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Original Research Article

Wheat straw and alfalfa hay alone or combined in a high-concentrate diet alters microbial-host interaction in the rumen of lambs

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ABSTRACT

The inclusion of various forages in a normal forage-to-concentrate ratio has widely been reported to reveal the changes that occur in the foregut tissues. However, the mechanism by which the wheat straw, alfalfa hay, or both alter the orchestrated crosstalk of microbiome and host-transcriptome in the rumen of lambs fed a high-concentrate diet is elusive. Sixty-three Hulunbuir lambs were randomly allotted to 3 dietary groups, and each dietary group had 3 pens with 7 lambs. The lambs were fed high-concentrate diets (70%) supplemented with either 30% wheat straw (30S), a mixture of 15% alfalfa hay and 15% wheat straw (30M), or 30% alfalfa hay (30A) over a 2-week adaptation period and a 12-week formal trial. Compared with the 30S and 30A groups, the 30M group had greater ($P < 0.05$) levels of plasma glucagon-like peptide (GLP-2), interleukin-2 (IL-2). Humoral immunity showed a tendency to increase in the 30M group, as evidenced by the greater levels of plasma immunoglobulins (Ig) A and IgG ($P > 0.05$). The 16S rRNA result showed that the abundance of *Lachnospiraceae* (*NK3A20 group* and *unclassified*), *Olsenella*, *Shuttleworthia*, and *Succiniclasticum* were enriched in the 30M group. Meanwhile, the abundances of *Ruminococcaceae NK4A214* and *prevetolla_7* were enriched in 30S and 30A, respectively. The RNA-seq identified 35 shared differentially expressed genes (DEGs) between the "30S vs. 30M" and "30S vs. 30A," enriched in lipid metabolism pathways, including glycerophospholipid and arachidonic acid metabolism. The weighted gene co-expression network analysis results revealed that the expression of genes in the darkred (194 genes) and darkgreen (134 genes) modules showed a strong positive correlation with phenotypic traits and bacterial genera, respectively. The genes in the darkgreen module were involved in carbohydrate, lipid, and amino acid metabolism and showed a wide range of associations with *Prevotella_7*, *Shuttleworthia*, and *Succiniclasticum*, indicating that ruminal microbes might act as a vital driver in the microbiome-host interaction, likely through fermentation of end-products or metabolites. In conclusion, the results indicate that microbiome enrichment in response to feeding wheat straw and alfalfa hay might drive microbiome-host crosstalk to regulate rumen function in lambs fed a high-concentrate diet.

1. Introduction

With the desire to achieve higher production performance and profitability, feeding energy-rich diets or high-concentrate diets for fattening or growing ruminants has become a common trend in the livestock industry. These feeding patterns may cause adverse effects on rumen function, such as lower pH, increased accumulation

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of volatile fatty acids (VFAs), and decreased fiber degradation, leading to a higher risk of various diseases or metabolic disorders (Elmhadi et al., 2022). In this regard, Angus cows fed a 65% concentrate diet showed greater dry matter intakes and growth performance at the cost of health disorders, revealed by a high concentration of rumen and plasma lipopolysaccharide (Chen et al., 2021). Furthermore, feeding high-concentrate diets has also been found to affect the rumen microbial community structure and function, as well as its interaction with the host animal. These shifts in microbial structure or composition in response to feeding a high-concentrate diet might cause a loss of functional capacity at the community level (McCann et al., 2016; Mu et al., 2022). Studies have also found that feeding high-concentrate diets depleted bacterial diversity and reduced functionality of the rumen, as evidenced by changes in the abundance and functional role of various bacterial genera (Khafipour et al., 2009). Such changes in microbial structure and function are also likely to alter host metabolic function.

The rumen microbiota plays a crucial role in regulating host metabolism, and it's estimated to influence over 10% of the host transcriptome (Malmuthuge et al., 2019). This regulation likely occurs through complex interactions between microbial and host metabolism, which evolve throughout different growth stages and in response to dietary supplementation. On top of that, the microbially-driven VFAs, such as butyrate and acetate, have been shown to mediate the biological processes essential for the growth of ruminal epithelium in lambs fed a starter diet (Lin et al., 2019a). This underscores the potential for dietary modifications to shape microbiome-host crosstalk and thereby influence various physiological functions. Furthermore, studies have also shown that goat kids fed a diet containing milk replacer with concentrate and alfalfa hay improved rumen papillae growth. This improvement was linked to changes in the microbiota composition, which affected gene expression involved in both rumen development and VFA production (Chai et al., 2021). Despite these insights, knowledge of microbial-host interaction and its impacts on host metabolism primarily focuses on starter diets or specific developmental stages. The underlying mechanism of how feeding a high-concentrate diet with wheat straw or alfalfa hay alters the microbial-host interaction and host metabolism function at a molecular level is unclear.

The inclusion of various forages as a source of neutral detergent fiber (NDF) in the diets of dairy cows has become trendy as part of promoting production and a healthy gastrointestinal environment. In this sense, feeding equivalent contents of NDF from various forages to dairy cows has shown comparable results in milk yield and digestibility (Eastridge et al., 2017). Likewise, feeding wheat straw and corn stover to dairy cows has more pronounced effects on intake, pH, and energy balance than corn stover and alfalfa hay in diets formulated to provide equivalent levels of undigested NDF (Kahyani et al., 2019). Furthermore, the inclusion of different levels of corn stover as fiber in high- and low-concentrate diets of heifers improved microbial protein synthesis and dry matter intake without affecting body weight (BW) (Lascano and Heinrichs, 2011). These results have indicated the contribution of fiber from various forages in modulating the rumen environment and phenotypic traits. To this end, replacing wheat straw with alfalfa hay in a concentrate-rich diet results in variable levels of potentially digestible and indigestible NDF and protein. Thus, we hypothesized that the differences in nutrient composition in the supplemented forages might have different effects on the phenotypic traits and crosstalk of ruminal microbial-host metabolism to regulate the rumen function in lambs fed a high-concentrate diet. Furthermore, the rumen epithelium is believed to be a unique site for the crosstalk of microbe-host metabolism due to its wide range of contributions to the growth of a given host. Thus, analysis of the host transcriptome and ruminal microbiome might offer deeper insight into the microbe-host interaction in lamb fed a high-concentrate diet with various forages. To achieve this, we identified the gene module that has a strong association with the bacterial taxa using the weighted correlation network analysis (WGCNA) approach.

2. Materials and methods

2.1. Animal ethics statement

The Institute of Subtropical Agriculture's Animal Care and Use Committee reviewed and approved all experimental protocols for this trial (ISA-202020). The lambs involved in the experiment were managed in accordance with these approved protocols. The feeding trial took place in Hulunbuir City, located in the Inner Mongolia Autonomous Region of China.

2.2. Animals and experimental design

Sixty-three fat-tailed male Hulunbuir lambs at 3 months of age with an initial mean BW of 18.55 ± 1.98 kg were allotted to 9 pens in a randomized complete block design based on initial BW. Pens were randomly assigned to 1 of the 3 treatment groups. The experimental diets were prepared with a forage-to-concentrate ratio of 30:70 on a dry matter (DM) basis. The lambs were fed a diet comprising 70% barley and corn-based concentrate supplemented with either 30% wheat straw (30S), a combination of 15% alfalfa hay and 15% wheat straw (30M), or 30% alfalfa hay (30A). The feeding trial was conducted for 14 weeks, including 2 weeks of acclimatization and a 12-week formal trial. The nutrient levels of the experimental diets are presented in Table 1. Lambs received their respective diets thrice daily at 07:30, 14:30, and 20:30 and had free access to water. The BW was taken at 2-week intervals before morning feeding, and average daily gain (ADG) was calculated as the slope of BW against time. Feed offered and refusals per pen were recorded daily to calculate the individual feed intake (IFI) during the formal trial period. The ratio method was implemented to compute the IFI as described elsewhere (Lee et al., 2016).

2.3. Sample collection

On day 90 before morning feeding, blood was drawn from the jugular vein of each lamb into 5-mL heparinized tubes (Changsha Yiqun Medical Equipment Co., Ltd., Hunan, China) to measure the concentrations of cytokines and immunoglobulins. After centrifugation at 3000 × *g* for 15 min at room temperature, the aliquots were collected and frozen at −80 °C until analysis. Seven lambs from each group with a final BW close to the group mean BW were selected, slaughtered by a registered veterinarian, and immediately eviscerated. Following dissection and separation of serosal and muscular layers, the rumen epithelial tissue was individually collected from the bottom side of the central sac, washed with sterile phosphate-buffered saline (pH 7.4), instantly frozen in liquid nitrogen, and stored at −80 °C for transcriptome analysis. Rumen contents (the mixed rumen digesta) were filtered using 4 layers of sterile cheesecloth, and about 10 mL of rumen content was aseptically collected into a sterile plastic tube, snap-frozen in liquid nitrogen, and stored at −80 °C for 16S rRNA sequencing.

2.4. Feed analysis

Experimental feed dry matter (DM, method 930.15), crude protein (CP, N × 6.25; method 984.13), ash (method 942.05), and calcium (Ca) and phosphorus (P) (method 935.13) were assessed

Table 1
Ingredients and chemical composition of diet fed during the experiment (DM basis).

