituent of ribosome” (seven genes, FDR B&H =  $3.966\text{E}-8$ ) for GO:Molecular function, “SRP-dependent cotranslational protein targeting to membrane,” “cotranslational protein targeting to membrane,” “nuclear-transcribed mRNA catabolic process, nonsense-mediated decay,” “protein targeting to ER” and “establishment of protein localization to endoplasmic reticulum” (six genes, FDR B&H =  $1.985\text{E}-7$ ) for GO:Biological Process and “ribosomal subunit” for GO:Cellular

Component (seven genes, FDR B&H = 7.833E-8). The highest enriched pathway was “Selenocysteine synthesis” (BioSystems: REACTOME, seven genes, FDR B&H = 2.052E-9).

Genes upregulated in control were significantly enriched in 30, 14, and 14 GO terms for the Molecular Function, Biological Process and Cellular Component categories, respectively, and 50 pathways (Supplementary Table S7). The top enriched GO terms for GO:Molecular function were “chromatin binding” and “transcription factor binding” (four genes, FDR B&H = 6.844E-3). For the GO:Biological Processes the highest enriched GO terms were “positive regulation of nucleic acid-templated transcription,” “positive regulation of transcription, DNA-templated,” and “positive regulation of RNA biosynthetic process” (six genes, FDR B&H = 1.801E-2). For the GO:Cellular Component the top enriched GO term was “Golgi trans cisterna” (two genes, FDR B&H = 1.166E-3), and for the pathway databases “Chromatin organization,” and “Chromatin modifying enzymes” (BioSystems: REACTOME, 4 genes, FDR B&H = 1.609E-3).

## Differentially expressed genes between RESPO+ and RESPO-

There were 18 genes differentially expressed ( $P_{adj} < 0.05$ ) between RESPO- and RESPO+ goats, 13 were upregulated in the RESPO+ animals and five in RESPO- (Supplementary Table S8). The top three differentially expressed genes were *DBI* ( $P_{adj} = 0.002, 0.56$ ), *PBXIP1* ( $P_{adj} = 0.006, -0.89$ ) and *ELMSAN1* ( $P_{adj} = 0.006, -0.63$ ). Results from the functional enrichment analyses for the upregulated genes in RESPO+ are detailed in Supplementary Table S9. The top enriched GO term among those significantly enriched was potassium channel inhibitor activity (two genes, FDR B&H = 1.323E-3) for the GO:Molecular function category (18 terms, FDR B&H < 0.05), regulation of potassium ion transmembrane transport (three genes, FDR B&H = 5.715E-3) for the GO:Biological Process category (50 terms, FDR B&H < 0.05), and anchoring junction (four genes, FDR B&H = 3.794E-2) for the GO:Cellular Component (16 terms, FDR B&H < 0.05). No pathways were significantly enriched.

All results for the functional enrichment analyses of genes upregulated in RESPO- are in Supplementary Table S10. For the five upregulated genes, 11 terms were enriched for the GO:Molecular function category; “oxidoreductase activity, acting on the CH-OH group of donors, quinone or similar compound as acceptor” and “glycerol-3-phosphate dehydrogenase (quinone) activity” (one gene, FDR B&H = 5.407E-3) being the highest enriched terms. For the GO: Biological Process category, we found 22 terms significantly

