

Role and mechanisms of moisture regulation in enhancing alfalfa silage quality

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Abstract

Background

Regulating raw material moisture is a key strategy for inhibiting undesirable microorganisms and improving the fermentation quality of alfalfa silage. However, the effects of post-wilting rehydration on the microbial community and its metabolic pathways remain unclear. This study therefore aimed to elucidate the microbial mechanisms by which this rehydration process enhances fermentation quality through the modulation of key microorganisms and metabolic pathways.

Results

This study investigated the effects of four moisture pretreatment treatments—ND50, ND65, PS50, and PS65—on microbial community dynamics and metabolomic changes after 90 days of silage fermentation. The results showed that the contents of DM, WSC, pH, AN/TN, AA, and BA in the PS65 treatment were significantly lower than those in ND65 ($P < 0.05$). In the PS50 treatment, the contents of DM, CP, WSC, and pH were significantly lower than those in ND50 ($P < 0.05$). Across all treatments, the relative abundance of lactic acid bacteria exceeded 97%, with *Lactiplantibacillus* and *Lentilactobacillus* being the dominant genera. Notably, the relative abundance of *Lentilactobacillus* in the spraying treatments was significantly higher than that in the corresponding wilting treatments. The yeast counts in the spraying treatments (PS65 and PS50) were significantly lower than those in the wilting treatments (ND65 and ND50) ($P < 0.05$). In addition, the spraying treatments reduced the relative abundance of certain pathogenic fungi, including *Fusarium* and *Aspergillus*. Metabolomic analysis showed that the water-sprayed treatment PS65 significantly reduced pH ($P < 0.05$) and increased lactic acid (LA) content ($P < 0.05$) by regulating multiple metabolic pathways, including ascorbate and aldarate metabolism and phenylpropanoid biosynthesis. Further pathway analysis indicated that the PS65 treatment may influence ascorbate and aldarate metabolism, phenylpropanoid biosynthesis, thereby promoting fiber degradation in plant tissues. Microbial traceability analysis revealed that the differential metabolites were mainly derived from *Lactiplantibacillus*, *Lentilactobacillus*, and *Weissella*.

Conclusion

Within an appropriate moisture range, water spraying after wilting modulates the microbial community of alfalfa silage. This practice increases the relative abundance of *Lentilactobacillus*, inhibits the proliferation of pathogenic fungi such as *Fusarium* and *Aspergillus*, and reduces mycotoxin accumulation. Consequently, the water spraying treatment significantly lowers silage pH and increases LA content by altering key metabolic pathways, including ascorbic acid and aldaric acid metabolism, phenylpropanoid biosynthesis, and phenylalanine metabolism.

1. Introduction

Solid-state fermentation refers to a process in which microorganisms are cultivated on insoluble solid substrates. Unlike liquid fermentation, the water required for solid-state fermentation is absorbed by the solid substrate, resulting in little to no free water[1]. Solid substrates serve not only as the carbon source for microbial growth and metabolism but also as the microecological environment in which microorganisms develop. The continuous phase is defined as the fluid phase that dominates in a multiphase system and is capable of accommodating and permeating other dispersed phases. The fundamental distinction between solid-state fermentation and liquid-state fermentation lies in whether the gas phase or the liquid phase constitutes the continuous phase.

Silage production, as a typical solid-state fermentation process, involves wilting, chopping, and compacting forage followed by anaerobic fermentation to enable long-term preservation of feed materials[2]. Previous studies have shown that excessively high moisture content in raw materials results in insufficient water-soluble carbohydrates in alfalfa, which facilitates the rapid proliferation of undesirable microorganisms such as *Clostridium*, ultimately leading to a decline in silage quality[3]. In contrast, pre-regulating the moisture content of raw materials to an appropriate range (50–65%) creates favorable conditions for homofermentative lactic acid fermentation dominated by lactic acid bacteria, thereby significantly increasing lactic acid production, lowering pH, and improving the preservation of nutrients such as proteins[4; 5]. The study further indicates that while excessively low moisture content may suppress harmful bacteria, it can adversely affect compaction density and fermentation initiation, thereby necessitating the use of additives[6]. In recent years, driven by advances in molecular biology techniques, research has expanded beyond traditional assessments of fermentation quality to elucidate microbial community composition and metabolic functions during silage production. High-throughput sequencing studies have demonstrated that putrefactive processes in high-moisture alfalfa silage are closely associated with specific clostridial taxa. In particular, *Clostridium* sp. *BTY5* and *Garciaella* sp. *GK3* have been identified as key pathogenic bacteria driving butyric acid fermentation[7]. However, the growth rate and metabolic activity of microorganisms are closely associated with the water activity of their environment, and substantial differences exist in water activity requirements among different microbial groups. Therefore, the physicochemical environment supporting microbial growth is a primary concern during alfalfa silage fermentation. In mature plant cells, over 90% of the water is stored as free water in the central vacuole, which serves as the primary reservoir of cellular free water. This vacuole is enclosed by the tonoplast, while the entire cell is surrounded by a cell wall rich in cellulose and pectin. This structural characteristic limits water availability to lactic acid bacteria and hinders nutrient dissolution and the diffusion of organic acids. To investigate how moisture regulates key metabolic pathways and microbial composition to improve fermentation quality, this study used wilted forage evenly sprayed with sterile water as the treatment group. Subsequently, four groups of samples were subjected to sealed fermentation for 90 days, and changes in their nutritional composition and fermentation quality were compared. In addition, bacterial and fungal communities were characterized using 16S rRNA and ITS sequencing, and metabolite profiles were determined by LC–MS. These analyses were used to evaluate the effects of supplemental free water on microbial diversity and metabolic characteristics in alfalfa silage.

2. Materials and methods

2.1 Silage preparation

The plant material used in this study was the alfalfa (*Medicago sativa* L.) cultivar WL343, which was provided by the Grassland Research Institute of the Chinese Academy of Agricultural Sciences and cultivated at the Mengniu Helin Base in Hohhot. The forage was harvested at the initial flowering stage on August 5, 2024. After mowing, the alfalfa was subjected to four moisture pretreatment treatments as follows: Natural drying to 65% moisture (ND65), in which alfalfa was naturally sun-dried until the moisture content reached approximately 65%; natural drying to 50% moisture (ND50), in which alfalfa was naturally sun-dried until the moisture content reached approximately 50%; spray to 65% moisture (SP65), in which alfalfa naturally sun-dried to approximately 50% moisture was sprayed with sterile water to increase the moisture content to approximately 65%; and spray to 50% moisture (SP50), in which alfalfa naturally sun-dried to approximately 35% moisture was sprayed with sterile water to increase the moisture content to approximately 50%. After pretreatment, the alfalfa was chopped into 1–2 cm lengths using a guillotine chopper. The silage inoculant (0.005 g/kg) and sucrose (2 g/kg) were separately dissolved in 5 mL/kg of sterile water. After thorough mixing, the solutions were evenly sprayed onto each treatment. The treated alfalfa was then packed into polyethylene bags (250 mm × 350 mm), vacuum-sealed, with each bag containing approximately 300 g of material. Each treatment was prepared in triplicate and stored at room temperature for 90 days. Samples were collected after ensiling for subsequent analyses.

2.2 Sample Collection and Index Measurement

After opening the silage bags, a portion of each sample was immediately frozen at – 20°C in sealed plastic bags for subsequent chemical analyses. For extract preparation, 20 g of silage was mixed with 180 mL of distilled water, allowed to stand at 4°C for 24 h, and then filtered through four layers of medical gauze. The filtrate was used for the determination of pH, ammonia nitrogen (NH₃-N), and organic acids (OA). The pH was measured using an electronic pH meter (LAQUAtwin-pH-22, Horiba, Japan), and NH₃-N concentration was determined using the phenol–hypochlorite method [8]. The filtrate (1500 µL) was filtered through a 0.22-µm aqueous filter, and oxalic acid (OA) was determined using a high-performance liquid chromatography system according to the method described in Wang et al.'s report [9]. For microbial enumeration, 20 g of silage was homogenized with 180 mL of sterile saline solution and serially diluted. The populations of yeasts and molds, lactic acid bacteria, and coliform bacteria were determined using the agar plate method following the procedures described by Yan et al [10]. The dry matter (DM) content of the remaining silage in each bag was determined after oven-drying at 65°C for 72 h. Dried samples were then ground and passed through a 1.0 mm sieve for further analysis. Crude protein (CP) content was analyzed using the Kjeldahl method [11]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to the method of Van Soest et al [12]. Water-soluble carbohydrate (WSC) content was determined using the method described by Thomas [13].

2.3 Bacterial community composition analysis

Upon completion of silage fermentation, all samples were collected and thoroughly homogenized within each treatment group. Total microbial DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega, USA). DNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer, and integrity was verified by 1% agarose gel electrophoresis. For bacterial and archaeal community analysis, the V3–V4 region of the 16S rRNA gene was amplified with the universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). For fungal community analysis, the ITS1 region was amplified using the fungus-specific primers ITS1F (5'-CTTGGTCATTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). All PCR products were examined on 2% agarose gels, and target bands were excised and purified with the AxyPrep DNA Gel Extraction Kit, followed by quantification using a Quantus™ Fluorometer (Promega, USA). Sequencing libraries were prepared with the TruSeq™ DNA Sample Prep Kit and subjected to paired-end sequencing on an Illumina MiSeq platform (Illumina, USA).

Using fastp (<https://github.com/OpenGene/fastp>, version 0.20.0) software to quality control of the original sequencing sequence, use FLASH (<http://www.cbcb.umd.edu/software/flash>, Version 1.2.7) software for Mosaic: use UPARSE software (<http://drive5.com/uparse/>, version 7.1), according to 97% of the similarity of sequence OTU clustering and eliminate chimeras. Species classification and annotation were performed for each sequence using RDP classifier (<http://rdp.cme.msu.edu/>, version 2.2), and the Silva 16S rRNA database (version 138) was compared. The comparison threshold was set at 70%.

2.4 Metabolite Analysis

Samples were vacuum freeze-dried using a freeze dryer (Scientz-100F). The dried samples were ground into a fine powder using a mixer mill (MM400, Retsch) at 30 Hz for 1.5 min. Metabolites were extracted with a precooled 70% methanol–water solution containing internal standards at – 20°C, followed by centrifugation at 12,000 rpm for 3 min. The resulting supernatant was then used for UPLC–MS/MS analysis. Metabolomic data acquisition was performed using an ultra-performance liquid chromatography system (UPLC; ExionLC™ AD, SCIEX, <https://sciex.com.cn/>) coupled with a tandem mass spectrometry (MS/MS) system. UHPLC separation was performed using an Agilent SB-C18 chromatographic column (1.8 µm, 2.1 mm × 100 mm). The mobile phases were: Phase A, ultrapure water with 0.1% formic acid; and Phase B, acetonitrile with 0.1% formic acid. The elution gradient was as follows: at 0.00 min, Phase B was at 5%; from 0.00 to 9.00 min, Phase B linearly increased to 95%, which was maintained for 1 min; from 10.00 to 11.10 min, Phase B decreased back to 5%, which was held until 14 min. The flow rate was 0.35 mL/min, and the column temperature was maintained at 40°C. The injection volume was 4 µL.

The metabolomics data of these samples were qualitatively analyzed using the MetWare database independently developed by MetWare Biotechnology Co., Ltd. (Jiaxing, China). The variable importance of predicted (VIP) ≥ 1.0 and absolute multiple change (FC) ≥ 5.0 was used as the criterion for the selection of differential metabolites. Compounds using KEGG database (<http://www.kegg.jp/kegg/compound/>) for identification of the metabolites of comments, and then map the annotation of metabolites to KEGG pathways database (<http://www.kegg.jp/kegg/pathway.html>).

2.5 Statistical analysis

The physical and chemical property data of silage were first organized using Microsoft Excel 2016 and then analyzed by analysis of variance (ANOVA) with IBM SPSS Statistics 26, Sample values were expressed as “mean ± standard deviation.” Microbial diversity data were analyzed using QIIME2 and visualized using R 4.4.2 and Python 3.5.