Item	30S	30M	30A
Ingredients, %			
Alfalfa hay	0.00	15.00	30.00
Wheat straw	30.00	15.00	0.00
Corn	30.01	30.01	30.01
Barley	10.87	10.87	10.87
Soybean meal	12.5	12.5	12.5
Wheat bran	8.72	8.72	8.72
Cottonseed meal	3.00	3.00	3.00
Beet molasses	1.00	1.00	1.00
Sodium bicarbonate	0.70	0.70	0.70
Calcium carbonate	0.50	0.50	0.50
Sodium chloride	0.50	0.50	0.50
Magnesium oxide	0.20	0.20	0.20
Premix[1]	2.00	2.00	2.00
Chemical composition, %			
Metabolizable energy[2], MJ/kg	10.04	10.13	10.22
Dry matter	90.12	89.62	89.98
Crude protein	13.92	17.01	20.20
Neutral detergent fiber	35.14	31.78	25.55
Acid detergent fiber	18.26	14.09	7.64
Ether extract[2]	2.32	2.77	3.22
Ash	7.84	8.84	7.35
Calcium	0.32	0.49	0.66
Phosphorus	0.11	0.14	0.17

[1] One kilogram of premix contained the following: 35.4 g $FeSO_4 \bullet 7H_2O$; 16.6 g $ZnSO_4 \bullet H_2O$; 3.3 g $CuSO_4 \bullet 5H_2O$; 11.5 g $MnSO_4 \bullet H_2O$; 104.2 g $MgSO_4 \bullet H_2O$; 9.0 g Na_2SeO_3; 6.5 g KI; 2.8 g $CoCl_2 \bullet 6H_2O$; 15, 000 IU, vitamin A; 2,000 mg vitamin D; 25 mg vitamin E.
[2] Calculated based on Ministry of Agriculture of China recommendations (MOA, 2004) and (Heuzé and LebasV, 2019).

using the standard protocol of the Association of Official Agricultural Chemists (AOAC, 2000). Acid-detergent fiber (ADF) and NDF of the experimental feed were measured following the methods of Van Soest et al. (1991).

2.5. Detection of plasma cytokines and immunoglobulins

The plasma levels of glucagon-like peptide 2 (GLP-2), interleukin-2 (IL-2), transforming growth factor β (TGF-β), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), immunoglobulin A (IgA), IgG, and IgM were detected using the sheep enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's directions (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). The levels of immunoglobulins and cytokines were expressed as ng/mL, μg/mL, or pg/mL. The intra-assay and inter-assay coefficient variation (CV) of ELISA kits used for cytokines and immunoglobulins were <10% and <12%, respectively.

2.6. Genomic DNA extraction and 16S rDNA gene sequencing

The E.Z.N.A. Stool DNA Kit (D4015, Omega Bio-tek, Inc., Norcross, GA, USA) was used to isolate the microbial DNA from the rumen content biopsies following the manufacturer's guidelines. The quantity and purity of isolated DNA were verified using a NanoDrop 2000 UV–vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. The universal primer set 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) along with 12 bp unique barcodes were used to amplify the bacterial 16S rRNA gene in the V3–V4 hypervariable region and to construct an amplicon library (Fadrosh et al., 2014). The PCR reactions were performed as described by Gebeyew et al. (2021). A 2% agarose gel electrophoresis was used to verify the PCR products. The AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) were then used to purify the PCR products. The Qubit 3.0 (Invitrogen, Waltham, MA, USA) was used to quantify the PCR products, which were pooled into 1 sample based on equimolar concentration to construct Illumina paired-end libraries. The quantity and size of the amplicon library were determined using the Illumina Library Quantification Kit (Kapa Biosciences, Woburn, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The libraries were sequenced on the NovaSeq PE250 platform (Illumina Technologies Co., Ltd, San Diego, CA, USA).

2.7. Computational approaches for 16S rRNA sequencing data

The QIIME data analysis package (version 1.9.1) was used to perform the quality control of raw data in FASTQ files as described in our previous study (Yang et al., 2021). Clean reads were then clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff using USEARCH (version 10). The representative read of each OTU was annotated and aligned to the SILVA 16S rRNA database (version 138.1) using the RDP classifier algorithm with a confidence threshold of 80% (Quast et al., 2013). The population richness (Chao1) and evenness (Shannon index) were computed using QIIME2 (available at https://library.qiime2.org/). Principal coordinate analysis (PCoA) based on the weighted UniFrac distance was used to assess beta diversity across the 3 treatment groups.

2.8. RNA extraction, sequencing, and library preparation

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from each rumen epithelium tissue as per the manufacturer's direction. The ND-1000 NanoDrop Spectrometer and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA) were used to quantify the total RNA concentration and purity, respectively. The RNA integrity was checked using agarose-formaldehyde (1%) gel electrophoresis. Samples with an RNA integrity number (RIN) value of >7 were used for RNA-seq library construction. TruSeq RNA Library Prep Kit v2 (Illumina, CA, USA) was used to construct the library for RNA seq and enrich poly(A)-tailed host mRNA with oligo(dT) beads. The BGIseq 500 platform was used to sequence the library at BGI Tech Solutions Co., Ltd. (BGI-Shenzhen, China) and generate single-end 50-base reads as per the manufacturer's directions.

2.9. Quality control analysis and mapping

The Trimmomatic module in SOAPnuke (v1.5.2) was used to filter the raw reads (Li et al., 2008). Raw reads were flagged as low-quality and excluded from the datasets if they: (1) contained an adapter; (2) had greater than 50% of ambiguous sequences labeled as N; (3) had more than 20% of bases with a quality score below 10. High-quality reads were mapped against the *Ovis aries* reference genome (Oar V3.1) using HISAT2 (v2.1.0) (Kim et al., 2015) with the default parameters. The expression levels of host genes were calculated based on the number of fragments per kilobase of exon per million fragments mapped (FPKM).

2.10. Identifying differentially expressed genes

The bioinformatics tool DESeq2 (v3.15) was used to identify the differentially expressed genes (DEGs) between the 3 dietary groups (Love et al., 2014). The DEGs were declared with log2 fold change (logFC) greater than 1 and adjusted *P*-values (using the Benjamini-Hochberg [BH] method for controlling the false discovery rate [FDR]) less than 0.05 (Benjamini and Hochberg, 1995). The DEGs were presented based on the pairwise comparison among the dietary groups (30S vs. 30M, 30S vs. 30A, and 30M vs. 30A).

2.11. WGCNA

We used WGCNA approaches to uncover the interaction of the lamb phenotypic traits (body weight changes [BWC], IFI, cytokines, and immunoglobulins) and metagenomics with the rumen tissue transcriptome (generated from the same samples) (Langfelder and Horvath, 2008). All expressed protein-coding genes (9322, FPKM >1.0) in rumen tissue samples from all experimental lambs were used in the WGCNA (R package v3.4.1). Briefly, the pickSoftThreshold function in the WGCNA package was used to build a gene co-expression network according to the co-expression/correlation modes among genes. We generated 16 (RNA-seq vs. phenotype traits) and 15 (RNA-seq vs. bacterial genera) modules using the blockwiseModules function based on the following parameters: minimum module size of 20. Merged cutoff of 75%, and soft-thresholding powers of 10 and 7, respectively. And the identified clusters of densely interconnected genes (gene modules) were presented using a hierarchical clustering method. We then computed the Pearson correlation coefficients between the targeted traits and eigengenes of each gene module. The gene modules were declared statistically associated with the targeted traits at $P < 0.05$. Thus, we found that the darkred (RNA-seq vs. phenotype traits) and darkgreen (RNA-seq vs. bacterial genera) modules were highly associated with the phenotypic traits and bacterial genera, respectively, and these modules were used for further correlation analysis to identify the degree of association between respective traits and each gene module.

2.12. Association of plasma immune indexes and abundance of bacteria with the gene module

The bacterial genera and genes from the darkgreen module, as well as plasma cytokines and immunoglobulin, and genes from the darkred module — both negatively and positively associated with the respective traits — were subjected to correlation analysis. The analysis was performed using Spearman's rank correlation coefficient, and the results were visualized using a heatmap generated by the R package in the Omics studio (LC-Bio Technology Co., Ltd., Hangzhou, China). The correlation results with the $P < 0.01$ and $|r| \geq 0.6$ were declared as statistically significant or strongly associated.

2.13. The functional analysis

Database for Annotation, Visualization, and Integrated Discovery (DAVID) (v2022q3) was used to perform the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment and Gene Ontology (GO) terms for the DEGs, the darkgreen and darkred modules, which highly correlated with lamb phenotype traits (Nishihara et al., 2023). The KEGG pathways and GO terms were declared as significantly enriched biological functions at $P < 0.05$.

2.14. Statistical analysis

Statistical analyses were applied using the SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) and OriginPro 2023b. Each pen was used as an experimental unit for measuring BW and feed intake. Meanwhile, an individual lamb was considered as an experimental unit for the remaining variables, which were measured for each animal. All data were subjected to the analysis of variance (ANOVA) using the GLM procedure. First, the normality and homoscedasticity of data were verified using Shapiro–Wilk and Levene's tests, respectively. Then, the differences between group means were determined using Tukey's post hoc tests. Given that the relative abundances of bacterial phyla and genera were not normally distributed, we used the Wilcoxon rank-sum test to analyze the differential abundances between the dietary groups. The statistical differences and tendencies were regarded at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively. The mean ± SEM was used to present the results.

The model used in the experiment was:

$$Y_{ijk} = \mu + \tau_i + \beta_j + \varepsilon_{ijk},$$

where Y_{ijk} was the response variable, μ was the grand mean, τ_i was the i^{th} treatment effect, β_j was the j^{th} block effect, and ε_{ijk} was the error term.

3. Results

3.1. Growth performance and immune profiles

As shown in Table 2, IFI and ADG were unaffected by the dietary treatments ($P > 0.05$). The 30M group had greater ($P = 0.001$) IL-2 compared with the 30S and 30A groups (Table 3), highlighting resistance to high-concentrated diet-induced inflammation as IL-2 has anti-inflammatory characteristics. The level of GLP-2 was greater ($P = 0.007$) in the 30M group than that in the 30S group but was comparable with the 30A group ($P > 0.05$). The levels of IgA and IgG tended to be higher in the 30M group than in the other 2 groups ($P > 0.05$), indicating feeding a mixture of alfalfa hay and wheat straw may improve humoral immunity. Levels of IgM showed no significant variation across the 3 groups ($P = 0.218$).