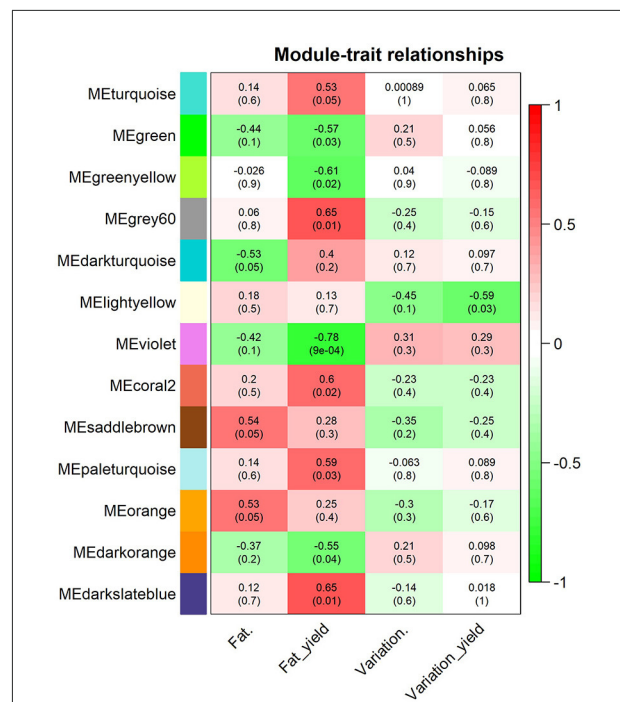


FIGURE 3

Significant module-trait associations found between the module eigengenes (ME) and the milk-fat traits analyzed [fat percentage (Fat.), fat yield (Fat\_yield), variation in fat percentage (Variation.), and variation in fat yield (Variation\_yield)]. Each row corresponds to a module eigengene labeled with colors and each column to a milk fat trait. Individual cells contain Pearson's correlation coefficients (outside parentheses) and the  $P$ -values of the correlation (within parentheses). The red to green color within the cells represents the positive (red) or negative (green) correlation of the modules with the milk fat traits.

enriched; “positive regulation of CoA-transferase activity,” “regulation of CoA-transferase activity,” “glycerol-3-phosphate catabolic process,” and “negative regulation of protein lipidation” (one gene, FDR B&H = 2.958E-2) being the top enriched ones. For the GO:Cellular component category, four terms were significantly enriched (FDR B&H < 0.05), with “glycerol-3-phosphate dehydrogenase complex” (one gene, FDR B&H = 1.361E-2) being the top enriched term. Moreover, we found 22 pathways significantly enriched, “Triglyceride Biosynthesis” (BioSystems: REACTOME, two genes, FDR B&H = 2.887E-3) being the top enriched pathway.

## Co-expressed gene modules and correlations with milk fat yield, milk fat percentage and milk fat variation traits

Using the WGCNA approach, we identified 67 modules of co-expressed and highly interconnected genes, each module being assigned to different colors (Figure 3). Among the

modules identified, three were significantly correlated to milk fat percentage (orange, saddlebrown, and darkturquoise), nine significantly correlated to milk fat yield (darkorange, green, violet, greenyellow, turquoise, paleturquoise, darkslateblue, grey60, and coral2) and 1 significantly correlated to the variation in milk fat yield due to MFD (lightyellow). The module with the highest correlation with any of the traits was the violet, with a correlation of  $-0.78$  with milk-fat yield ( $P$ -value = 0.0009, 46 genes). The unique module associated with milk fat yield variation due to MFD was lightyellow with a correlation of  $-0.59$  ( $P$ -value = 0.025, 95 genes). None of the modules was significant for all traits together. The list of all modules and associated genes is provided in [Supplementary Table S11](#).

The functional enrichment analyses performed with the training list of genes ([Supplementary Table S2](#)) confirmed their relevant function in processes related to milk fat synthesis. The highest enriched terms for the GO:Molecular function, GO:Biological Processes, GO:Cellular Component, Pathway and Gene family categories were “acyltransferase activity” (31 genes, FDR B&H =  $4.255E-11$ ), “lipid metabolic process” (130 genes, FDR B&H =  $5.813E-46$ ), endoplasmic reticulum subcompartment (64 genes, FDR B&H =  $3.769E-11$ ), Metabolism of lipids and lipoproteins (BioSystems:REACTOME, 103 genes, FDR B&H =  $3.769E-11$ ), and Acyl-CoA dehydrogenase family (six genes, FDR B&H =  $7.984E-7$ ), respectively. After the functional prioritization analyses, 106 genes from the test gene set (genes from modules significantly correlated to milk-fat phenotypes) overlapped with genes in the training set, and 745 genes were functionally prioritized ([Supplementary Table S12](#)). In total, 25% of the genes in our test set were functionally associated to milk fat synthesis or MFD.

Once we had the milk fat gene list ([Supplementary Table S3](#)), we performed the overrepresentation analyses for the gene modules significantly correlated to the milk-fat phenotypes. The results are summarized in [Table 1](#). There were 10 modules overrepresented; the turquoise one was the highest enriched. The lightyellow module, was the only one significantly correlated to the variation in fat yield due to MFD, and was also enriched with 21 genes functionally related to milk fat synthesis ([Figure 4](#)).