3. Results

3.1 Nutrient and fermentation characteristics

The nutritional and fermentation parameters of the alfalfa silage are presented in Table 1. The CP content in the PS50 treatment was significantly lower than that observed in the other treatments ($P < 0.05$). Significant differences were also found in WSC concentrations among the groups ($P < 0.05$), which followed the descending order of ND50 > PS50 > PS65 > ND65. In contrast, no significant differences were detected for NDF, ADF, or Ash content among the treatments ($P > 0.05$). In addition, Moisture content significantly affected all fermentation parameters of alfalfa silage. Compared with a moisture content of 65%, a moisture content of 50% significantly increased lactic acid (LA) concentration, while significantly reducing acetic acid (AA), pH, ammonia nitrogen to total nitrogen ratio (AN/TN), propionic acid (PA), and butyric acid (BA) concentrations. In addition, the numbers of lactic acid bacteria and yeasts were also reduced at 50% moisture content. Distinct differences also emerged when comparing the water-spraying and wilting group. For both moisture levels, the water-spraying treatments (PS65 and PS50) resulted in significantly higher LA content and, significantly lower pH and yeast counts compared to their wilted counterparts ($P < 0.05$). In the PS65 treatment, AA, PA, and BA contents were all significantly lower than in the corresponding wilting group ($P < 0.05$). In contrast, at the lower moisture level of PS50, these parameters (AN/TN, AA, and BA) did not differ significantly from the wilting treatment ($P > 0.05$).

Table 1
Fermentation characteristics of alfalfa silage

Item	65%	50%		
	ND65	PS65	ND50	PS50
DM (g/kg FM)	291.05 ± 3.96d	341.60 ± 7.81c	525.71 ± 9.74a	504.88 ± 15.03b
CP (g/kg DM)	216.33 ± 4.41a	216.72 ± 4.15a	222.11 ± 2.24a	206.11 ± 2.30b
NDF (g/kg DM)	347.96 ± 18.95a	335.11 ± 12.95a	332.98 ± 16.64a	350.93 ± 18.72a
ADF (g/kg DM)	258.52 ± 13.59a	241.45 ± 14.99a	246.28 ± 11.41a	258.26 ± 13.22a
WSC (g/kg DM)	23.13 ± 1.89d	28.71 ± 2.94c	43.39 ± 1.20a	38.58 ± 0.98b
ASH (g/kg DM)	108.51 ± 2.06a	108.82 ± 1.38a	106.00 ± 1.08a	105.47 ± 2.16a
pH	5.29 ± 0.02a	4.83 ± 0.02b	4.69 ± 0.03c	4.40 ± 0.01d
NH3-N (g/kg total N)	53.17 ± 5.32a	41.58 ± 2.42b	25.62 ± 3.83c	24.21 ± 1.92c
LA (g/kg DM)	43.64 ± 2.72d	58.04 ± 1.84b	49.81 ± 4.01c	66.42 ± 2.4a
AA (g/kg DM)	70.61 ± 3.76a	52.43 ± 5.75b	31.68 ± 1.02c	26.22 ± 1.82c
PA (g/kg DM)	40.87 ± 0.78a	35.91 ± 0.1b	26.78 ± 1.88d	30.57 ± 0.35c
BA (g/kg DM)	4.72 ± 1.1a	3.26 ± 0.16b	1.18 ± 0.3c	1.62 ± 0.28c
Lactic acid bacteria, log10 cfu/g	7.27 ± 0.3a	7.53 ± 0.24a	6.45 ± 0.09b	6.69 ± 0.22b
Coliform bacteria, log10 cfu/g	ND	ND	ND	ND
Yeasts, log10 cfu/g	7.06 ± 0.21a	6.51 ± 0.06b	3.24 ± 0.23c	2.67 ± 0d
Molds, log10 cfu/g	ND	ND	ND	ND
Note: ND65: Naturally wilted to 65% moisture content; PS65: Naturally air-dried to 50% moisture content, then sprayed to 65%; ND50: Naturally wilted to 50% moisture content; PS50: Naturally air-dried to 35% moisture content, then sprayed to 50%; DM: Dry matter; CP: Crude protein; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; WSC: Water-soluble carbohydrates; ASH: Ash; LA: Lactic Acid; AA: Acetic Acid; PA: Propionic Acid; BA: Butyric Acid; NH3-N: ammonia nitrogen. Different lowercase letters denote significant differences between values in the same row. ND, not detected.				

3.2 Bacterial and fungal community

3.2.1 Bacterial diversity and community composition

The bacterial and fungal communities of alfalfa silage were characterized by sequencing 16S rDNA and ITS regions. As shown in Fig. 1(a,b), the Shannon indices of the water spray treatments (PS50 and PS65) were higher than those of the corresponding wilting treatments (ND50 and ND65), indicating that water spray treatment improved the evenness of the bacterial community. The composition of the bacterial community in alfalfa silage is shown in Fig. 1(c,d). At the phylum level, Firmicutes was the dominant phylum in all treatments, with a relative abundance exceeding 97%. The relative abundance of Proteobacteria was highest in the PS50 treatment (2.0%). *Lactobacillus* was the dominant genus across all treatment groups, with its relative abundance being higher in the 65% moisture content group than in the 50% moisture content group. As shown in Fig A.1, lactic acid bacteria in the treatment groups were predominantly composed of *Lactiplantibacillus* and *Lentilactobacillus* (relative abundance > 97% each). Principal coordinate analysis (PCoA) based on

the Bray–Curtis distance matrix is shown in Fig. 1e. The first two principal coordinates explained 93.88% of the total community variation, with PC1 accounting for 84.22% and PC2 accounting for 9.66%. Samples within the same treatment clustered closely, indicating high repeatability among replicates. ND65 and PS65 were clearly separated along PC1, reflecting pronounced differences in bacterial community composition between these treatments. Venn diagrams were used to illustrate the numbers of shared and unique bacterial OUTs among treatments. As shown in Fig. 1f, a total of 147 bacterial OUTs were detected. Compared with ND65, the PS65 treatment contained 26 shared OUTs and 51 unique OUTs. Similarly, compared with ND50, the PS50 treatment shared 55 OUTs, with 64 OUTs being unique to PS50. Significant differences in the relative abundances of *Weissella*, *Exiguobacterium*, *Enterococcus*, *Microbacterium*, *Pseudomonas*, *Staphylococcus*, *Salana*, *Pseudoclavibacter*, and *Tianweitan* were observed among treatments (Fig. 1g). Pairwise comparisons at the genus level (Fig. 1h) showed that the relative abundance of *Weissella* in the PS65 treatment reached 0.3726, which was significantly higher than that in the other treatments ($P < 0.05$). The relative abundance of *Exiguobacterium* in the PS50 treatment (0.06867) was significantly lower than that in the ND50 treatment (0.09771). In addition, the relative abundances of *Microbacterium*, *Pseudomonas*, *Staphylococcus*, *Salana*, *Pseudoclavibacter*, and *Tianweitan* in the PS50 treatment were significantly higher than those in the other treatments ($P < 0.05$).

Figure A.1. Advanced Enrichment Circle Plots. (a) Plot between groups ND65 and PS65; (b) Plot between groups ND50 and PS50; (c) Plot between groups ND50 and ND65; (d) Plot between groups PS50 and PS65.

3.2.2 Fungal diversity and community composition

As shown in Fig. 2(a,b), The ace and shannon indices of the PS65 group were higher than those of the other groups, indicating a higher species richness and a more uniform species distribution. The fungal community composition Fig. 2(c,d) was dominated by the phyla Ascomycota and Basidiomycota. The relative abundance of Ascomycota was highest in the PS65 treatment, followed by PS50, ND65, and ND50. In contrast, for Basidiomycota, the ND50 treatment exhibited the highest relative abundance (42.50%), while the abundance in the PS65 group (32.35%) was significantly lower than that in the corresponding ND65 treatment ($P < 0.05$). The dominant fungal genera in alfalfa silage included *Apiotrichum*, *Leciterra*, *unclassified_f_Didymellaceae*, *Cutaneotrichosporon*, *Phoma*, *Gibberella*, *Colletotrichum*, *Cladosporium*, *unclassified_k_Fungi*, and *Filobasidium*. The relative abundance of *Apiotrichum* was significantly higher in the wilting treatment groups (ND65, ND50) than in the water-spraying groups (PS65, PS50), while *Leciterra* showed the opposite trend. The relative abundance of *Cutaneotrichosporon* was higher in the 50% moisture content treatments (ND50, PS50) than in the 65% moisture content treatments. The abundance of *Cladosporium* was highest in the ND50 group, and the abundance of *unclassified_k_Fungi* was higher in both the ND50 and PS50 groups. Principal coordinate analysis (PCoA) based on fungal OUTs composition is shown in Fig. 2e. The first two principal coordinates explained 45.63% of the total community variation, with PC1 accounting for 31.42% and PC2 accounting for 14.21%. ND65, ND50, and PS65 were clearly separated along PC2, indicating distinct differences in fungal community composition among treatments. As shown in the fungal Venn diagram (Fig. 2f), a total of 347 fungal OUTs were detected across all treatments. Compared with ND65, the PS65 treatment shared 166 OUTs and contained 103 unique OUTs. Compared with ND50, the PS50 treatment shared 61 OUTs, with 96 OUTs being unique. Differences in fungal taxa among treatments are illustrated in Fig. 2g, where significant differences in the relative abundances of *Cladosporium*, *Coprinellus*, *Lysurus*, and *Malassezia* were observed among multiple groups. Further pairwise comparisons at the genus level (Fig. 2h) showed that the relative abundances of *Coprinellus*, *unclassified_c_Sordariomycetes*, and *unclassified_f_Psathyrellaceae* were significantly higher in the PS50 treatment than in the corresponding ND50 treatment. In addition, the relative abundance of *Lysurus* in the PS50 treatment was significantly higher than that in ND50.

3.2.3 Correlation analysis between microorganisms and silage fermentation quality

Correlation analysis between bacterial genera and physicochemical parameters of silage (Fig. 3a) showed that *Lactobacillus* was significantly positively correlated with pH ($P < 0.05$). Both pH and WSC contents were significantly positively correlated with *Lactococcus*, *Enterobacter*, *Enterococcus*, and *Leuconostoc*. *Staphylococcus*, *Devosia*, and *Microbacterium* were all significantly positively correlated with LA ($P < 0.05$). Conversely, significant negative correlations were found between AA and *Exiguobacterium*, *Nocardioides*, *Microbacterium*, and *Pseudomonas* ($P < 0.05$). Similarly, *Exiguobacterium*, *Nocardioides*, *Paracoccus*, *Rhodococcus*, *Myroides*, *Delftia*, and *Sphingomonas* were all significantly negatively correlated with BA ($P < 0.05$). Additionally, *Exiguobacterium* and *Nocardioides* were positively correlated with DM but negatively correlated with $\text{NH}_4^+\text{-N}$ and PA ($P < 0.05$). Regarding fungal genera Fig. 3b, *Fusarium* showed significant positive correlations with pH, AA, PA, BA, $\text{NH}_4^+\text{-N}$, and Ash, but negative correlations with DM and water-soluble WSC ($P < 0.05$). In contrast, *unclassified fungi* exhibited the opposite pattern, being negatively correlated with pH, AA, PA, BA, and $\text{NH}_4^+\text{-N}$, while positively correlated with DM and WSC ($P < 0.05$). Finally, *Cutaneotrichosporon* was positively correlated with NDF and ADF, while CP was positively correlated with *Cladosporium* but negatively correlated with *unclassified_c_Sordariomycetes* ($P < 0.05$).