3.2. Ruminal microbiota profiles

The effects of wheat straw and alfalfa hay alone or combined on the profiles of ruminal bacterial taxa were investigated. The results of the rumen content microbiota analysis showed that neither the Chao1 index nor the Shannon index was affected by the dietary treatments ($P > 0.05$, Fig. S1). However, the compositions of ruminal phyla and genera were significantly altered across the 3 dietary treatments (Fig. 1). Notably, lambs in the 30M group had greater ($P < 0.05$) relative abundances of Firmicutes and Actinobacteria and lower ($P < 0.05$) relative abundance of Proteobacteria than those in the other two dietary groups. The 30S group had a greater abundance of Fibrobacteres compared with the 30M ($P = 0.082$) and 30A ($P < 0.05$) groups. At the genera level, lambs in the 30M group had greater ($P < 0.05$) relative abundances of *Eubacterium_coprostanol_genes_group*, *Lachnospiraceae NK3A20 group*, *Lachnospiraceae unclassified*, and *Olsenella* than those in the 30S and 30A groups (Fig. 2). The 30A group had a lower abundance of *Ruminococcaceae NK4A214* group compared with the 30S ($P = 0.055$) and 30M ($P = 0.018$) groups (Fig. 3). Conversely, lambs in the 30A group had ($P = 0.037$) a greater abundance *prevetolla_7* than that in the 30S group but comparable with the 30M group

Table 2
The effects of wheat straw and alfalfa hay alone or combined on growth performance in growing lambs[1].

Item	30S	30M	30A	*P*-value
IBW, kg	19.0 ± 1.02	18.0 ± 0.64	18.5 ± 0.59	0.431
FBW, kg	35.9 ± 1.68	37.0 ± 0.66	35.9 ± 0.67	0.754
BWC, kg	16.8 ± 0.92	19.0 ± 0.82	17.4 ± 0.87	0.254
ADG, kg/d	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.255
IFI, kg/d	1.8 ± 0.16	1.6 ± 0.09	1.9 ± 0.15	0.349

IBW = initial body weight; FBW =, final body weight; BWC = body weight changes; ADG = average daily gain; IFI = individual feed intake.
[1] 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. Values are expressed as means ± SEM.

Table 3
The effects of wheat straw and alfalfa hay alone or combined on the concentrations of plasma cytokines and immunoglobulins in growing lambs[1].

Item	30S	30M	30A	*P*-value
IL-2, pg/mL	503.3 ± 40.14[b]	722.3 ± 24.54[a]	597.0 ± 30.46[b]	0.001
IFN-γ, pg/mL	586.6 ± 30.13	547.1 ± 29.55	548.0 ± 27.83	0.561
TNF-α, pg/mL	133.6 ± 6.00	145.6 ± 7.49	145.5 ± 8.27	0.428
TGF-β, ng/mL	30.0 ± 1.58	28.7 ± 1.54	29.5 ± 1.26	0.826
GLP-2, μg/mL	4.6 ± 0.28[b]	6.1 ± 0.30[a]	5.5 ± 0.24[ab]	0.007
IgA, μg/mL	400.7 ± 9.75	437.4 ± 12.65	418.0 ± 10.38	0.086
IgG, μg/mL	49.6 ± 2.90	58.1 ± 2.58	51.4 ± 1.93	0.065
IgM, μg/mL	1904.4 ± 65.50	2107.8 ± 79.41	1966.2 ± 95.29	0.218

IL-2 = interleukin-2; INF-γ = interferon-γ; TGF-β = transforming growth factor β; GLP-2 = glucagon-like peptide; TNF-α = tumor necrosis factor-α; Ig = immunoglobulin.
[1] 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. Values are expressed as means ± SEM. Means in the same row with different superscript letters are significantly different among groups ($P < 0.05$).

($P > 0.05$). Compared with the 30S group, the 30M group had a higher ($P = 0.028$) abundance of *Shuttleworth*, which was comparable ($P > 0.05$) to that in the 30A group. Lambs in the 30S group had a lower abundance of *Succiniclasticum* compared with the 30M ($P = 0.065$) and 30A ($P = 0.037$) groups.

3.3. Profiling the transcriptome of rumen epithelium

About 16,319 ± 93 genes expressed across all sample tissues across the 3 treatments were profiled using the RNA-seq-based transcriptome approach (Table S1). We observed a higher number of DEGs (FC > 1) when comparing between the 30S vs. 30A and 30S vs. 30M groups and a low number of DEGs between the 30M vs. 30A group (Table S2). Of them, 89 and 257 up-and-downregulated DEGs were identified in the 30A compared with the 30S group, respectively (Fig. 4A). Likewise, about 32 and 84 up-and-downregulated DEGs were detected in the 30M compared with the 30S group, respectively (Table S3). Equal numbers of up-and-down DEGs were observed between the 30M vs. 30A groups (Table S4). As shown in Fig. 4B, 109 and 80 unique DEGs were detected between the 30S vs. 30M and 30S vs. 30A groups, respectively, whereas 35 shared DEGs were also observed between 30S vs. 30M and 30S vs. 30A groups.

To identify the significantly enriched pathways associated with the unique and shared DEGs, we performed functional analysis using KEGG pathway enrichment analysis. The significantly enriched pathways for the unique DEGs between the 30S vs. 30A groups were the "glycolysis/gluconeogenesis (*PGAM2* and *HK2*)," "biosynthesis of amino acids (*PYCR1* and *PGAM2*)," "Fructose and mannose metabolism (*PFKB3*)," and "complement and coagulation cascades (*MASP2* and *C4BPA*)" (Fig. 4C and F). Likewise, several pathways were significantly enriched for the common DEGs between the 30S vs. 30M and 30S vs. 30A, including "Glycerophospholipid metabolism" and "Arachidonic acid metabolism (*PLA2G4D* and *PLA2G4B*)" (Fig. 4D). The *PLA2G4D* and *PLA2G4B* genes were down-regulated by the supplementation of wheat straw and alfalfa hay mixture or alfalfa hay alone compared to the wheat straw alone (Fig. 4G). The significantly enriched pathways

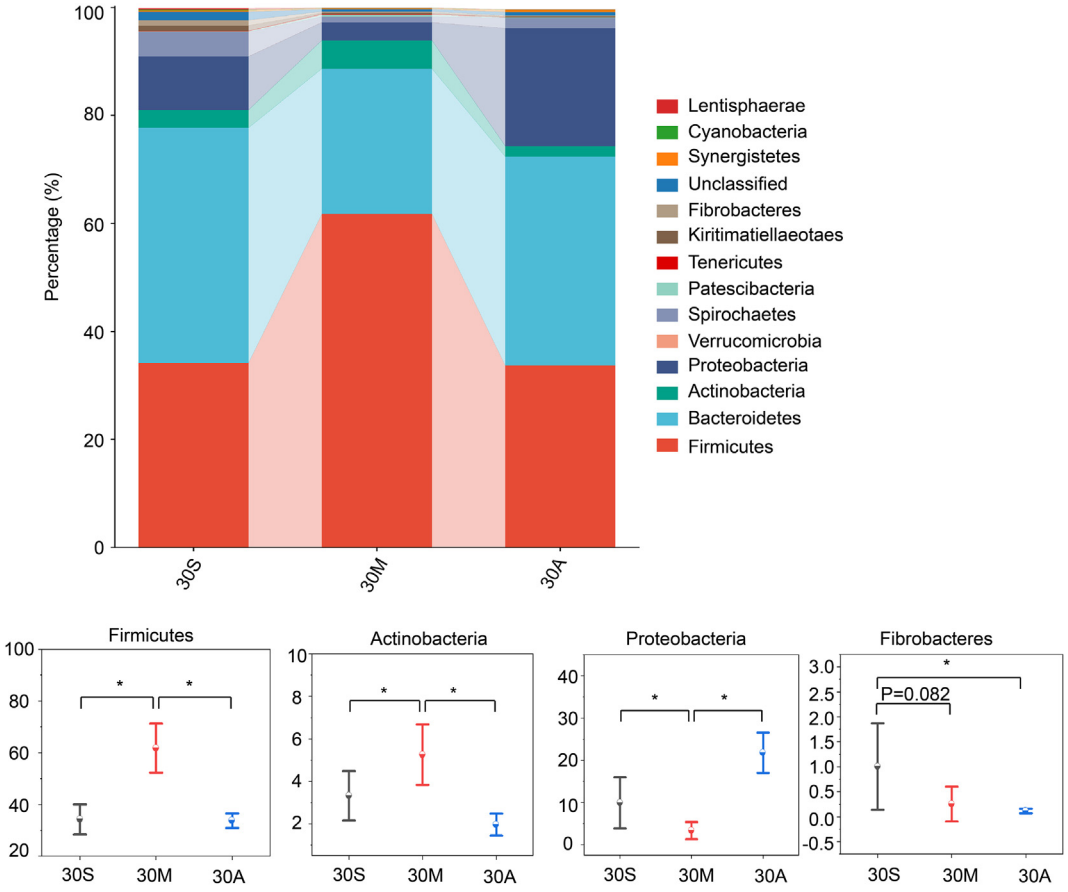


Fig. 1. The relative abundances of bacterial phyla. The effects of wheat straw and alfalfa hay alone or in combination on the relative abundance of bacterial phyla in the rumen content of growing lambs (A). The comparison of the relative abundances of bacterial phyla among the 3 groups was analyzed using the Kruskal–Wallis rank-sum test (B). 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. Values are expressed as means ± SEM, indicated by vertical bars. *Significantly different means ($P < 0.05$).