## Discussion

We investigated changes in the milk somatic cell transcriptome in goats subjected to a diet known to induce MFD through two RNA-Seq analytical approaches to determine genes and gene networks involved in individual differences in the degree of MFD. First, the DEA allowed us to determine those genes differentially expressed between the analyzed conditions (control vs. MFD and RESPO- vs. RESPO+). Second, the WGCNA allowed us to gain insights into the

genetic signatures related to complex quantitative phenotypes such as milk fat synthesis and individual MFD susceptibility. Although DEA is the methodology commonly chosen to discriminate DEGs between different experimental conditions, this approach can have several limitations. In addition to the fact that genes are studied individually, the application of this approach can be challenging when molecular differences between the analyzed conditions are small (31). On the other hand, the WGCNA constructs networks of genes based on the pairwise correlations between their expression (18) such that genes contributing to the same biological process are usually under the same transcriptional regulation and tend to have a correlated expression performance (32). Thus, it has been shown that the combination of DEA and WGCNA approaches can be advantageous for identifying genes involved in functional differences between similar tissues where gene expression levels do not differ much (31). The complementarity of these two methodologies helped us gain insights into the genetic basis of a quantitative phenotype, diet-induced MFD in the lactating mammary gland, where most reads (~90%) account for major proteins involved in milk synthesis, and only subtle differences in gene expression exist across animals (absolute  $\log_2$ FoldChange hardly surpass 2).

In DEA, a total of 45 genes exhibited differences in expression. Among them, *SREBF1-like* and *SREBF* genes were upregulated in the control group. Sterol regulatory element-binding transcription factors (SREBFs) act as main regulators in controlling enzymes involved in de novo synthesis of fatty acids and cholesterol (33, 34). Our findings agree with reports in cattle and sheep, where decreases in *SREBF1* expression have been associated with MFD (5, 35, 36). Although a recent study in goats under starch-induced MFD found no variation in *SREBF* abundance, a decrease in *INSIG1* expression was detected, which may affect SREBP activity (15). Similarly, a previous study performed by our research group, aiming to identify differentially expressed genes from the MSCs transcriptome of FO-induced MFD in dairy sheep, did not detect differential expression for *SREBF1*, but the *SREBP signaling pathway* was enriched (11). Among the GO (Molecular Function and Biological Processes) terms and biological pathways that were enriched in the present study, several contained genes involved in the regulation of RNA transcription (*SREBF1*, *HIVEP3*, *ZNF827*, *PPRC1*, *DOT1L*, and *ASH1L*), chromatin-modifying enzymes (*NCOR2*, *CHD4*, *DOT1L*, and *ASH1L*), and histone methyltransferase activity (*DOT1L* and *ASH1L*).

Dietary lipids have been postulated as regulators of gene expression in a hormonal-independent manner through regulation of activity and/or abundance of transcription factors and nuclear receptors affecting the transcription of many genes (37, 38). Moreover, epigenetic changes in gene expression due to modifications in the chromatin have been attributed to dietary unsaturated fatty acids in non-ruminant animal models (39). For upregulated genes during MFD, we mainly observed



TABLE 1 Results from the overrepresentation analyses among genes in modules significantly correlated to milk fat phenotypes and the milk fat gene list<sup>a</sup>.

Module name	Number of genes in the module	Common genes (modules vs. milk fat gene list)		P-value	FDR B&H	Bonferroni
		Number	%			
Turquoise	2,014	564	28.00	1.40E−246	1.82E−245	1.82E−245
Green	552	114	20.65	5.77E−26	3.75E−25	7.50E−25
Greenyellow	210	50	23.81	8.96E−15	3.88E−14	1.16E−13
Grey60	110	24	21.82	2.56E−07	8.32E−07	3.33E−06
Darkturquoise	83	20	24.10	3.45E−07	8.98E−07	4.49E−06
Lightyellow	95	21	22.11	9.48E−07	2.05E−06	1.23E−05
Violet	46	12	26.09	1.64E−05	3.05E−05	0.0002
Coral2	23	6	26.09	0.0009	0.0015	0.0123
Saddlebrown	53	10	18.87	0.0013	0.0019	0.0173
Paleturquoise	47	9	19.15	0.0018	0.0023	0.0232
Orange	65	8	12.31	0.0459	0.0497	0.5968
Darkorange	64	8	12.50	0.0422	0.0497	0.5482
Darkslateblue	30	4	13.33	0.0641	0.0641	0.8338