3.3 Effects of moisture regulation on differential metabolites

As shown in Fig. 4, based on the UPLC–MS/MS platform, a total of 1,023 metabolites were detected in the 12 alfalfa silage samples in this experiment. These metabolites included 238 flavonoids, 157 phenolic acids, 113 lipids, 99 amino acids and their derivatives, 90 terpenoids, 89 alkaloids, 84 organic acids, 34 nucleotides and their derivatives, and 26 lignans and coumarins. These compounds endow alfalfa with a wide range of biological activities. The inclusion of alfalfa silage in the diet has been reported to increase feed intake and digestibility in dairy cows and to improve their production performance and metabolic capacity, which may be associated with the abundance of bioactive compounds in alfalfa. However, no tannin-like compounds were detected in the present study, which may be related to the characteristics of the raw materials used. The principal component analysis (PCA) of metabolites is shown in Fig. 4a. Samples within each treatment group were well clustered, indicating good experimental repeatability. Clear separation was observed among the different treatment groups. The two moisture content groups were distinctly separated from each other. Principal component 1 (PC1) explained 40.53% of the total variance, indicating that moisture content had a significant effect on the metabolite profiles of silage alfalfa. Differences were also observed between the natural drying and water spraying treatment groups. Principal component 2 (PC2) explained 18.36% of the total variance, suggesting that water spraying treatment also influenced silage metabolites, with a greater impact observed in the treatment groups with a moisture content of 65%.

After 90 days of ensiling, comparisons between alfalfa silage subjected to natural drying and water spraying revealed substantial differences in metabolite profiles. In the ND65 and PS65 treatment groups, a total of 988 differential metabolites were detected, of which 81 were up-regulated and 89 were down-regulated (Fig. 4b). In the ND50 and PS50 treatment groups, 1,014 differential metabolites were identified, including 32 up-regulated and 69 down-regulated metabolites (Fig. 4c). These results indicate that, under the same moisture content, the chemical composition of alfalfa silage differed markedly between water spraying and natural sun-drying treatments. Among the different water spraying treatments, 210 metabolites were increased and 73 metabolites were decreased (Fig. 4d). When comparing natural sun-drying treatments with moisture contents of 65% and 50%, 228 metabolites were increased and 100 metabolites were decreased (Fig. 4e). The results indicated that water spraying treatment reduced the influence of moisture content on differential metabolites. In this study, when the moisture content was 65%, lipid compounds showed the greatest up-regulation among the differential metabolites in water-sprayed silage, whereas flavonoids exhibited the greatest down-regulation. When the moisture content was 50%, phenolic acids were the most prominently up-regulated as well as down-regulated differential metabolites. When comparing moisture contents of 65% and 50%, regardless of whether water spraying was applied, flavonoids represented the most increased differential metabolites, while terpenoids showed the greatest decrease.

3.4 KEGG Pathway Enrichment Analysis

In this study, metabolic pathways were annotated based on the identified differential metabolites, and KEGG enrichment analysis was performed. As shown in Fig. 5, when the moisture content was 65%, water spraying treatment resulted in significant enrichment of the pathways “ascorbate and aldarate metabolism” and “pyruvate metabolism” ($P < 0.05$) (Fig. 5a). When the moisture content was 50%, water spraying treatment led to significant enrichment of “arginine biosynthesis” ($P < 0.05$) (Fig. 5b). Compared with the 50% moisture content, the most significantly enriched metabolic pathways of differential metabolites at 65% moisture content were “biosynthesis of secondary metabolites,” “tricarboxylic acid (TCA) cycle,” and “phenylpropanoid biosynthesis” ($P < 0.05$) (Fig. 5c). Furthermore, when comparing the water spraying treatments at moisture contents of 65% and 50%, the significantly enriched pathways included “ubiquinone and other terpenoid-quinone biosynthesis,” “phenylalanine metabolism,” “monobactam biosynthesis,” “isoquinoline alkaloid biosynthesis,” and “arginine biosynthesis” ($P < 0.05$), whereas the “TCA cycle” pathway was not significantly enriched ($P > 0.05$) (Fig. 5d).

As shown in Fig B.1 and 6, several metabolic pathways were altered. In the ascorbate and aldarate metabolism, D-Glucuronate was significantly downregulated, while its lactone form and the downstream product Tartaric acid semialdehyde were significantly upregulated. Conversely, the branch metabolite 2-Dehydro-3-deoxy-L-arabinonate was significantly downregulated. In the phenylalanine metabolic pathway, most metabolites showed no significant changes. However, phenylpyruvate, trans-Cinnamate, and phenylpropanoate were significantly downregulated. In contrast, succinate, which connects this pathway to central metabolism, was significantly upregulated. In the phenylpropanoid biosynthesis pathway, cinnamic acid, ferulic acid, and zingerone were significantly downregulated, whereas coumarol and sinapyl alcohol was upregulated. Within the α -linolenic acid metabolic pathway, stearoyl acid, 9(S)-HpOTrE, and 12-OPDA were significantly upregulated, while 9-hydroxy-12-oxo-10(E),15(Z)-octadecadienoic acid was significantly downregulated. Finally, in the nicotinic acid and nicotinamide metabolic pathway, nicotinamide was significantly downregulated, whereas maleic acid and succinic acid were significantly upregulated. In the phenylpropanoid biosynthesis pathway, cinnamic acid, ferulic acid, and sinapic acid were significantly downregulated, whereas p-coumaryl alcohol and sinapyl alcohol was upregulated. Within the α -linolenic acid metabolic pathway, stearoyl acid, 9(S)-HpOTrE, and 12-OPDA were significantly upregulated, while 9-hydroxy-12-oxo-10(E),15(Z)-octadecadienoic acid was significantly downregulated. Finally, in the nicotinic acid and nicotinate and nicotinamide metabolism pathway, nicotinamide was significantly downregulated, whereas maleic acid and fumarate were significantly upregulated.

Figure B.1. Advanced enrichment circle plots. (a) Comparison between groups ND65 and PS65; (b) Comparison between groups ND50 and PS50; (c) Comparison between groups ND50 and ND65; (d) Comparison between groups PS50 and PS65.

3.5 WGCNA Analysis

First, hierarchical clustering analysis was performed on all samples. No outlier samples were identified or removed, and all samples were retained for subsequent weighted gene co-expression network analysis (WGCNA). Based on metabolite expression data from all samples, a topological overlap matrix (TOM) was calculated, followed by hierarchical clustering. As shown in Fig. 7a, all metabolites were classified into seven distinct co-expression modules using the dynamic tree-cutting method. Each module was assigned a unique color, representing a group of metabolites with highly coordinated expression patterns (Table 3). The TOM network heat map was used to evaluate the overall structure of the co-expression network. The heat map displayed several distinct bright squares along the diagonal, each corresponding to a highly interconnected module, whereas the regions between modules were relatively darker [14]. This clear “module–diagonal” pattern confirmed that the metabolite co-expression network exhibited a pronounced modular structure, indicating that metabolites involved in different biological processes or regulatory programs formed relatively independent functional units. Weighted gene co-expression network analysis (WGCNA) was applied to the metabolomics data to identify metabolite modules associated with ripening-related traits [15]. Correlation analysis between the identified modules and treatment groups showed that the Green, Brown, Blue, and Red modules were significantly associated with specific treatments (Fig. 7b). Specifically, ND65 was strongly negatively correlated with the Green module ($r = -0.97$, $P < 0.01$) and strongly positively correlated with the Brown module ($r = 0.97$, $P < 0.01$). In addition, PS65 showed a significant positive correlation with the Red module ($r = 0.92$, $P < 0.01$), PS50 was positively correlated with the Turquoise module ($r = 0.63$, $P < 0.01$), and ND50 exhibited a strong positive correlation with the Blue module ($r = 0.88$, $P < 0.01$). The network structures of the individual modules are presented in Fig. 7c.

As shown in Fig. 7e, pH was significantly positively correlated with the brown and yellow modules ($r = 0.94$, $P < 0.01$; $r = 0.85$, $P < 0.01$) and significantly negatively correlated with the green and turquoise modules ($r = -0.90$, $P < 0.01$; $r = -0.85$, $P < 0.01$). Lactic acid (LA) was significantly positively correlated with the green module ($r = 0.82$, $P < 0.01$) and negatively correlated with the brown module ($r = -0.79$, $P < 0.01$). Acetic acid (AA), propionic acid (PA), and butyric acid (BA) were significantly negatively correlated with the green module ($r = -0.79$, -0.69 , and -0.73 , respectively; $P < 0.01$) and with the turquoise module ($r = -0.95$, -0.90 , and -0.90 , respectively; $P < 0.01$). In contrast, AA, PA, and BA showed significant positive correlations with the brown module ($r = 0.87$, 0.81 , and 0.81 , respectively; $P < 0.01$) and the yellow module ($r = 0.94$, 0.92 , and 0.90 , respectively; $P < 0.01$).

3.6 Tracing Analysis of Key Metabolites

Based on WGCNA and pathway enrichment analyses, key metabolites with significant differential expression were identified. Traceability analysis of these metabolites revealed (Table 3) (Fig. 8), Ferulic and sinapic acids were derived exclusively from *g_Lactiplantibacillus*. A broader range of genera, including *g_Enterococcus*, *g_Lentilactobacillus*, *g_Weissella*, *g_Pediococcus*, *g_Myroides*, and *g_Lactiplantibacillus*, were identified as sources of L-citrulline, L-arginine, glutaric acid, fumaric acid, and xanthine; glutaric acid and xanthine were also associated with *g_pseudoflavobacterium*. Similarly, D-glucuronic acid and nicotinamide originated from *g_Enterococcus*, *g_Lentilactobacillus*, *g_Weissella*, *g_Myroides*, and *g_Lactiplantibacillus*. D-galacturonic acid and N- α -acetyl-L-ornithine were primarily derived from *g_Enterococcus*, *g_Lentilactobacillus*, and *g_Weissella*, although N- α -acetyl-L-ornithine was also sourced from *g_Myroides* and *g_Lactiplantibacillus*. Finally, D-glucurono-6,3-lactone and dehydroascorbic acid were derived from *g_Myroides*.

Figure 8. Sankey diagram illustrating the results of the microbial source tracking analysis. Nodes in the diagram represent different taxonomic levels, and the thickness of the edges corresponds to the proportional contribution or flow between these taxa.

2

module	CAS	Compounds	Class II	Kegg_map
Blue	C01494	Ferulic	Phenolic acids	ko00940,ko01100,ko01110
	C00482	Sinapic acid	Phenolic acids	ko00940,ko01100,ko01110
	C00327	L-Citrulline	Amino acids and derivatives	ko00220,ko01100,ko01110,ko01230
	C00062	L-Arginine	Amino acids and derivatives	ko00220,ko00261,ko00330,ko00472,ko00970,ko01100,ko01110,ko01230,ko02010
Brown	C00191	D-Glucuronic acid*	Saccharides and Alcohols	ko00040,ko00053,ko00520,ko00562,ko01100,ko01240
	C02670	D-Glucurono-6,3-lactone	Saccharides and Alcohols	ko00053,ko01100
	C00333	D-Galacturonic acid*	Saccharides and Alcohols	ko00040,ko00053,ko00520,ko01100,ko01240,ko02010
	C00437	N- α -Acetyl-L-ornithine	Amino acids and derivatives	ko00220,ko01100,ko01110,ko01210,ko01230
Green	C00489	Glutaric acid	Organic acids	ko00071,ko00310,ko01100
Turquoise	C00122	Fumaric acid*	Organic acids	ko00020,ko00190,ko00220,ko00250,ko00350,ko00360,ko00620,ko00650,ko00760,ko01100,ko
Yellow	C00385	Xanthine	Nucleotides and derivatives	ko00230,ko00232,ko01100,ko01110
others	C00153	Nicotinamide	Vitamin	ko00760,ko01100,ko01240
	C05422	Dehydroascorbic acid	Vitamin	ko00053,ko00480,ko01100

4. Discussion

4.1 Impact of Water Spraying on the Fermentation Profile and Nutritional Quality of Alfalfa Silage

Significant differences in organic acid content were observed among the different moisture treatment groups. pH and AN/TN are important indicators reflecting the fermentation quality of silage. Lower pH values and AN/TN levels indicate greater inhibition of harmful microorganisms and reduced crude protein degradation. In the present study, silage with 50% moisture had significantly lower pH and AN/TN contents than silage with 65% moisture ($P < 0.05$),

which is consistent with the reduced abundance of undesirable microorganisms, such as yeasts, under low-moisture conditions. Butyric acid is the main product of *Clostridium* fermentation, and *Clostridium* species tend to proliferate in silage with high moisture content. When the butyric acid content in silage exceeds 5 g/kg DM, it can negatively affect feed intake in livestock[13]. In the present study, the butyric acid contents in all treatments were below 5 g/kg DM, indicating that the silage quality in each experimental group was acceptable. Kung et al. reported that wilting forage to achieve a higher dry matter content can reduce the incidence of *Clostridium*[16]. Therefore, lower moisture content is more favorable for improving silage fermentation quality, which is consistent with the results of the present study. The LA content in the spraying treatments (PS65 and PS50) was significantly higher than that in the corresponding wilting treatment groups ($P < 0.05$), whereas both pH values and yeast counts were significantly lower than those of the corresponding wilting treatments ($P < 0.05$). These effects may be attributed to two main factors. First, water spraying can regulate environmental osmotic pressure, thereby influencing the production of microbial enzymes involved in plant cell wall degradation and interfering with the transformation of plant cell wall components. Second, water spray treatment alters the medium environment in which microorganisms utilize substrates during ensiling, facilitating the transition from a solid to a more liquid phase. This change creates more suitable water activity conditions for lactic acid bacteria fermentation while simultaneously promoting substrate transformation.