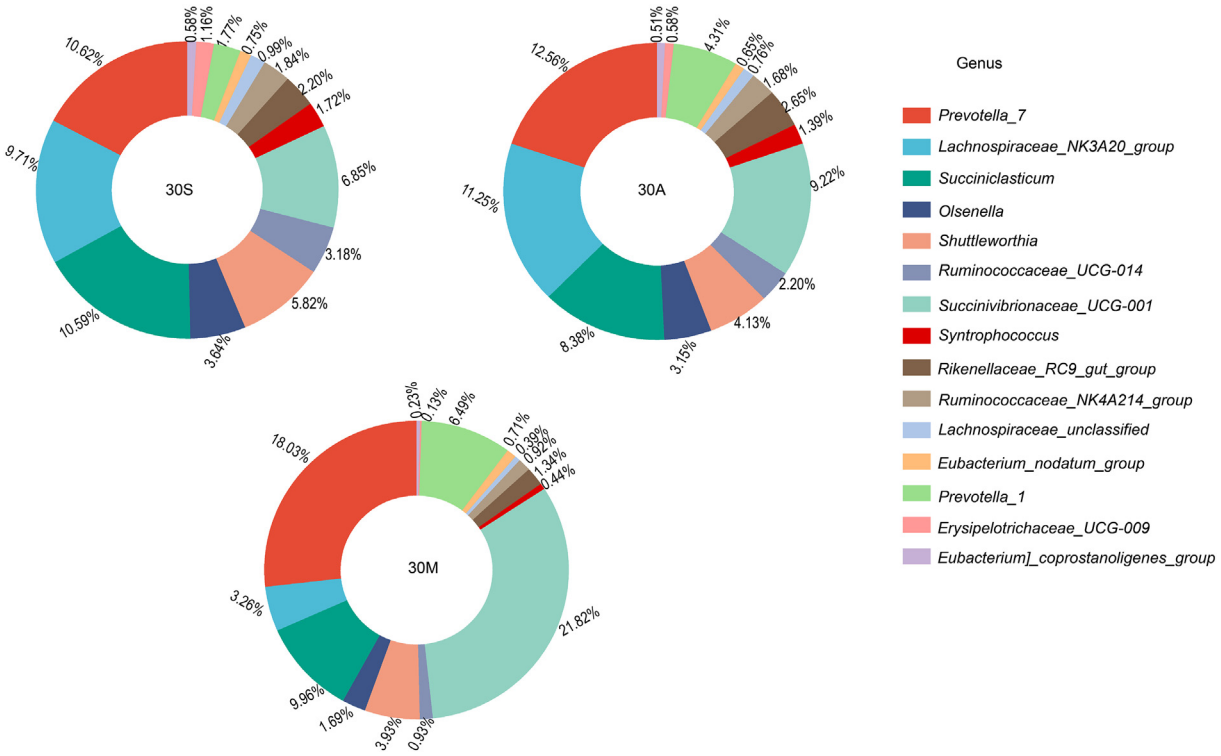


Fig. 2. The effects of wheat straw and alfalfa hay, alone or combined, on the relative abundance of bacterial genera in the rumen content of growing lambs. 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay.

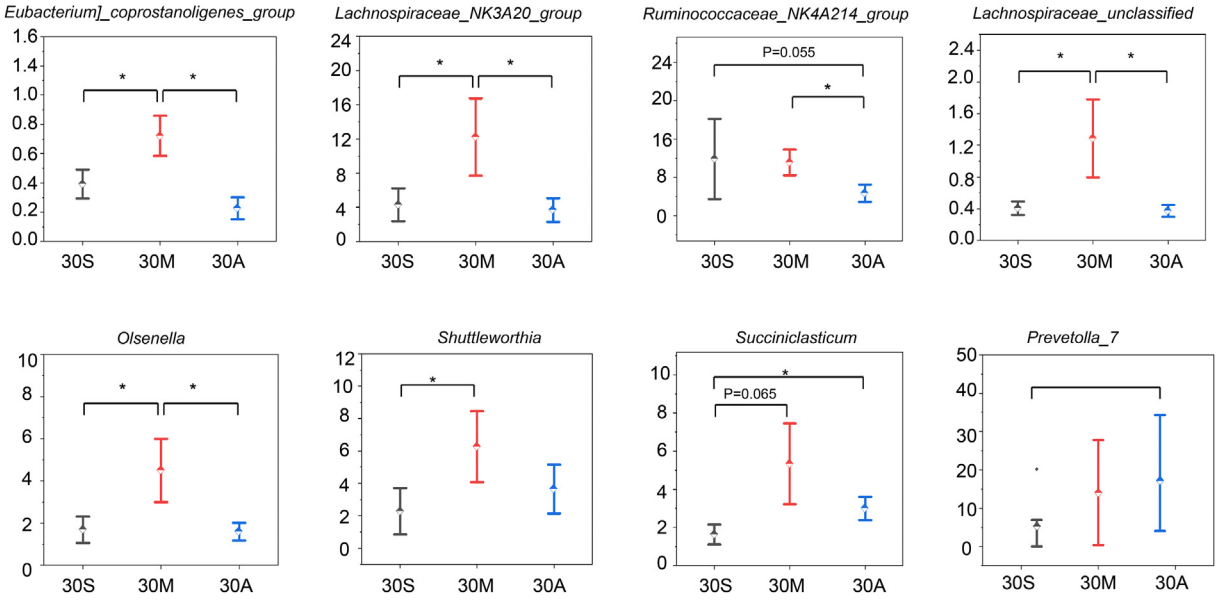


Fig. 3. Comparisons of the relative abundances of bacterial genera among the 3 groups were analyzed using the Kruskal–Wallis rank-sum test. 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. Values are expressed as means ± SEM, indicated by vertical bars. *Significantly different means ($P < 0.05$).

for unique DEGs between the 30S vs. 30M groups were "Biosynthesis of unsaturated fatty acids (*ACOT7* and *ELOVL4*)," "Mineral absorption (*SLC9A3* and *SLC26A9*)," NOD-like receptor signaling pathway (*P2RX7*, *RNASEL*, and *TRPM2*)", and "NF-kappa B signaling pathway (*CARD14* and *LBP*)" (Fig. 4E–H).

3.4. The WGCNA revealed the host transcriptome interaction with the rumen microbiome and plasma immune indexes

To gain a better insight into the interaction between the host transcriptome and phenotypic traits and microbiome, we identified

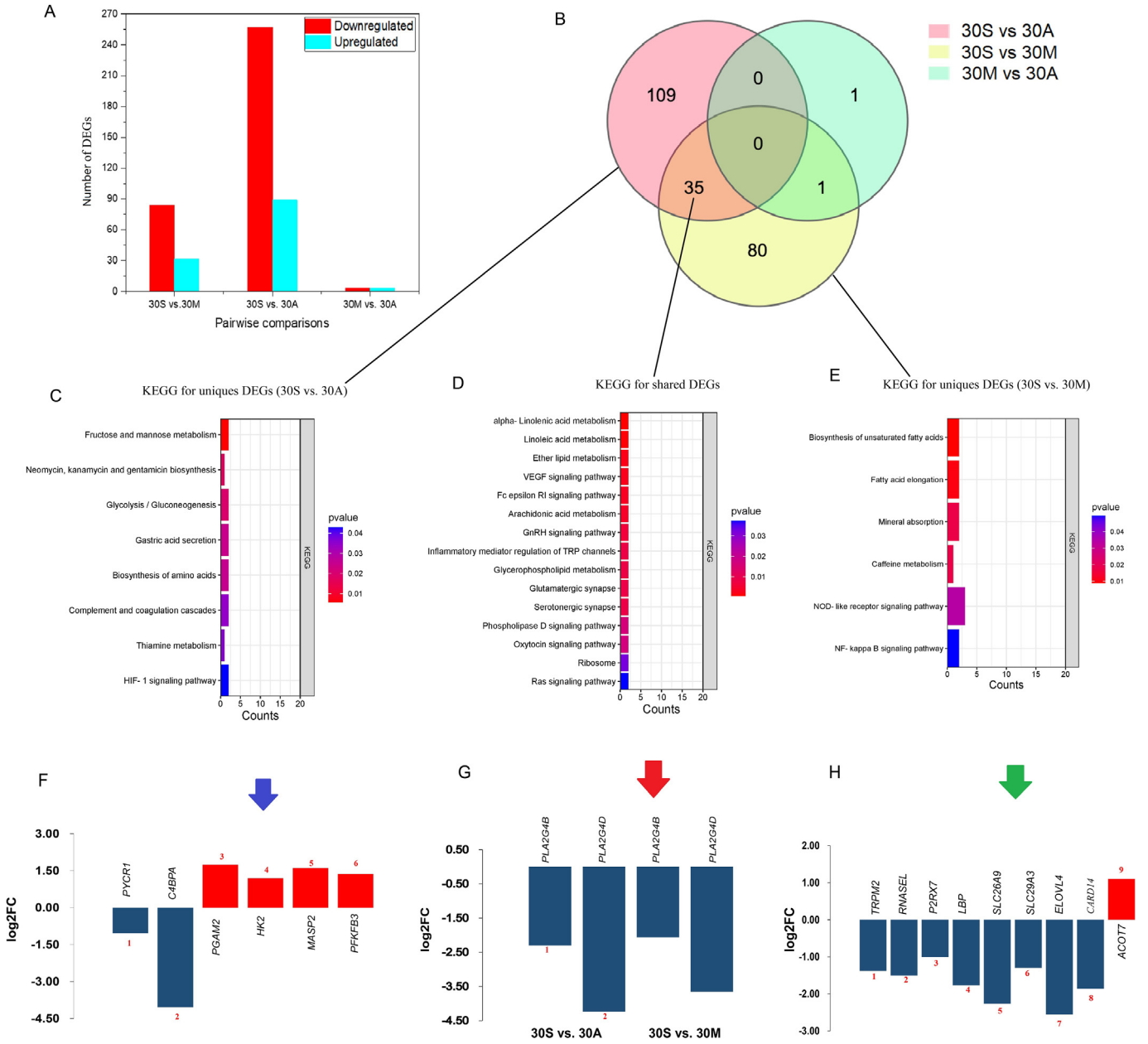


Fig. 4. Transcriptome profile of rumen epithelium. The number of differentially expressed genes between each pairwise comparison (A). The number of unique and shared differentially expressed genes (DEGs) identified in the rumen tissue among 3 groups (B). Significantly enriched pathways for the unique and shared DEGs among the groups (C to E). Gene expression levels in significantly enriched pathways for the unique and shared DEGs (F to H). 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. Values are expressed as means ± SEM, indicated by vertical bars. *Significantly different means ($P < 0.05$). The pathways with a $P < 0.05$ were considered significant, and the top significantly enriched pathways are presented.