<sup>a</sup>The milk fat gene list (obtained from the literature and prioritization analyses) is detailed in [Supplementary Table S3](#).

genes encoding proteins that are structural constituents of the ribosome (*MRPL27*, *RPL24*, *RPLP0*, *RPL3*, *RPS7*, *RPL13*, and *RPS8*), supporting the hypothesis that *RPL* genes are targets of dietary long-chain polyunsaturated fatty acids in rodents (40). On the contrary, these results contrast with the previously reported stability of *RPLP0* expression in the mammary gland of ruminants (41), which prompted its use as a reference gene in nutritional trials, including the study of mammary lipogenesis in goats under marine lipid-induced MFD conditions (42).

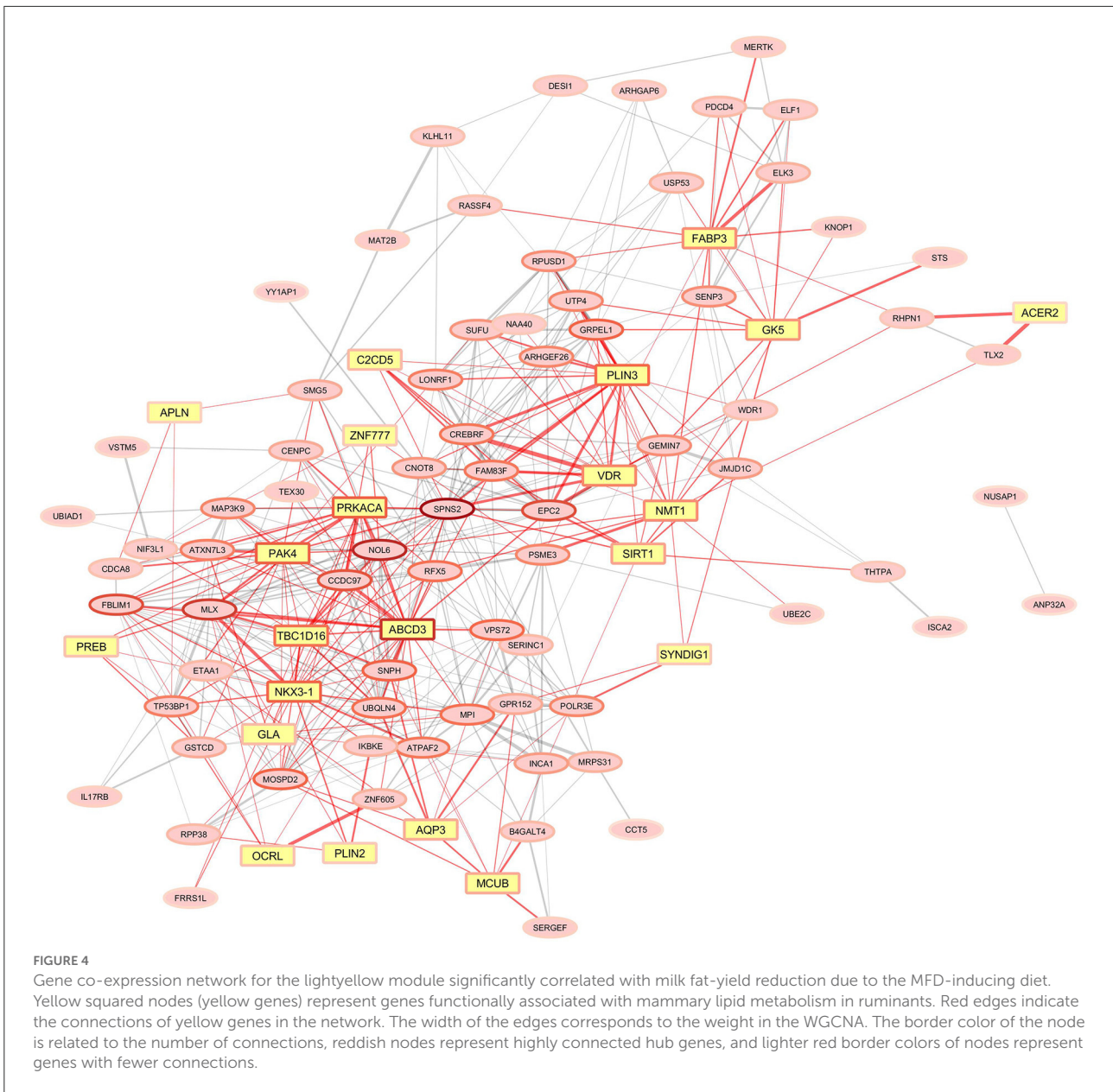
Once the main DEGs due to the MFD-inducing diet were determined, we identified those differentially expressed in RESPO+ and RESPO− goats. Only 18 genes were deemed differentially expressed. The upregulated GO terms clustering genes upregulated in RESPO+ goats were mainly related to the regulation of potassium ion transmembrane transport (*WNK4*, *BINI*, and *CAV1*), specifically, potassium channel inhibitor activity. Supplementation with potassium-based products has been suggested to improve milk fat concentration in dairy ruminants. However, this effect has not been related to a direct effect in the mammary gland, but to decreased production of ruminal *trans*-10 18:1 and *trans*-10, *cis*-12 CLA (43). In the companion study (8), FO supplementation tended to induce a greater increase in milk *trans*-10 18:1 concentration in RESPO+ than RESPO−, but no relationship between milk *trans*-10, *cis*-12 CLA and responsiveness to the MFD-inducing diet was detected.