4.2 Impact of Water Spraying on the Diversity of the Microbial Community

Among all treatments, ND65 exhibited the highest Ace index value, suggesting greater species richness. Yang et al. reported that the diversity of the bacterial community decreased after 90 days of ensiling, and regarded the reduction in microbial diversity as an indicator of successful silage fermentation [17]. *Lactiplantibacillus* and *Lentilactobacillus* were the dominant genera in all treatment groups, with a combined relative abundance exceeding 97%. Moreover, the abundance of *Lentilactobacillus* was significantly higher in the water-sprayed group than in the corresponding wilted group, this may be because the water-spraying treatment altered the nutrient medium environment required for microbial growth. *Pantoea* is a facultative anaerobic bacterium. In the present study, the relative abundance of *Pantoea* was slightly higher in the PS50 treatment, which may be attributed to the longer wilting time. Under anaerobic conditions, *Clostridium* can ferment lactic acid, a product of primary fermentation by lactic acid bacteria (LAB), and convert it into butyric acid, acetic acid, carbon dioxide, and hydrogen[18]. Its spores can survive in the gastrointestinal tract of dairy cows, and some species can produce highly pathogenic toxins that contaminate raw milk, resulting in off-flavors and excessive gas formation in cheese[19]. However, *Clostridium* was not detected in the present study. Overall, the dominant bacterial genera exhibited a high degree of similarity among treatments. This similarity may be attributed to adequate nutrient availability and the predominance of lactic acid bacteria, which create favorable conditions for successful ensiling. However, microbial community composition showed significant separation along the PC1 axis among different treatment groups. This pattern may be attributed to the water-spray treatment, which could have induced changes in the rare species—a taxonomically minor yet numerically substantial fraction of the community—thereby driving the observed divergence in overall community structure.

At the fungal phylum level, Ascomycota was the dominant fungal group in alfalfa silage. Liu et al. reported in barley silage that the relative abundance of Ascomycota increased, whereas that of Basidiomycota decreased after ensiling[20]. At the genus level, the dominant fungal genera observed in this study were consistent with those previously reported for forages such as alfalfa, sweet sorghum, corn, and barley [21]. *Fusarium* species can produce a wide range of mycotoxins, such as fusaric acid, fumonisins, beauvericin, nivalenol, culmorin, moniliformin, zearalenone, and enniatins[22], whereas *Aspergillus* species are capable of producing aflatoxins and ochratoxin[23]. In the present study, the relative abundance of *Alternaria* did not differ significantly among treatments, whereas *Fusarium* showed a lower abundance in the ND50 treatment, and *Aspergillus* exhibited a lower abundance in the water spray treatments. These results suggest that the spraying treatment can influence the composition of the fungal community and effectively reduce the relative abundance of certain pathogenic fungi, thereby helping to lower the risk of mycotoxin exposure from silage during feeding. Overall, water spray treatment not only altered the composition of dominant microbial genera among treatments but also reduced the relative abundance of toxic and pathogenic fungi within the fungal community, thereby lowering the potential feeding risk associated with silage use.

4.3 Correlations Between Silage Fermentation Quality and the Microbial Community

Studies have shown that *Lactobacillus* produces LA by consuming large amounts of WSC, thereby lowering the pH. The positive correlation observed in this study may be due to a time lag between microbial growth and the accumulation of metabolic products during the early stages of silage fermentation[24]. *Staphylococcus* and *Microbacterium* were significantly positively correlated with LA ($P < 0.05$), whereas a low-pH environment can inhibit the growth of *Staphylococcus*[25]. *Exiguobacterium* and *Nocardioide*s were significantly positively correlated with WSC contents ($P < 0.05$). These results are consistent with the findings of Lu et al. [26] In this study, *Fusarium* showed a significant negative correlation with WSC ($P < 0.05$). This suggests that *Fusarium* can proliferate under conditions of organic acid accumulation as WSC is consumed. In addition, *Cutaneotrichosporon* was significantly positively correlated with NDF and ADF contents ($P < 0.05$), but significantly negatively correlated with Ash content ($P < 0.05$). Based on previous studies, synergistic degradation of NDF and ADF occurs between *Pichia* and *Cutaneotrichosporon*, suggesting that this fungus may play an important role in fiber degradation[27].

4.4 Differences in silage metabolites

4.4.1 Effects of Moisture Regulation on Phenolic Compounds and Organic Acids in Alfalfa Silage

Flavonoids are a class of polyphenolic secondary metabolites with anti-inflammatory, antimicrobial, and antioxidant properties that promote animal growth and development. In the present study, flavonoids were the most up-regulated differential metabolites under the 50% moisture content treatment. Consistent with this, Zhan et al. reported that dietary supplementation with alfalfa flavonoids could alter milk composition and enhance the immunity of dairy cows by modifying the lymphocyte-to-neutrophil ratio[28]. In the treatment group with a moisture content of 65%, lipid compounds showed the greatest up-regulation among the differential metabolites following water spraying treatment, including 11 free fatty acids, 5 glycerides, and 2 lysophosphatidylethanolamine

species. Among these, α -linolenic acid is an essential fatty acid that cannot be synthesized endogenously by mammals and must be obtained from plant sources.

Phenolic acids, including cinnamic acid, ferulic acid, caffeic acid, and quinic acid, exhibit strong antibacterial activity[29]. Ferulic acid is esterified to lignin in plant cell walls, and lactic acid bacteria inoculation promotes its release from the cell wall matrix.[30]. Hydroxycinnamates can be hydrolyzed by ferulic acid esterases to produce hydroxycinnamic acids. In the present study, all of these phenolic acid compounds were up-regulated in the treatment group with 50% moisture content, which may be associated with microbial enzymatic activity[31]. Furthermore, mandelic acid was detected in all water-sprayed silage treatments but was absent in the naturally dried silage. Mandelic acid possesses antibacterial properties and can inhibit certain pathogenic microorganisms. Jeon et al. reported that in a 2 mg/mL mandelic acid solution containing 1.0 M NaCl at pH 5.0, foodborne pathogenic bacteria such as *Escherichia coli* O157:H7 and *Staphylococcus aureus* KCCM40881 were inhibited, whereas *Lactobacillus* species were not affected[32].

Organic acids are commonly used indicators for evaluating silage fermentation quality. They not only create a low-pH environment that inhibits the growth of harmful microorganisms but also exert additional functions, such as antimicrobial activity. Previous studies have shown that lactic acid bacteria utilize citric acid through at least two distinct metabolic pathways. In the first pathway, citric acid is converted into succinic acid via the reduction of oxaloacetic acid, mediated by enzymes such as fumarase, fumarate reductase, and malate dehydrogenase. In the second pathway, citric acid is metabolized through the pyruvate route to produce acetic acid and formic acid[33]. It has also been reported that certain lactic acid bacteria strains are unable to convert citric acid into pyruvate; instead, they generate succinic acid through malic acid and fumaric acid via reverse transport protein systems[34]. In the present study, the fumaric acid content in the PS65 treatment showed an increasing trend compared with that in the ND65 treatment. As a key intermediate linking malic acid and succinic acid, the accumulation of fumaric acid reflects an adequate upstream supply of malic acid and enhanced metabolic flux, indicating that the microbial community in the early stage of ensiling tends to channel metabolism toward organic acid production via the “first pathway.” This may partly explain the relatively higher lactic acid content observed in the PS65 treatment. This metabolic feature was consistent with the increased availability of alfalfa substrates rich in malic acid and the higher abundance of genera such as *Weissella* in this treatment[35]. The relatively higher acetic acid content in the ND65 treatment may be attributed to the decarboxylation of L-Malic Acid to pyruvate by soluble cytoplasmic malic enzyme in *Lactobacillus plantarum*[36]. In addition, the level of 2-propylmalic acid was also up-regulated in the PS65 treatment, suggesting that cells enhanced their resistance to biological stress during the wilting process through the accumulation of this compound[37]. Notably, although the relative abundance of *Lactobacillus* in the PS65 treatment was lower than that in the ND65 treatment, the higher abundance of *Weissella* and the significantly greater total number of lactic acid bacteria resulted in significantly higher lactic acid and acetic acid contents in PS65 compared with ND65.

4.4.2 Effects of moisture regulation on metabolic pathways in alfalfa silage

In the ascorbate and aldarate metabolism pathway, glucuronate, a major uronic acid, was significantly down-regulated, whereas its lactone form, D-glucurono-1,4-lactone, and the cleavage intermediate tartronate semialdehyde were correspondingly up-regulated. These changes indicated that water spraying treatment promoted the release of uronic acids from the plant cell wall. In addition, the down-regulation of 2-dehydro-3-deoxy-L-arabinonate further suggested that carbon flux was redirected toward acid production rather than pentose rearrangement metabolism. These results imply that water spraying treatment may regulate the osmotic conditions of the raw materials, facilitating microbial conversion of plant cell wall-derived uronic acids into pyruvate, which favors subsequent lactic acid fermentation. Previous studies have reported that *Lactiplantibacillus plantarum*, possessing cellulase and hemicellulase activities, can significantly reduce silage pH by down-regulating butyrate and pentose phosphate pathways while up-regulating ascorbate and aldarate metabolism [38] [39]. Within the phenylpropanoid biosynthesis pathway, cinnamic acid, ferulic acid, and sinapic acid were significantly down-regulated, whereas their downstream lignin monolignol precursors, p-coumaryl alcohol and sinapyl alcohol, exhibited an up-regulation trend. Given that microorganisms lack the complete capacity for de novo phenylpropanoid synthesis under silage conditions, these changes more likely reflected the redistribution of plant-derived phenylpropanoid metabolites during fermentation rather than an overall activation of the pathway. Previous studies have shown that the addition of *Lactobacillus buchneri* can up-regulate secondary metabolites associated with phenylpropanoid metabolism, including sinapic acid and dihydroferulic acid, which influence lignin synthesis in the cell wall [40]. These findings suggest that anaerobic fermentation may promote fiber degradation in plant tissues [41]. Overall, water spraying treatment appeared to enhance the antioxidant potential of soluble carbohydrates and improve fiber digestibility through modulation of phenylpropanoid metabolism.