about 9,322 genes as core transcriptome, using criteria that included genes expressed in all sample tissues and FPKM >1 across the 3 groups (Table S5). These genes were subjected to the WGCNA approach. The results highlighted that a total of 16 and 15 modules showed various degrees of association with the lamb's phenotypic traits (BWC, IFI, and plasma immune indexes) and the abundance of bacterial genera, respectively (Fig. 5A and B; Figs. S2 and 3). The expression of genes in the darkred (194 genes) and darkgreen (134 genes) modules showed a strong positive correlation with the respective traits (Tables S6–8). About 9 genes in the darkgreen and darkred modules overlapped with the DEGs, including *SRI*, *ISYNA1*, *S100A14*, *ID1*, *PYCR1*, *ACOT8*, *ACOT7*, *WDR34*, and *TPT1*. The functional analysis for the selected modules was performed using KEGG pathway enrichment analysis. The genes in the darkred module were enriched with biological functions, including "cell cycle," "aminoacyl-TRNA biosynthesis," "intestinal immune network for IgA production," "adipocytokine signaling pathway," and "AMPK signaling pathway" (Fig. 6A). The genes in the darkgreen module were related to amino acid, carbohydrate, and lipid metabolism, including "biosynthesis of amino acids," "amino sugar and nucleotide sugar metabolism," and "biosynthesis of unsaturated fatty acids," and "cysteine and methionine metabolism" (Fig. 6B). These major genes, associated with various pathways, showed different expression levels across the 3 dietary treatment groups (Fig. 7).

To further identify the degree of association of each gene from the darkred and darkgreen modules with the immune indexes and

bacterial taxa, we performed Spearman's rank correlations of the gene from the selected modules with plasma cytokines and immunoglobulins or bacterial genera. The plasma level of IgA had strong correlations ($P < 0.01$) with *SRA1*, *RBM8A*, and *RUVBL2* (Fig. S4). Likewise, the plasma level of GLP-2 showed a significant correlation ($P < 0.01$) with *CCT7*, *OAZ1*, *LAMTOR2*, *MLF2*, *TMEM223*, *GALT*, *CDKZAP1*. The abundance of *Prevotella_7* had positive correlations ($P < 0.01$) with 48 genes, including *GOT2*, *TECR*, *MPI*, and *CBS* (Fig. S5). Likewise, the abundance of *Shuttleworthia* showed positive correlations ($P < 0.01$) with 48 genes, including *PYCR1* and *GMPPB*. The abundance of *Succiniclasticum* had a positive association ($P < 0.01$) with 19 genes, including *MIF* and *ACOT8*. The abundance of *Olsenella* showed a positive association with *CIAPIN1*. The abundance of *Prevotella_1* had negative correlations ($P < 0.01$) with *NTMT1* and *GPS1*. The abundance of *Rikenellaceae_RC9_gut_group* showed negative correlations ($P < 0.01$) with *MLF2*, *PGP*, and *PIH1D1* and a positive association with *SMYD2*. The abundance of the *Ruminococcacee NK4A214_ group* had a negative correlation ($P < 0.01$) with *TRABD* and a positive correlation ($P < 0.01$) with *SMYD2*.

4. Discussion

It is well documented that wheat straw has low protein and starch levels but a high NDF with a slow digestion rate. In contrast, alfalfa hay is characterized by a high proportion of protein, moderate starch levels, and digestible NDF with a high digestion rate (Kahyani et al., 2019). Thus, the experimental diets were planned to provide different levels of nutrients from wheat straw and alfalfa hay alone or combined in a concentrate-rich diet. These differences in nutrient compositions were supposed to modulate the rumen, thereby affecting phenotypic traits and molecular features, including growth performance and host-microbial consortia. However, a partial or full replacement of alfalfa hay for wheat straw in a high-concentrate diet did not significantly improve the growth performance and feed intake of lambs. The findings from the present study are in close agreement with the results of a previous study (Lascano and Heinrichs, 2011). In the cited study, both ADG and feed intake failed to show improvement in response to the inclusion of various levels of corn stover as a fiber source in concentrate-rich diets (forage-to-concentrate ratio of 20:80) in heifers. The results of our study can be explained by the small gap in fiber and starch compositions between wheat straw and alfalfa hay. Alternatively, the high proportion of concentrate in the total ration potentially limited the influence of forages on the phenotype traits. Feeding alfalfa hay as a replacement for wheat straw for an extended period is expected to result in noticeable differences in phenotypic traits. Despite a lack of substantial improvement in phenotypic traits, feeding alfalfa hay — either as a partial or full replacement for wheat straw — positively affects the immune profile and enriches beneficial bacteria.

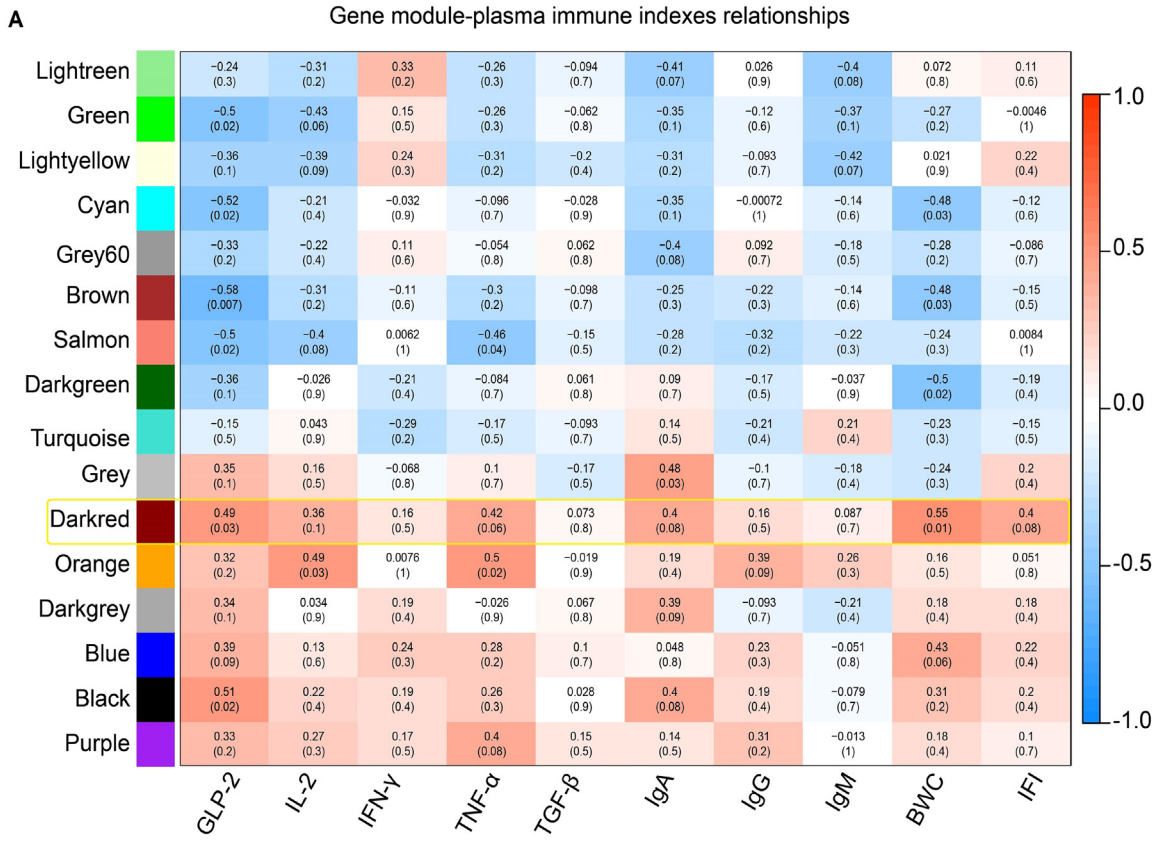
4.1. Wheat straw and alfalfa hay mixture positively affect the plasma immune profile

Oral administration of 0.75 g of mannan-oligosaccharide to calves and inclusion of 3% of fermented wheat bran polysaccharides in the diet of lambs has been shown to improve the circulating IgA and IgM levels, respectively (Lazarevic et al., 2010; Wang et al., 2020), suggesting that an active component of carbohydrates can enhance humoral immunity. In this study, the increases in plasma IgA and IgG levels in response to feeding wheat straw and alfalfa hay mixture were likely due to the differences in starch and fiber components in the supplemented forages. A greater level of plasma IL-2, which has been shown to promote immunoglobulins production from the B cells (Collins and Oldham, 1993), was consistent with the higher concentration of plasma immunoglobulins of lambs from the 30M group. It can also highlight the anti-inflammatory property of IL-2 (Bachmann et al., 2007), as reported in the previous study that showed that ewes fed basal diets with rumen-protected methionine and lysine have greater expression of IL-2 in the neutrophils (Tsiplakou et al., 2018). The observed resistance to inflammation induced by a high-concentrate diet, indicated by the higher IL-2 expression and improved humoral immunity in lambs fed the two forages, suggests that incorporating wheat straw and alfalfa into a concentrate-rich diet could enhance the immune profile without adverse effects. In alignment with the observed effects on cytokines and immunoglobulins, lambs fed a diet containing wheat straw and alfalfa hay mixture showed a higher level of plasma GLP-2 than those in the other two groups. Previous research has indicated that GLP-2, stimulated by a high-energy diet, contributes to the maintenance of intestinal barrier integrity and exhibits anti-inflammatory characteristics (Sun et al., 2018). The enhancement of circulatory GLP-2 in the 30M group could be associated with the specific composition of the diet, particularly its starch, lipid, and fiber content. Overall, lambs in the 30M group had better systemic immune status than those in the other two groups. This improvement in cytokine, immunoglobulins, and GLP-2 in the 30M group was matched with the enrichment of relative abundances of bacterial genera, including the *Lachnospiraceae, Olsenella,* and *Eubacterium_coprostanol_genes group*. The latter two genera have been shown to contribute to the immune system function (Mager et al., 2020) and lower the risk of metabolic disease by reducing the level of cholesterol, respectively (Li et al., 1998).