In RESPO− goats, two upregulated genes (*GPD1* and *DBI*) explained the enrichment in the pathway “Triglyceride biosynthesis,” suggesting that less susceptible goats had higher synthesis of triglycerides in mammary cells. The *GPD1* encodes

for glycerol-3-phosphate dehydrogenase 1, which acts in the reversible conversion of dihydroxyacetone phosphate (DHAP) and reduces nicotinic adenine dinucleotide (NADH) to glycerol-3-phosphate (G3P) and NAD+, having a critical role in carbohydrate and lipid metabolism. A study in dairy cows and goats fed diets supplemented with starch, plant oil, or fish oil showed no differences in the expression of *GPD1* in the mammary gland due to changes in milk fat content in response to diets (44). This agrees with our findings when comparing control vs. MFD. However, the *GPD1* gene was differentially expressed when we compared RESPO+ and RESPO−, suggesting a relevant role of this gene in the individual differences in MFD severity.

Bernard et al. (44) reported a negative correlation between *GPD1* gene expression and the milk content of *cis*-11 16:1, a fatty acid that is present in FO. Although there is no research deciphering the biological meaning of this correlation, milk concentrations of *cis*-11 16:1 seem associated with reduced milk fat content (g/kg) (44–46). However, in the companion paper by Della Badia et al. (8), we observed a higher *cis*-11 16:1 proportion in RESPO− goats ( $P = 0.028$ ), which would not support the negative correlation between *GPD1* gene and this fatty acid. In any event, further research is necessary to elucidate the mechanisms underlying these links and to verify whether they denote causation (e.g., a biological effect of *cis*-11 16:1 on gene expression) or not (i.e., a simple reflection of *cis*-11 16:1 supply with FO).

The other gene upregulated in RESPO− animals and linked to lipid metabolism was *DBI*. This gene, also known as *ACBP*,



encodes for acyl-CoA binding protein, which plays a role in modulating the regulatory functions and utilization of long-chain fatty acyl-CoA, preventing the inhibitory effects of these compounds on enzyme activity (e.g., on mitochondrial acyl-CoA synthetase and acetyl-Coa carboxylase) (47). The *DBI* gene was downregulated in dairy cows and sheep displaying MFD due to the consumption of diets rich in unsaturated fatty acids (11, 35). In this study, *DBI* was not among the differentially expressed genes when comparing control vs. MFD groups, but when evaluating more and less susceptible animals (i.e., RESPO+ vs. RESPO-). Thus, it could be speculated that changes in the expression of *DBI* gene might represent a mechanism that protected mammary cells of RESPO- goats

from excessive accumulation of long-chain fatty acyl-CoA due to FO supplementation (47).