In this study, the overall levels of phenylalanine in the PS65% and ND65% groups remained relatively stable, whereas the synthesis levels of its key downstream intermediates—phenylpyruvate, trans-cinnamic acid ester, and phenylpropionic acid ester—were all downregulated, indicating inhibition of the secondary metabolic pathway of aromatic amino acids. Accumulation of phenylpyruvate generally results from deamination, a process accompanied by ammonia production that adversely affects silage quality. Meanwhile, lactic acid bacteria can generate intermediate products via phenylalanine and tyrosine metabolic pathways, which subsequently react with alcohols to form aromatic esters that contribute to improved silage aroma [42]. Trans-cinnamic acid ester and phenylpropionic acid ester are associated with lignin synthesis and specific secondary metabolic pathways; their reduction may indicate increased diversion of substrates toward energy metabolism. Phenylalanine, an essential aromatic amino acid, is oxidized to tyrosine in vivo, and phenylalanine hydroxylase participates in carbohydrate metabolism [43]. In this study, the upregulation of fumarate suggested that the carbon skeleton of phenylalanine was increasingly utilized for energy metabolism, potentially contributing to fermentation system stability. Fatty acids play a crucial role in supplying energy to lactic acid bacteria and maintaining their key physiological activities. In the α -linolenic acid metabolic pathway, α -linolenic acid itself did not show significant differences among treatments; however, several downstream oxidized derivatives exhibited significant changes. Specifically, stearic acid, 9(S)-HOTRE, and 12-OPA were significantly upregulated, whereas 9-hydroxy-12-oxo-10(E),15(Z)-octadecadienoic acid was significantly downregulated, indicating metabolic redistribution within this pathway during silage fermentation. Unlike previous reports in mixed oat–pea silage, in which arachidonic acid and linolenic acid increased simultaneously, α -linolenic acid remained relatively stable as the initial substrate in this study [44]. This stability may reflect its reserve capacity in plant tissues, suggesting that pathway regulation mainly occurred at the level of oxidative conversion. Changes in the accumulation of downstream lipooxygenation products further indicated activation of fatty acid oxidation-related reactions during fermentation, potentially associated with interactions

between lipid metabolism and silage microorganisms. Inoculating *Lactiplantibacillus plantarum* into silage corn significantly increased alpha-linolenic acid [45]. within the nicotinate and nicotinamide metabolism pathway, nicotinamide was significantly down-regulated, whereas fumarate and maleate were significantly up-regulated, indicating metabolic redistribution within this pathway during silage fermentation. Nicotinamide is an important precursor in NAD⁺ metabolism, and its decreased abundance may reflect continuous utilization during fermentation to meet the demand for cofactors involved in microbial energy metabolism and redox reactions [46]. Nicotinate and nicotinamide participate in anti-inflammatory processes and energy metabolism, and their metabolic pathways can be regulated by various bioactive compounds, such as saikosaponins [47]. Fumarate and maleate are downstream metabolites that can be linked to the tricarboxylic acid (TCA) cycle, and their significant accumulation suggests enhanced metabolic flux between nicotinate/nicotinamide metabolism and central energy metabolism.

As shown by the correlation heatmap between modules and treatment groups in the WGCNA analysis, In both the brown and turquoise modules, amino acids and their derivatives constituted the core metabolites. Most volatile compounds in silage originate from essential nutrients such as amino acids and fatty acids, which directly influence silage aroma and flavor [48]. In the present study, most amino acids and their derivatives were more abundant in silage with a moisture content of 50% than in silage with 65% moisture, which may be associated with the activity of plant enzymes and microorganisms. Specifically, sweet-tasting amino acids (L-serine, L-proline, D-valine, D-leucine, and L-threonine), bitter-tasting amino acids (L-cysteine, L-arginine, L-leucine, and L-phenylalanine), and sour-tasting amino acids (L-aspartic acid) were up-regulated. The contribution of amino acids to feed flavor depends on both their taste thresholds and taste activity values [49]; Guo et al. reported that inoculation of *Lactobacillus buchneri* into alfalfa silage increased the contents of polyols such as arabitol, erythritol, mannitol, and threitol, which differed from the sugar alcohols (maltitol, xylitol, mannitol, and threitol) detected in the present study [50]. This discrepancy may be related to differences in the lactic acid bacterial inoculants used. Biogenic amines produced via amino acid decarboxylation can pose risks to animal and human health, as they may react with nitrites to form carcinogenic nitrosamines [51]. In this study, the contents of biogenic amines, including histamine, putrescine, cadaverine, spermidine, tyramine, and tryptamine, were reduced in water-sprayed silage, thereby contributing positively to silage quality.

4.4.3 Traceability Association between Key Metabolites and Microorganisms

Traceability analysis of these metabolites indicated that metabolic changes in phenolic acids were closely correlated with the genus *Lactiplantibacillus*. The metabolism of phenolic acids by lactic acid bacteria is strain-specific, and phenolic acid decarboxylase, which catalyzes the decarboxylation of caffeic acid and ferulic acid, has been identified in *Lactobacillus plantarum* [52] [53]. In the present study, within the 50% moisture content treatment, phenolic acids represented the most significantly up- and down-regulated differential metabolites. This may be explained by the fact that water spray treatment altered the microbial community composition, which in turn influenced the activity of key enzymes such as phenolic acid decarboxylase, ultimately resulting in pronounced changes in phenolic acid metabolism. During the early stages of silage fermentation, *Enterococcus* often acts as a dominant bacterial group, playing a crucial transitional and beneficial role [54]. The present study confirmed that *Enterococcus* is capable of metabolizing D-glucuronic acid, which is consistent with previous reports [55]. Some *Weissella* species can utilize α -cyclodextrin, fumaric acid, and glycyl-L-glutamine as carbon sources [56]. In this study, *Weissella* was the differential microorganism with the most significant change in relative abundance, which was notably increased in the water spray treatment (PS65) [35]. This finding might explain the observed difference in fumaric acid production in this treatment group. Furthermore, *Weissella* is associated with the generation of arginine, suggesting that the differences in arginine levels among treatments may be attributed to the varying abundance of this genus. In addition, specific lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecalis*, and *Lactobacillus plantarum*, are also capable of degrading arginine [57]. In plant-associated lactic acid bacteria, this degradation primarily occurs via the arginine deiminase pathway. During this process, arginine is enzymatically degraded into citrulline and ornithine, which releases ammonia and ATP, thereby helping to alleviate environmental acid stress. For instance, a limited arginine supply can promote cell growth and enhance the acid tolerance of *Lactobacillus sanfranciscensis* CB1 during fermentation [58]. In the present study, differential metabolites between the water spray and wilting treatments were significantly enriched in the arginine biosynthesis pathway. However, as the core strain identified in this study, *Lactobacillus plantarum*, is unable to synthesize arginine de novo and must rely on exogenous sources, the water spray treatment did not directly promote arginine synthesis. Instead, it significantly altered the initial fermentation environment, leading to the accumulation of upstream intermediate metabolites in this pathway. Two mechanisms may explain this observation. On one hand, water spraying likely caused the rapid lysis of fragile, wilted plant cells, resulting in a large-scale release of intracellular storage proteins and enzymes. The subsequent hydrolysis of these proteins would have increased the availability of direct precursors for arginine biosynthesis. On the other hand, water spraying accelerated the release of soluble carbohydrates, which stimulated vigorous fermentation by lactic acid bacteria. The resulting rapid acidification would have inhibited the growth of arginine-degrading microorganisms, thereby slowing the overall rate of arginine decomposition. Additionally, the low expression of pathways related to β -nicotinamide mononucleotide in *Lactobacillus plantarum* affects its utilization of exogenous nicotinamide [59]. This metabolic characteristic might be an important factor contributing to the observed differences in the relative abundance of this bacterium among treatments.

4.5 Effects of Spray Treatment on Plant Physiology and Microorganisms

Recent studies have shown that cell wall integrity can significantly affect the fermentation performance of leguminous forages [60–62]. The intact cellular structure provides an initial barrier against microbial access to entrapped nutrients [63]. The purpose of ensiling is to induce orderly cell death under anaerobic conditions, thereby facilitating the release and transformation of nutrients. During the first two days of fermentation, the rapid decrease in water-soluble carbohydrate (WSC) content indicates [64] that lactic acid bacteria primarily utilize soluble sugars from intercellular spaces and the cytosol, along with nutrients released from cell wall disintegration driven by acidification and mechanical compression. Plant cells do not die immediately at the onset of ensiling; they can continue to undergo respiration and enzymatic hydrolysis for up to 12 hours. During this period, residual oxygen within the silo is consumed through the aerobic respiration of the plant cells [65]. In the present study, it is hypothesized that in the ND65 treatment, plant cells remained in a partially viable state during the initial stage of ensiling. Cations such as potassium and calcium within the alfalfa cells likely formed buffering systems with organic acids, enabling the cells to resist the acidic environment and delaying widespread cell lysis. In contrast, for the PS65 treatment, the reintroduction of free water to wilted forage would have disrupted the osmotic balance across the cell membrane, leading to a loss of selective permeability and subsequent

leakage of a large volume of ions and small organic molecules into the intercellular spaces. Consequently, the medium for microbial growth transitioned from a solid to a semi-liquid phase. This change would have significantly increased the contact efficiency between microorganisms and nutrients compared with solid-state fermentation, as concentration gradients are eliminated and there is no initial requirement for the secretion of large amounts of enzymes to degrade a solid substrate [66]. Therefore, the inoculated lactic acid bacteria had access to sufficient substrate for rapid proliferation. This mechanism likely explains why the pH of PS65 was significantly lower than that of ND65, and why the lactic acid concentration was significantly higher.

It is well-established that bacteria generally require higher water activity for growth compared to yeasts, while molds have the lowest requirement [67]. Therefore, materials with the same total moisture content can differ in their ability to support microbial growth, depending on the ratio of free to bound water, which defines their water activity [68]. In the present study, the observed differences in bacterial and fungal diversity between the water spraying and wilting treatments may be attributed to variations in water activity. Water spray treatment likely increased the free water content within the silage system, creating water activity conditions that were more favorable for the growth of lactic acid bacteria while inhibiting fungal proliferation. Although the optimal moisture range for *Clostridium* can overlap with that of lactic acid bacteria, water spray treatment at 65% moisture significantly reduced both the ammonia nitrogen/total nitrogen ratio and butyric acid content in this study. These findings suggest that the proliferation of *Clostridium* could be managed by a combination of inoculating with lactic acid bacteria, maintaining a moisture content below 65%, and applying free water via spraying. In conclusion, under appropriate moisture conditions, water spray treatment can significantly improve the fermentation quality of silage.

5. Conclusion

Within an appropriate moisture range, spraying water on wilted alfalfa modulates the microbial community, increasing the relative abundance of *Lentilactobacillus*, inhibiting the growth of pathogenic fungi such as *Fusarium* and *Aspergillus*, and reducing mycotoxin accumulation. These effects ultimately improve the nutritional quality and feed safety of alfalfa silage. Spray water treatment significantly reduced pH and increased LA concentrations ($P < 0.05$) by modulating metabolic pathways, including ascorbic acid and aldonic acid metabolism, phenylpropanoid biosynthesis, and phenylalanine metabolism. Future studies should investigate the effects of supplemental free water on the activity of cell wall-degrading enzymes from lactic acid bacteria during the early stage of ensiling. It is also recommended to determine the optimal growth ranges of key microorganisms, such as *Lactobacillus plantarum* and *Clostridium* species, under varying water activity conditions. Additionally, a systematic analysis of microbial community dynamics during the initial plant cell disintegration phase of fermentation is warranted, with particular attention to the successional patterns of lactic acid bacteria. These investigations will provide a more precise theoretical foundation for elucidating the mechanisms by which water spraying regulates silage fermentation.

Abbreviations

DM	Dry Matter
CP	Crude Protein
NDF	Neutral Detergent Fiber
ADF	Acid Detergent Fiber
WSC	Water Soluble Carbohydrates
NH ₄ ⁺ -N	Ammonia Nitrogen
LA	Lactic Acid
AA	Acetic Acid
PA	Propionic Acid
BA	Butyric Acid
OA	Organic Acids

Declarations

Data availability

Sequence data that support the findings of this study have been deposited in NCBI SRA under accession number PRJNA1444882.

Ethics approval and consent to participate

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication

All authors have read and approved the final manuscript. They consent to its publication in BMC Plant Biology.