4.2. Wheat straw and alfalfa hay mixture enrich the prevalence of fiber and starch-degraded bacteria

The microbiota analysis showed that the dominant bacterial phyla, including Firmicutes, Actinobacteria, Proteobacteria, and Fibrobacteres, were shifted across the 3 dietary groups, indicating the specific preferences of bacterial phyla in the degradation of dietary fiber and fermentable substrate released from various plant polysaccharides present in the supplemented forages, which is in line with the previous report (Pandit et al., 2018). In this regard, *Prevotella* has the ability to utilize a wide range of substrates, such as polysaccharides and proteins (Betancur-Murillo et al., 2022). The enrichment of *Prevotella_7* in the 30A and 30M groups can be associated with the content of xylan, protein, starch, and pectin in the alfalfa hay. Given the role of *Lachnospiraceae* in maintaining the intestinal barrier and suppressing gut inflammation via modulating the production of butyrate (Meehan and Beiko, 2014), the greater abundance of the *Lachnospiraceae* (*NK3A20_group* and *unclassified*) in the 30M group of the present study is correlated with the observed improvements in immune status, as evidenced by the slight increases in IgA, IgG, and IL-2 levels. Likewise, considering the roles of *Eubacterium_coprostanol-genes and Olsenella* in reducing cholesterol levels and improving the immune system, the enrichments of these two bacterial genera in the rumen of lambs from the 30M group are further attributed to the alteration of immune profiles.

Although the exact role of the *Ruminococcaceae_NK4A214_group* is not clarified so far, the majority of bacterial genera belonging to the family Ruminococcaceae have been associated with lipid metabolism through modulating rumen biohydrogenation of long-chain fatty acids (Wang et al., 2019) and degradation of fiber and plant cell-wall polysaccharides (Zhang et al., 2022). We thus speculated that a greater abundance of *Ruminococcaceae_NK4A214_group* in the 30S group might alter the lipid


A
Gene module-plasma immune indexes relationships
Lightreen
Green
Lightyellow
Cyan
Grey60
Brown
Salmon
Darkgreen
Turquoise
Grey
Darkred
Orange
Darkgrey
Blue
Black
Purple
GLP-2
IL-2
IFN-γ
TNF-α
TGF-β
IgA
IgG
IgM
BWC
IFI
1.0
0.5
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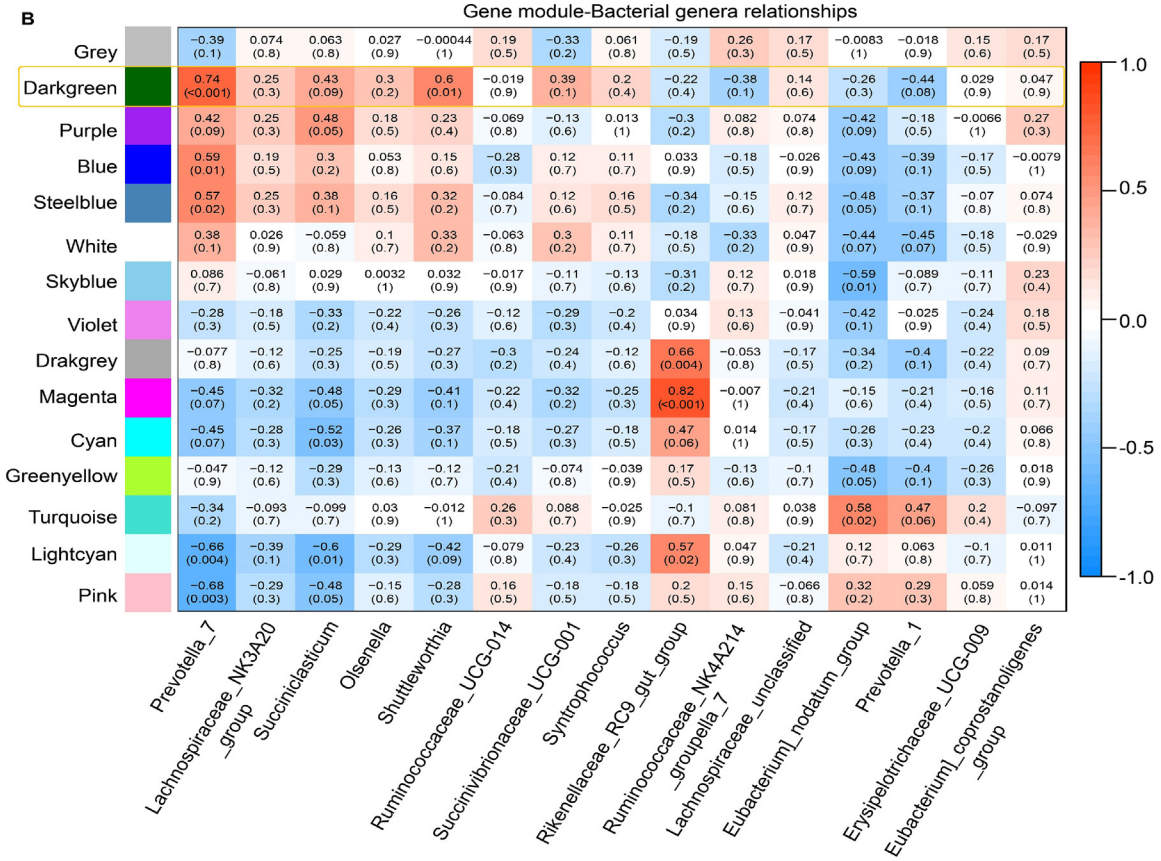
-0.5
-1.0


B
Gene module-Bacterial genera relationships
Grey
Darkgreen
Purple
Blue
Steelblue
White
Skyblue
Violet
Drakgrey
Magenta
Cyan
Greenyellow
Turquoise
Lightcyan
Pink
Prevotella_7
Lachnospiraceae_NK3A20_group
Succiniclasticum
Olsenella
Shuttleworthia
Ruminococcaceae_UCG-014
Succinivibrionaceae_UCG-001
Syntrophococcus
Rikenellaceae_RC9_gut_group
Ruminococcaceae_NK4A214_group
Prevotella_7
Lachnospiraceae_unclassified
Eubacterium]_nodatum_group
Prevotella_1
Erysipelotrichaceae_UCG-009
Eubacterium]_coprostanoligenes_group
1.0
0.5
0.0
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-1.0