In the WGCNA, most modules significantly correlated with milk fat phenotypes were related to milk fat yield, which would suggest that transcriptomic changes associated to milk fat decrease are subtle in comparison with those observed for milk fat synthesis. Our companion study postulated that some pre-existing variations in some traits, such as milk fat yield, may influence the susceptibility of sheep to dietary components inducing MFD, but this trend was not confirmed in goats (8). The relevance of this trait might explain that none of the modules correlated to milk fat yield was also correlated to variations in milk fat yield in response to diet, the latter variable

having only one significantly correlated module. Regarding milk fat percentage traits, none of the gene modules correlated with milk fat percentage was correlated with milk fat yield. In small ruminants, milk fat yield and milk yield are usually positively correlated, whereas milk fat percentage is negatively correlated with milk yield, which has often been attributed to a dilution effect (48, 49). This fact supports the idea that different gene correlated networks might drive both traits. One of the genes included in the turquoise module, which positively correlated to milk fat yield, was *LALBA*. This gene encodes alpha-lactalbumin, an essential protein for lactose synthesis, a major milk osmolyte influencing milk volume (50, 51), which may account for the relationship between this module and milk fat yield. Although there were also some discrepancies in significant modules and their correlations to different phenotypic traits, nearly all modules were enriched in genes functionally related to milk fat synthesis, which would support their biological relevance in relation to the evaluated traits.

As the main objective of the work was to identify genes and gene networks related to the individual susceptibility of goats to MFD, we paid particular attention to the lightyellow module of co-expressed genes, which was negatively correlated to MFD. Among the 95 genes in this network, 21 functionally-related to milk fat traits were enriched (Figure 4). Within these, *FABP3* has been shown as the intracellular fatty acid transporter with the greatest expression in the lactating mammary gland of cows, together with the previously discussed *DBI/ACBP* (28). Moreover, *FABP3* has been demonstrated to be a target gene of *SREBP1* and *PPARG*, the central regulators of lipid metabolism (52). A study in cows demonstrated that oleic acid increases lipid droplet accumulation in mammary epithelial cells by affecting the expression of *FABP3* (53). In line with this, we detected within the lightyellow gene co-expressed network two members of perilipin family proteins (*PLIN2* and *PLIN3*) related to lipid droplets (54). Specifically, *PLIN2* is involved in mammary lipid droplet formation, stabilization and secretion in goat mammary epithelial cells and regulates triglyceride accumulation (55). Although further research is necessary, these findings suggest that alterations in fatty acid transport might play an important role in inter-animal differences in susceptibility to diet-induced MFD in goats.

## Conclusions

The combination of two approaches, DEA and WGCNA, helped elucidate MSC transcriptomic changes caused during diet-induced MFD in dairy goats. Results supported that MFD in dairy goats is mediated by *SREBF* downregulation and variations in additional transcription factors and chromatin-modifying enzymes. Regarding individual susceptibility to MFD, we uncovered several DEGs between RESPO+ and RESPO- goats (e.g., *DBI* and *GPD1*) and a co-related gene network

linked to the decrease in milk fat. Although all candidate genes potentially related to individual susceptibility to MFD severity should be further investigated, alterations in fatty acid transport seem to play an important role.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-11581?accession=E-MTAB-11581>.

## Ethics statement

The animal study was reviewed and approved by Research Ethics Committees of the Instituto de Ganadería de Montaña, the Spanish National Research Council (CSIC), and the Junta de Castilla y León (Spain).

## Author contributions

GH, PF, and PT designed the study with the cooperation of J-JA. GH, PF, and PT coordinated all tasks involved in animal maintenance, diet design and phenotype recording. PT did RNA extractions. AS-V carried out the bioinformatic analyses. AS-V and BG-G wrote the first draft of the paper with the cooperation of PT, PF, J-JA, and JL. All authors read and approved the content of the paper.

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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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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2022.1037764/full#supplementary-material>



- machine learning methods to multi-tissue transcriptome data. *Front Genet.* (2021) 12:619857. doi: 10.3389/fgene.2021.619857
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