Authors' contributions

Guolin Yang: Conceptualization, Formal analysis, Methodology, Investigation, Data curation, Writing – original draft. **Heng Jiang:** Investigation, Data curation. **Haoran Wang:** Investigation, Data curation. **Zhennan He:** Methodology, Data curation. **Zhaoming Wang:** Investigation. **Si-Yi Wang:** Data curation. **Yuan-Yuan Jing:** Investigation, Data curation, Writing – review & editing. **Feng-Qin Gao:** Supervision, Investigation, Data curation, Funding acquisition, Writing – review & editing.

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Compliance statement

The plant experimental research conducted in this study complied with all relevant guidelines of the authors' institution and China, as well as international standards.

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Figures

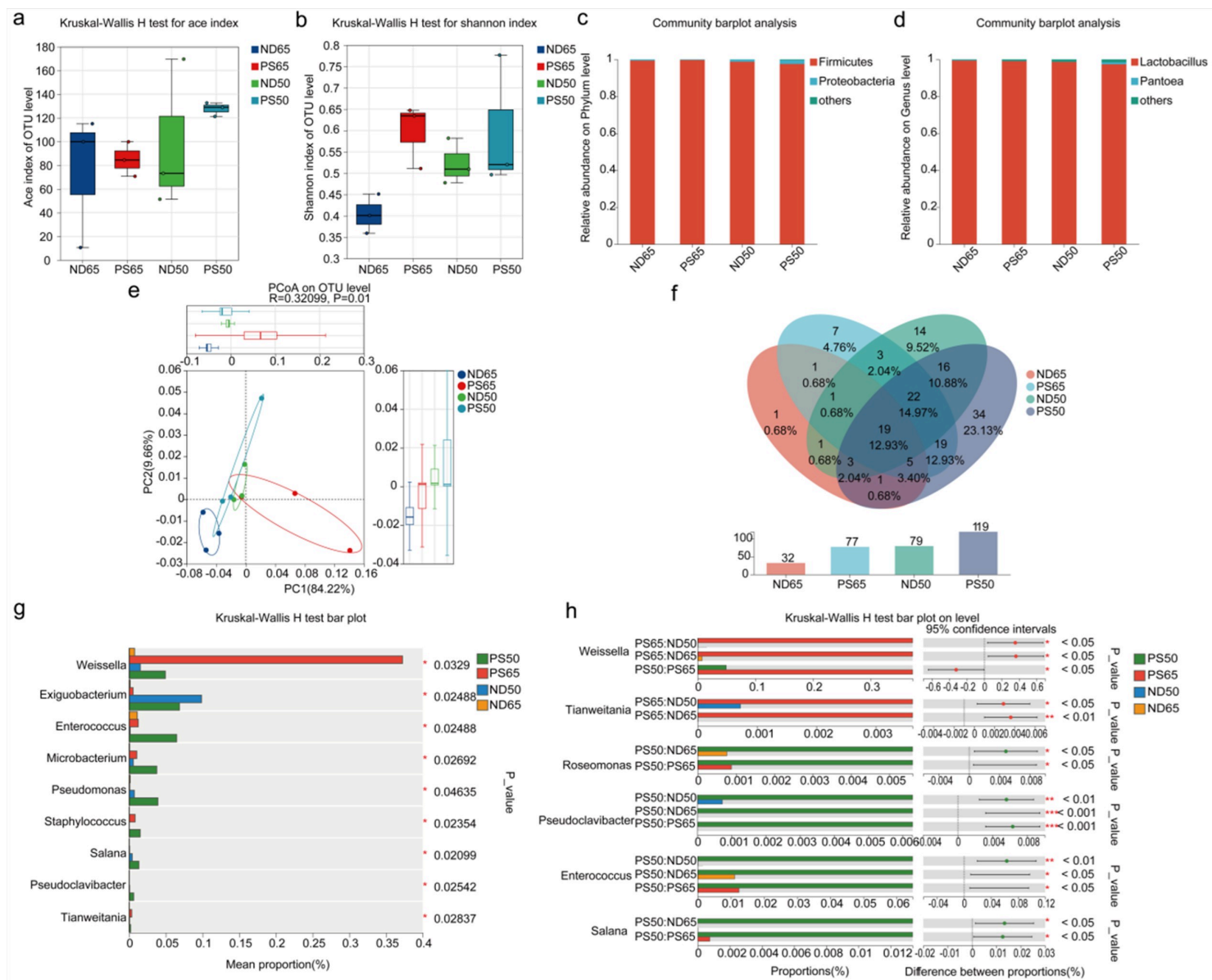


Figure 1

Bacterial community diversity and composition of alfalfa silage after 90 days of ensiling, showing: (a) α -diversity indices; (b) β -diversity visualization based on PCoA; (c) relative abundance of bacterial communities at the phylum level; (d) relative abundance of bacterial communities at the genus level; (e) Principal Coordinate Analysis (PCoA) plot; (f) Venn diagram of shared and unique ASVs; (g) comparison of the relative abundances of key genera among all treatments; and (h) pairwise comparison of the relative abundances of specific genera. ND65, natural wilting to 65% moisture content; PS65, natural wilting to 50% moisture content followed by water spraying to reach 65%; ND50, natural wilting to 50% moisture content; and PS50, natural wilting to 35% moisture content followed by water spraying to reach 50%. In Figure, asterisks and "NS" on the right side of the bar charts indicate significant differences based on the Kruskal-Wallis test: *** denotes $p < 0.001$; ** denotes $p < 0.01$; * denotes $p < 0.05$; NS indicates no significant difference.

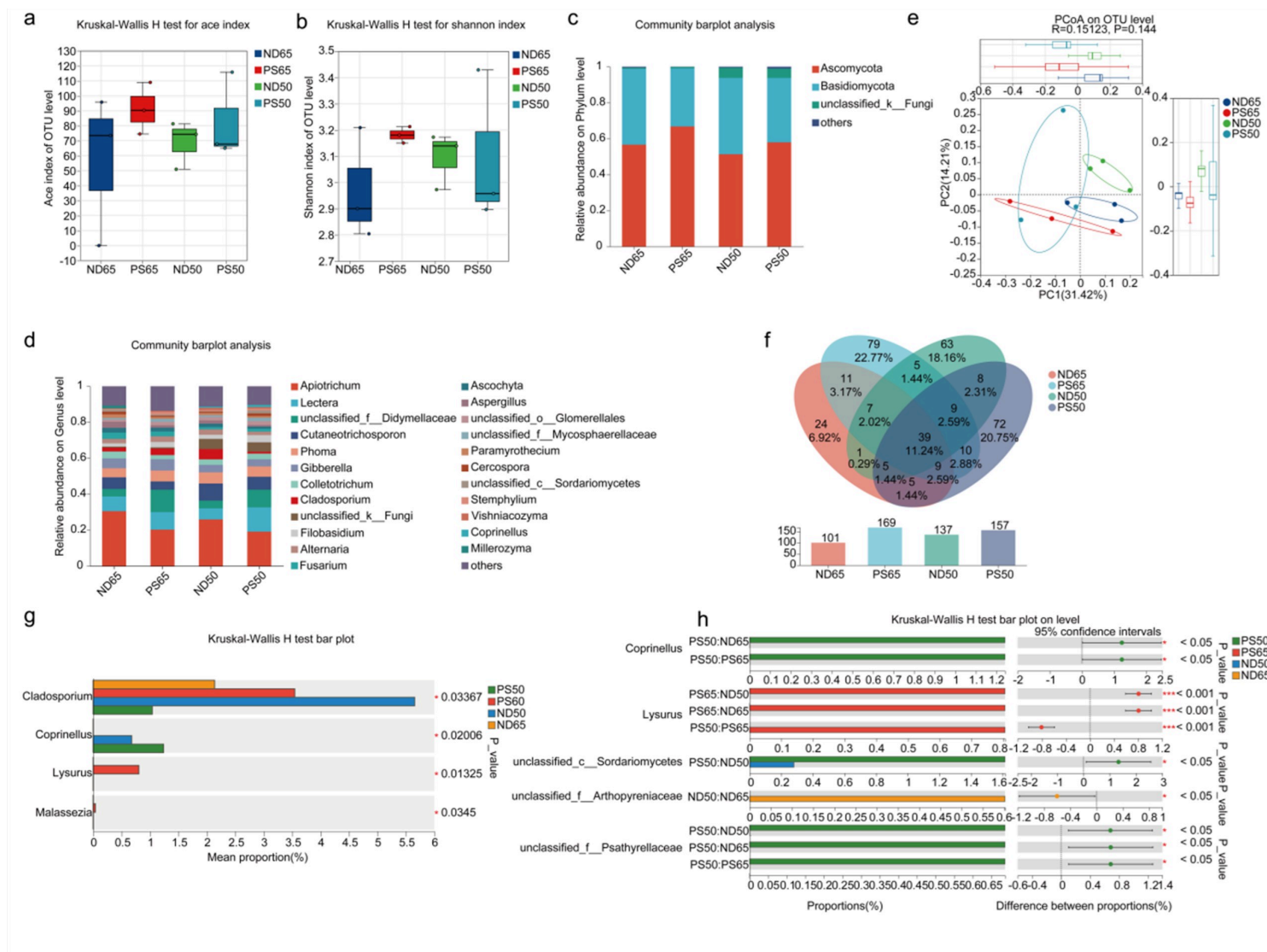


Figure 2

Fungal community diversity and composition of alfalfa silage after 90 days of ensiling, showing: (a) α -diversity indices; (b) β -diversity visualization based on PCoA; (c) relative abundance of fungal communities at the phylum level; (d) relative abundance of fungal communities at the genus level; (e) Principal Coordinate Analysis (PCoA) plot; (f) Venn diagram of shared and unique ASVs; (g) comparison of the relative abundances of key genera among all treatments; and (h) pairwise comparison of the relative abundances of specific genera. Treatments were defined as: ND65, natural wilting to 65% moisture content; PS65, natural wilting to 50% moisture content followed by water spraying to reach 65%; ND50, natural wilting to 50% moisture content; and PS50, natural wilting to 35% moisture content followed by water spraying to reach 50%. In Figure, asterisks and "NS" on the right side of the bar charts indicate significant differences based on the Kruskal-Wallis test: *** denotes $p < 0.001$; ** denotes $p < 0.01$; * denotes $p < 0.05$; NS indicates no significant difference.