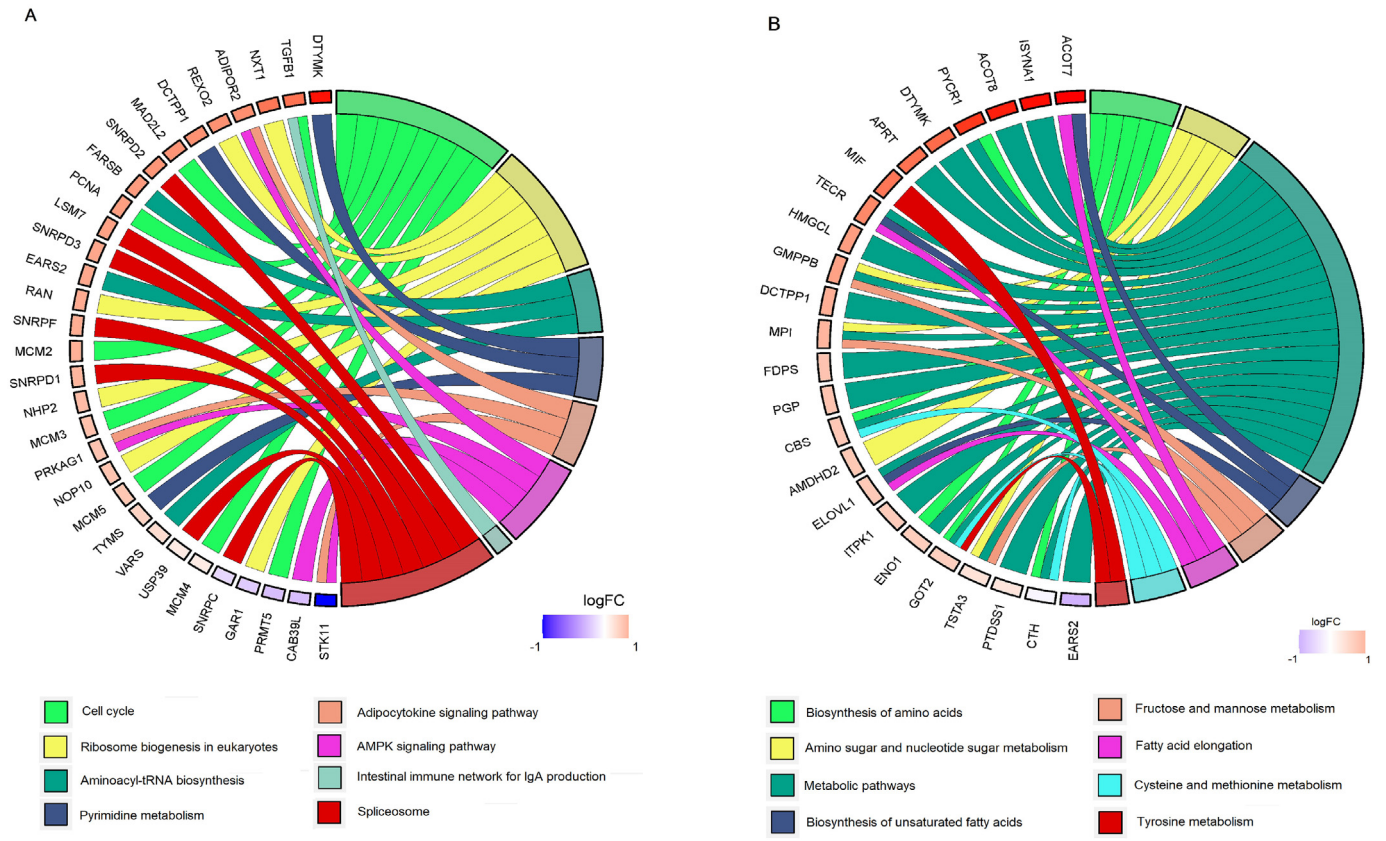

Fig. 6. Enriched pathways in the darkred (A) and darkgreen (B) modules, as identified by Database for Annotation, Visualization, and Integrated Discovery (DAVID). The pathways with a $P < 0.05$ were considered significant.

metabolism or fatty acid profiles. *Succiniclasticum* has a crucial role in the synthesis of ruminal propionate, a main precursor to gluconeogenesis, from succinate via the degradation of dietary fiber (van Gylswyk, 1995). Thus, the higher prevalence of *Succiniclasticum* in the rumen of lambs from the 30M group might improve the production of propionate. Considering the attribution of *Shuttleworthia* in the butyrate production (Zhou et al., 2021), the greater abundance of this bacterium in the 30M group could enhance the synthesis of butyrate, which has a crucial role in microbial and host epithelial cell growth. Overall, the enrichments of *Prevotella_7, Shuttleworthia,* and *Lachnospiraceae* (*NK3A20_group* and *unclassified*) *are* likely to alter rumen fermentation, thereby enriching the concentrations of butyrate, propionate, and the total VFAs, which is in line with the previous study (Mu et al., 2022).

4.3. The responses of various biological pathways to wheat straw, alfalfa hay, or both

To obtain clearer insights into how biological pathways in the rumen tissues of lambs respond to a high-concentrate diet supplemented with wheat straw, alfalfa hay, or both, we performed functional analysis using KEGG pathway enrichment analysis for the unique and shared DEGs. The results showed that most enriched biological pathways were associated with carbohydrate, lipid, amino acid, and immune metabolism. The *PLA2G4D* and *PLA2G4B* genes have been shown to be involved in various metabolic functions, including the regulation of prostaglandin biosynthesis, cellular inflammation, glycerophospholipid hydrolysis, gut membrane permeability, and oxidative stress (Tithof et al., 2007). These two shared DEGs decreased with the supplementation of wheat straw and alfalfa hay mixture or alfalfa hay alone, compared to the wheat straw alone, which agrees with the findings of Sun et al. (2021), who showed that a decrease in the expression of *PLA2G4D* and *PLA2G4B* occurred with the introduction of alfalfa hay. The complement system plays a vital role in innate immunity, serving as the first line of defense against pathogens and infection, and *MASP2* and *C4BPA* have been reported to be involved in complement activation via the lectin pathway (García-Laorden et al., 2020). These two unique DEGs (30S vs. 30A) exhibited differing expression patterns, likely contributing to the balance of immune function in rumen tissue. Additionally, *C4BPA* may perform multiple functions beyond immune function in the bovine rumen, such as lipid metabolism (Iqbal et al., 2021). Supplementing alfalfa hay may improve the fatty acid and amino acid profiles, as evidenced by the greater expression of *PYCR1* and *C4BPA* genes, which are involved in the biosynthesis of amino acids and lipid metabolism.

Fig. 5. Association of the host transcriptome with phenotypic traits using the WGCNA approach. The correlation between the host transcriptome and plasma immune indexes (A); The correlation between the host transcriptome and bacterial genera (B). Each module name appears on the left side, each trait at the bottom, and the strength of the correlation is represented by a bar on the right in varying intensities of orange (indicating positive correlation, $r > 0$) or blue (indicating negative correlation, $r < 0$). The numerical values within each square represent the Pearson correlation coefficient (upper value) and *P*-value (lower value). WGCNA = weighted correlation network analysis; BWC = body weight changes; ADG = average daily gain; IFI = individual feed intake; GLP-2 = glucagon-like peptide 2; IL-2 = interleukin-2; TGF-β = transforming growth factor β; TNF-α = tumor necrosis factor-α; INF-γ = interferon-γ; Ig = immunoglobulin.

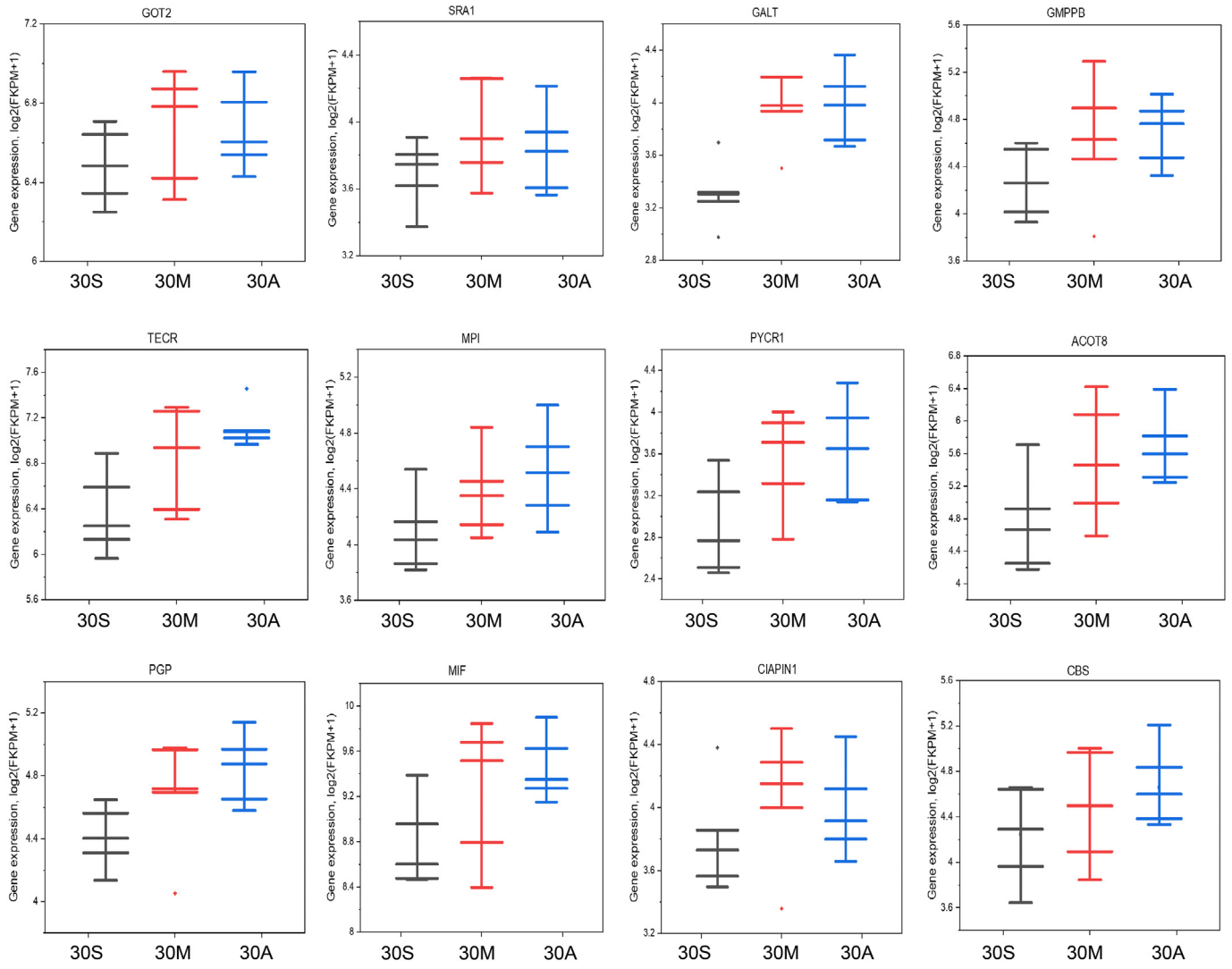

Fig. 7. The expression (log_2(FPKM +1)) of major genes associated with phenotypic traits and bacterial genera. 30S: 30% wheat straw; 30M: 15% alfalfa hay and 15% wheat straw mixture; 30A: 30% alfalfa hay. FPKM = fragments per kilobase of exon per million fragments mapped.