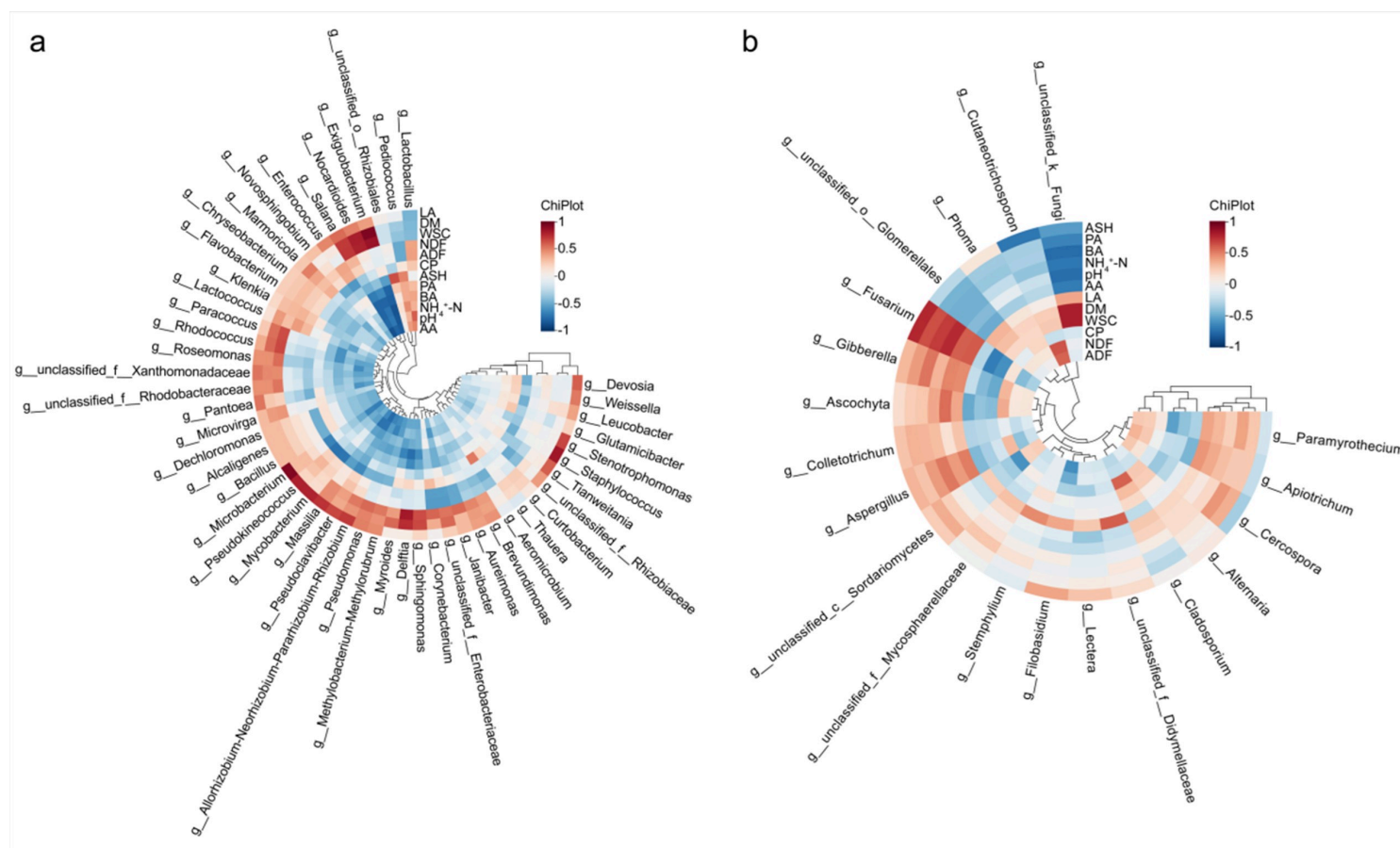


Figure 3

Heatmap analysis showing correlations between fermentation quality parameters and the relative abundances of (a) bacterial genera and (b) fungal genera.

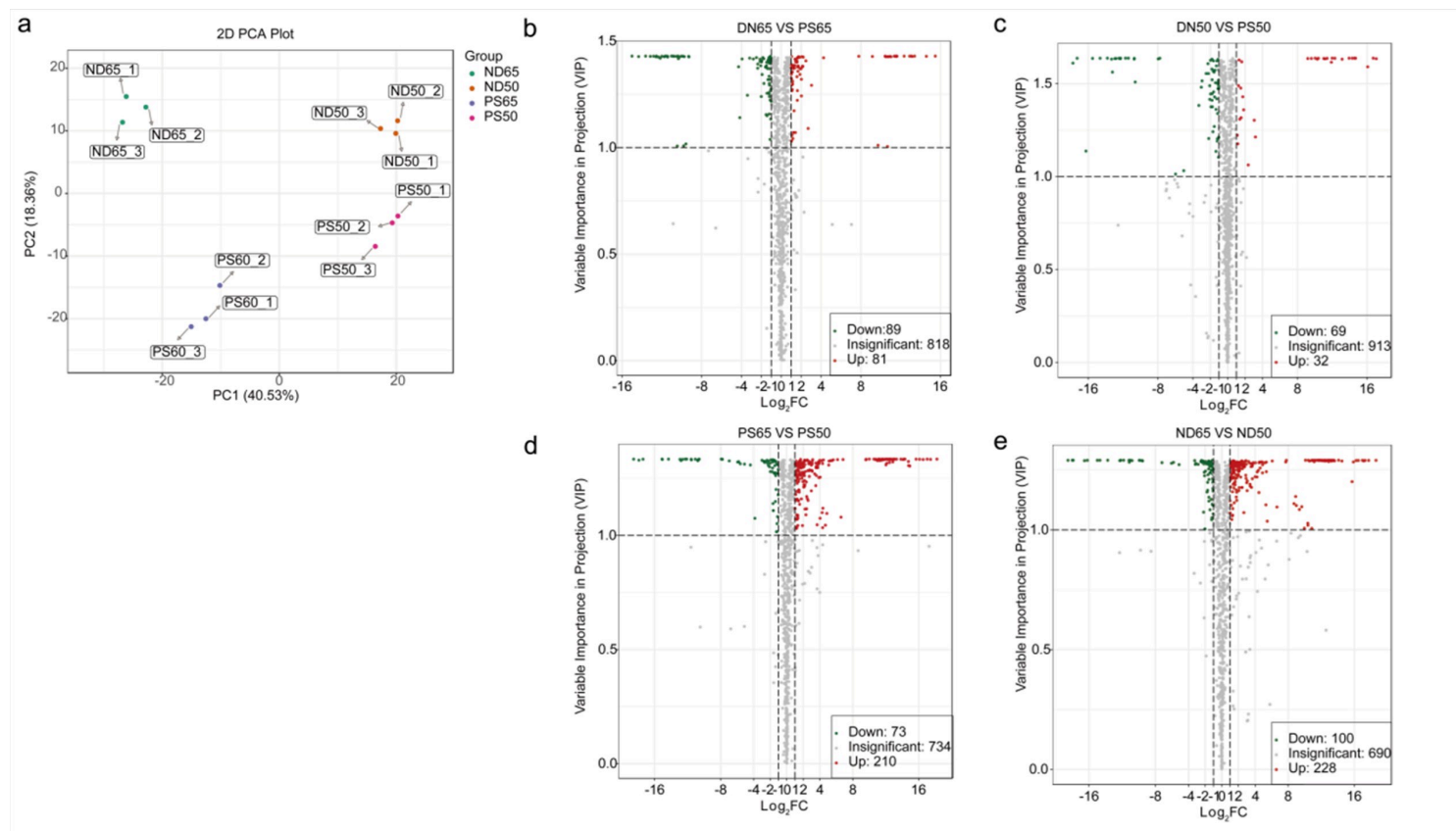


Figure 4

Metabolomic analysis of alfalfa silage samples. (a) Principal Component Analysis (PCA) plot of metabolite profiles. The percentage of variance explained by each principal component (PC1 and PC2) is indicated on the axes. Each point represents an individual sample, with different colors denoting different treatment groups. (b-e) Volcano plots showing differential metabolites between treatment groups: (b) ND65 vs. PS65, (c) ND50 vs. PS50, (d) PS65 vs. PS50, and (e) ND65 vs. ND50. In the volcano plots, red points denote significantly up-regulated metabolites, green points denote significantly down-regulated metabolites, and gray points represent non-significantly changed metabolites. The x-axis represents the $\log_2$ (Fold Change) of relative metabolite content, while the y-axis represents the Variable Importance in Projection (VIP) value.

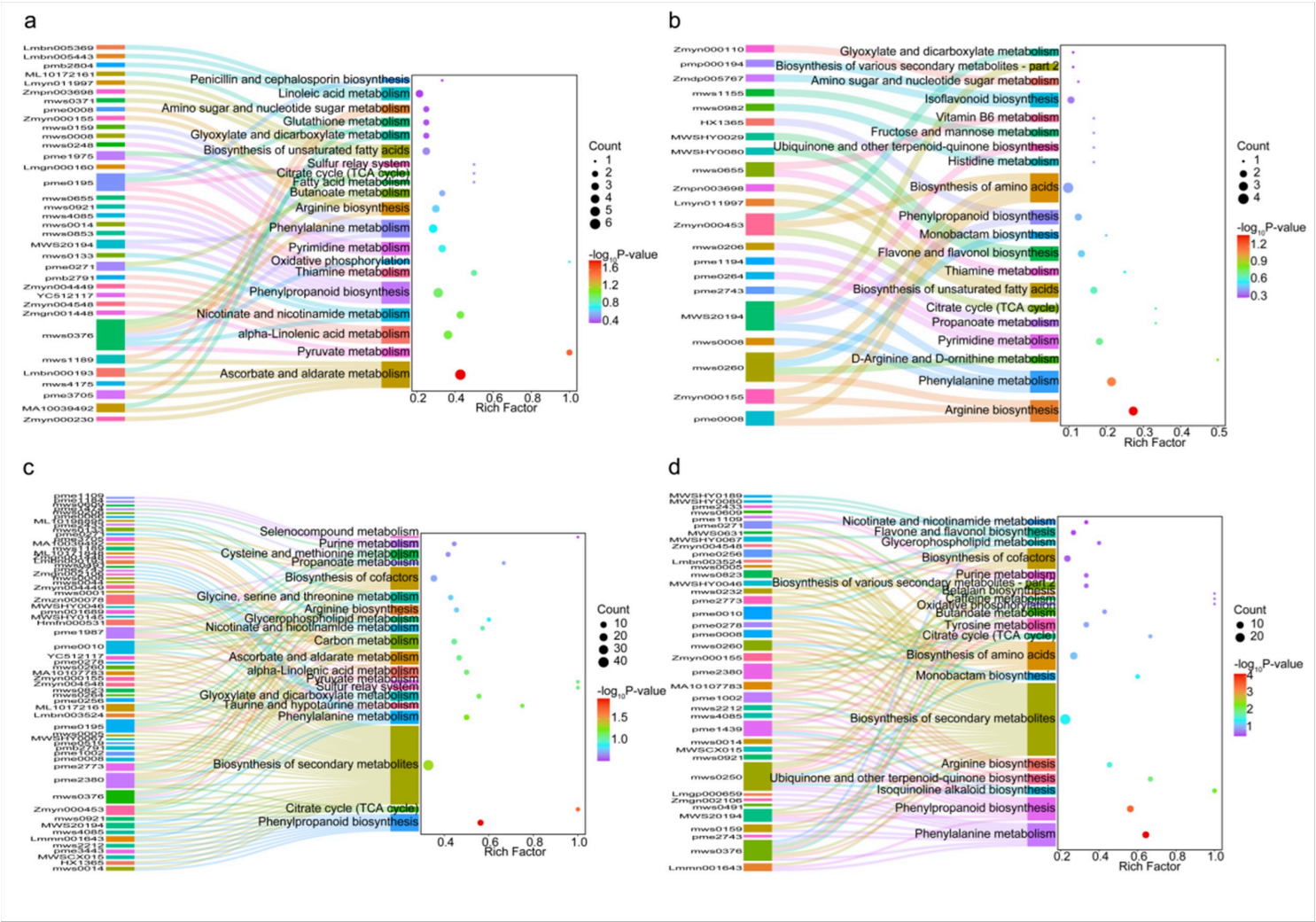


Figure 5

Sankey bubble diagrams visualizing metabolic pathway enrichment and metabolite flow. Panels show comparisons between treatment groups: (a) ND65 vs. PS65, (b) ND50 vs. PS50, (c) ND50 vs. ND65, and (d) PS50 vs. PS65. In the bubble plots, the x-axis represents the Rich Factor, the y-axis lists the pathway names, point size corresponds to the number of differential metabolites in a pathway, and point color indicates the enrichment significance (as $-\log_{10}(P\text{-value})$), with redder colors denoting higher significance. In the Sankey diagrams, nodes represent individual metabolites, and the thickness of the edges represents the metabolic flow between them.