Given the roles of *PGAM2* and *HK2* in glycolysis and their facilitation of the connection between glycolysis and physiological homeostasis through various mechanisms, the upregulation of these two unique DEGs (30S vs. 30A) might indicate that glycolysis is more profoundly influenced by the wheat straw than alfalfa hay. *SLC26A9* has been implicated in regulating cellular pH and interacting with VFAs (Sun et al., 2021). *SLC9A3* plays a crucial role in conveying protons into the rumen and sodium into the cell, thereby keeping the homeostasis of the ruminal epithelium (Beckett et al., 2021). The upregulation of *SLC26A9* and *SLC9A3* in lambs fed with wheat straw and alfalfa hay might be associated with the concentrations of VFAs, which can contribute to intraepithelial homeostasis, as indicated by the greater abundance of bacterial genera involved in VFA production. Thus, the rumen maintains homeostasis by enhancing the mechanism of discharging protons into the ruminal lumen and regulating pH balance. The upregulation of *ACOT7* in response to supplementing a high-concentrate diet with wheat straw, rather than a mixture of wheat straw and alfalfa hay, is partly consistent with the findings of Sun et al. (2021). Considering the roles of *CARD14* and *LPB* genes in activating inflammatory transcription factors and the innate immune response, respectively, decreased expression of both *CARD14* and *LPB* indicates that the lambs in the 30S group did not experience inflammation. In general, feeding either alfalfa hay or wheat straw alone likely improves various individual amino and fatty acid profiles, whereas a mixture of wheat straw and alfalfa hay enhances immune profiles and fermentation end-products, indicating the unique effects of wheat straw, alfalfa hay, or both on the molecular processes of the rumen.

4.4. The WGCNA uncovers the coordinated response of microbes and host transcriptomes in the rumen

The rumen tissue profoundly influences numerous metabolic functions, including lipid and amino acid metabolism, neuroendocrine regulation, and immune homeostasis. These metabolic functions are supposed to be regulated by the cross-talk between rumen tissue and microbial population and may also be altered in response to various feeding patterns. Therefore, various metabolic pathways linked with lipid and amino acid metabolism and immune homeostasis are anticipated to be annotated in response to feeding a high-concentrate diet supplemented with wheat straw, alfalfa hay, or both. In this regard, a previous study has shown that the rumen microbiota could regulate more than 10% of the host transcriptome (Malmuthuge et al., 2019). Given the enrichment of

metabolic pathways related to glycolysis/gluconeogenesis and innate immunity in the rumen among genes uniquely expressed in lambs fed a high-concentrate diet supplemented with either wheat straw, alfalfa hay, or a combination of both, the gene modules showing a strong association with the microbial taxa and immune indexes may offer deeper insights into the interaction between the host transcriptome and microbiota in regulating the rumen metabolism. Thus, we assumed that genes involved in lipid and amino acid metabolism of rumen tissue, along with microbial taxa in the rumen content, may coordinate to regulate rumen function in response to feeding a high-concentrate diet supplemented with various forages.

Based on the WGCNA analysis, we found genes involved in the lipid, carbohydrate, and amino acid metabolism enriched in the darkgreen module, which shows both positive and negative correlations with various bacterial genera. The saccharolytic bacteria *Prevotella_7* and *Shuttleworthia* showed a wide range of interaction with various genes from the darkgreen module, indicating these two bacterial genera might have a profound contribution to the host-microbial interaction under the present feeding conditions. Specifically, the *TECR, MPI, GOT2*, and *CBS* genes had a positive correlation with *Prevotella_7,* which has a vital role in the degradation of starch, protein, and xylan. The *TECR* gene is believed to catalyze the reduction of trans-2,3-enoyl-CoA to saturated acyl-CoA, and *CBS* is involved in redox homeostasis and mitochondrial bioenergetics, regulating homocysteine metabolism and biosynthesis of hydrogen sulfide (Zhu et al., 2018). *GOT2* takes part in the malate-aspartate shuttle, transferring electrons produced during glycolysis into the mitochondria for terminal oxidation or ATP generation. *MPI* is involved in glycosylation reactions by sustaining the supply of D-mannose derivatives (Shtraizent et al., 2017). These highlight that the substrate derived from the degradation of starch, protein, and xylan might contribute to homocysteine metabolism, fatty acid elongation, and glycosylation reactions via *TECR, MPI, GOT2*, and *CBS* genes.

PYCR1 plays a crucial role in the NAD(P)H-dependent synthesis of proline from pyrroline-5-carboxylate in a NAD(P)H-dependent. *GMPPB* facilitates the synthesis of the GDP-mannose pyrophosphorylase enzyme, essential for glycosylation pathways (Liu et al., 2021). *PYCR1* and *GMPPB* showed positive correlations with *Shuttleworthia,* which belongs to the Ruminococcaceae family. Considering the role of *Shuttleworthia* in short-chain fatty acid (SCFA) production and its family composed of members with a high content of odd-chain iso-fatty acid (OCIFA), *Shuttleworthia* is likely involved in fructose and mannose metabolism and redox metabolism by influencing proline and fatty acid synthesis, which has been shown to be involved in buffering cellular redox status. *ACOT8* is proposed to be involved in fatty acid oxidation and controlling peroxisomal lipid metabolism (Hunt et al., 2012). *MIF* has been shown to participate in inflammatory responses and maintain the gut barrier function (Sumaiya et al., 2022). *MIF* and *ACOT8* had a positive association with the *Succiniclasticum,* which is believed to promote butyrate production. Butyrate has a crucial role in innate immunity, gut barrier function, and lipid metabolism. Thus, an interaction of *Succiniclasticum* with *MIF* and *ACOT8*, mediated by butyrate, is likely, which can significantly impact fatty acid metabolism and innate immunity. These results indicate the coordinated interactions between the host transcriptome and microbes in a broad spectrum of metabolic pathways, regulating the rumen function under the present feeding condition.

We also speculated that genes involved in immune function and metabolism might respond to significantly altered plasma immune indexes. The WGCNA analysis of the host transcriptome and plasma immune indexes indicated that aminoacyl-tRNA biosynthesis, the adipocytokine signaling pathway, intestinal immune network for IgA production, and AMPK signaling pathway were enriched in the darkred module, which shows positive and negative correlation with plasma immune indexes. Particularly, IgA showed a positive association with *SRA1, RBM8A*, and *RUVBL2*. The *Ruvbl2* gene is reported as a prerequisite for T-dependent antibody responses as well as the development of T-cells (Arnold et al., 2012), and the expression of *SRA1* is linked with obesity and inflammation (Kochumon et al., 2021). *RBM8A* plays a vital role in synthesizing protein (RNA-binding motif protein 8A), which participates in various cellular functions, potentially including the IgA production process. These might highlight that host genes linked to humoral and innate immunity could positively respond to the plasma immune indexes of lambs fed a high-concentrate diet with wheat straw and alfalfa hay mixture. Furthermore, GLP-2 is involved in enhancing gut barrier function, increasing epithelial proliferation, and inhibiting apoptosis. It also shows a positive association with several genes, including *OAZ1* and *LAMTOR2*. *OAZ1* has a vital role in inhibiting the synthesis of inflammatory cytokines and regulating the synthesis of polyamine, which is involved in gut barrier function (Kang et al., 2017). Meanwhile, *LAMTOR2* is believed to participate in immune function by controlling endosomal biogenesis and activating ERK (Lin et al., 2019b). Taken together, the immune status of lambs fed a high-concentrate diet could be altered by supplementing wheat straw and alfalfa hay mixture, as evidenced by the improved plasma immune indexes and their positive association with the host genes involved in immune metabolism. These alterations stem from differences in the nutrient composition of wheat straw and alfalfa hay.

5. Conclusion

Feeding a high-concentrate diet with wheat straw alone is more likely to promote fatty acid and amino acid metabolism in rumen epithelial tissue. Combined supplementation of wheat straw and alfalfa hay enriches the humoral immunity and the prevalence of saccharolytic bacteria, such as *Prevotella_7* and *Shuttleworthia,* compared to wheat straw alone. This is likely due to differences in nutrient compositions, mainly substrates derived from the degradation of polysaccharides, proteins, xylan, and some other fibers. The inclusion of alfalfa hay in a high-concentrate diet alters the fatty acid synthesis metabolism and abundance of *Prevotella_7*. *Shuttleworthia, Prevotella_7,* and *Succiniclasticum* have shown a wide range of interactions with the host genes involved in amino acid and fatty acid metabolism and immune function. This indicates that ruminal microbes may act as a vital driver in the microbiome-host interaction, likely by altering fermentation end-products or metabolites. These findings shed light on the possible cross-talk between microbes and host transcriptome to regulate the rumen function of lambs fed a high-concentrate diet supplemented with forages. Future studies should evaluate the long-term effects of feeding a concentrate-rich diet, formulated to supply equivalent NDF and protein, with alfalfa hay replacing wheat straw, on host-microbial interaction. This provides unique insights into the role of fiber from different forages in regulating host-microbial interaction and rumen function in lambs fed a high-concentrate diet.

Availability of data and material

The transcriptome and 16S rRNA gene sequencing data for all samples were submitted to the National Center for Biotechnology Information Sequence Read Archive (SRA) under the accession numbers PRJNA996773 (https://www.ncbi.nlm.nih.gov/sra/PRJNA996773) and PRJNA996729 (https://www.ncbi.nlm.nih.gov/sra/PRJNA996729), respectively.

CRediT authorship contribution statement

Kefyalew Gebeyew: Conceptualization, Methodology, Data acquisition, Data analysis, and Writing - original draft; **Hui Mi**: Animal experiments and data acquisition; **Ruiping Du**, **Min Gao**, **Diriba Diba**, and **Shaoxun Tang**: Review and editing; **Zhixiong He** and **Zhiliang Tan**: Conceptualization, Methodology, Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix A. supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.08.010.

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