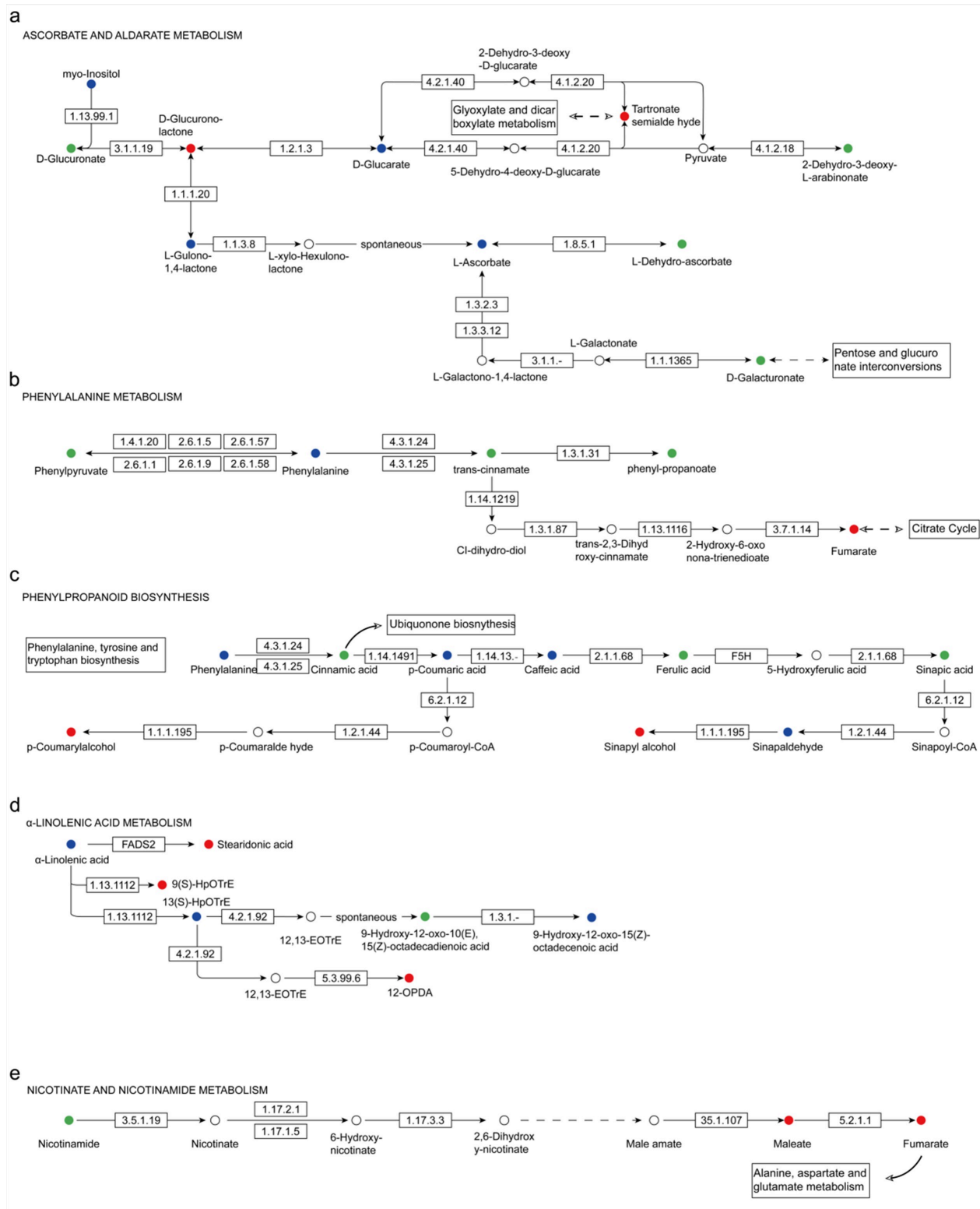


Figure 6

KEGG pathway maps illustrating key differential metabolic pathways: (a) ascorbate and aldarate metabolism, (b) phenylalanine metabolism, (c) phenylpropanoid biosynthesis, (d) α-linolenic acid metabolism, and (e) nicotinate and nicotinamide metabolism. Within the pathways, red indicates significantly up-regulated metabolites, green indicates significantly down-regulated metabolites, and blue indicates metabolites that were detected but showed no significant change.

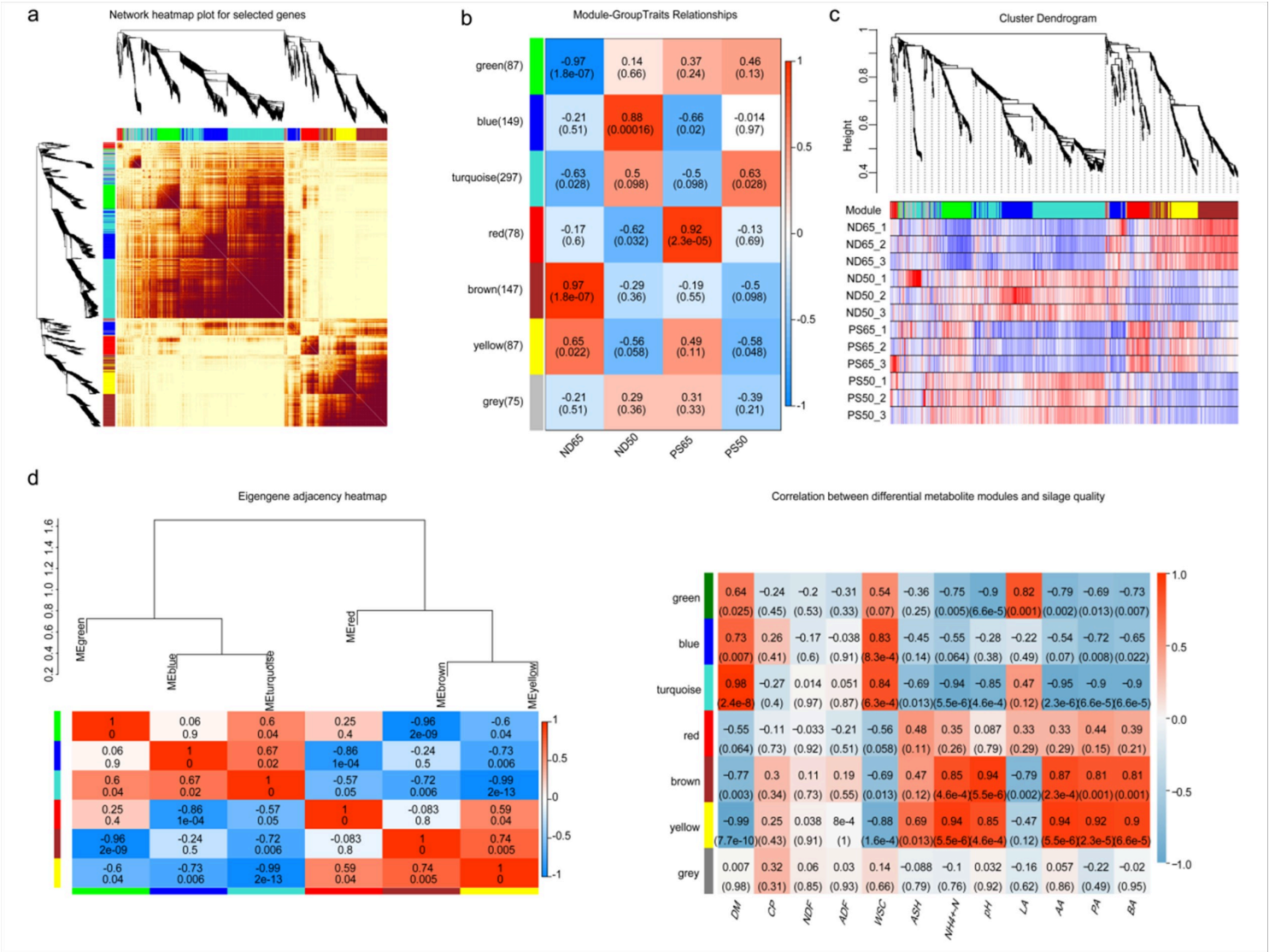


Figure 7

Weighted Co-expression Network Analysis (WGCNA) of metabolites in alfalfa silage. (a) Hierarchical clustering dendrogram of metabolites with module assignments and a corresponding heatmap of the topological overlap matrix, where color intensity from white to red indicates stronger connectivity. (b) Heatmap showing the correlations between module eigengenes and treatment groups. (c) Dendrogram of clustered module eigengenes. (d) Heatmap illustrating the correlations among module eigengenes. (e) Heatmap showing the correlations between module eigengenes and silage fermentation quality parameters. In the correlation heatmaps (b and e), color intensity represents the strength and direction of the correlation between modules and sample traits.

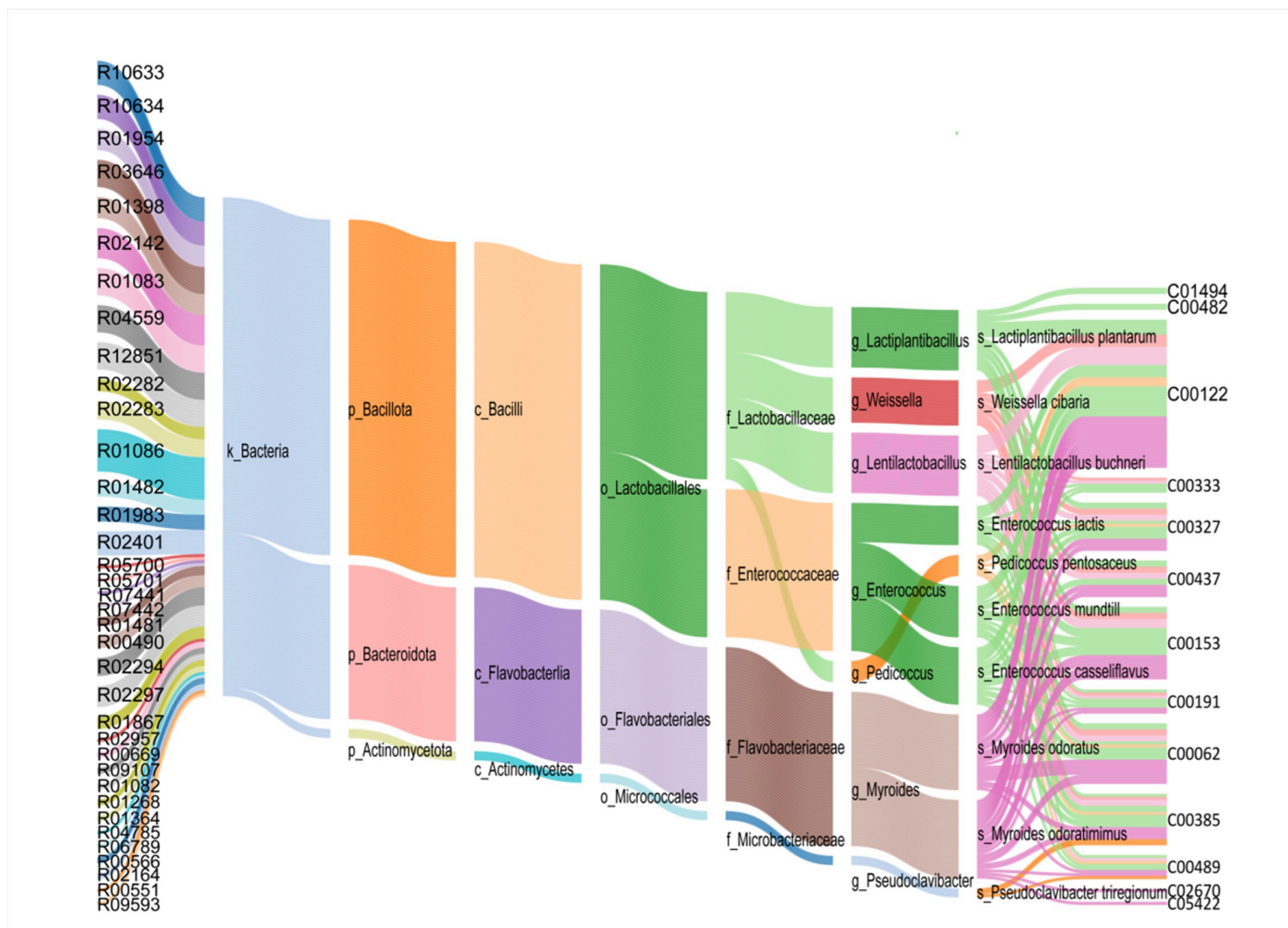


Figure 8

Sankey diagram illustrating the results of the microbial source tracking analysis. Nodes in the diagram represent different taxonomic levels, and the thickness of the edges corresponds to the proportional contribution or flow between these taxa.

Supplementary Files

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