

Recycling of *Lycium barbarum* by-products and bioactive substance application in silage - Insight into antioxidant activity and the regulation mechanism of anaerobic fermentation

Yajun Zhang

Ningxia University

Jiale Liao

Ningxia University

Yongxiang Pan

Ningxia University

Qing Zhang

South China Agricultural University

Qiang Lu

Ningxia University

Xiaodong Chen

Ningxia University

Wencan Ke

Ningxia University

Guijie Zhang

guijiezhong@nxu.edu.cn

Ningxia University

Research Article

Keywords: Silage, Plant extracts, Metabolic profile, Microbial community, Fermentation quality

Posted Date: April 22nd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-5960294/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Microbiology on July 11th, 2025. See the published version at <https://doi.org/10.1186/s12866-025-04033-0>.

Title Page

Title:

Recycling of *Lycium barbarum* by-products and bioactive substance application in silage - Insight into antioxidant activity and the regulation mechanism of anaerobic fermentation

Authors:

Yajun Zhang^a, Jiale Liao^a, Yongxiang Pan^a, Qing Zhang^b, Qiang Lu^a, Xiaodong Chen^c, Wencan Ke^{a*}, Guijie Zhang^{c*}.

Affiliations and addresses:

^a College of Forestry and Prataculture, Ningxia University, Yinchuan, Ningxia 750021, China

^b College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou 510642, China

^c College of Animal Science and Technology, Ningxia University, Yinchuan, Ningxia 750021, China

***Corresponding Author:**

Guijie Zhang

Wencan Ke

Email: guijiezhong@nxu.edu.cn

Email: kewencan@nxu.edu.cn

Tel: 86-9512062861

Fax: 86-9512062861

Address: College of Forestry and Prataculture, Ningxia University, Yinchuan, Ningxia 750021, China

ABSTRACT

Silage, a traditional anaerobically fermented feed primarily used for ruminants, can be enhanced by incorporating *Lycium barbarum* by-products (LBB) to improve its functional properties. LBB is rich in bioactive compounds with notable medicinal and nutritional benefits, positioning it as a promising alternative to conventional additives. This study investigates the effects of LBB and its bioactive components—*Lycium barbarum* polysaccharide (LBP), flavonoid (FLA), and betaine (BET) on fermentation parameters, bacterial communities and metabolites in alfalfa silages. LBB, LBP, FLA, and BET significantly increased lactic acid (LA) and water-soluble carbohydrates (WSC) production ($P < 0.05$), with LBP contributing the highest increases in LA (64.54%) and WSC (70.34%) compared to the control. Furthermore, LBB, LBP, FLA, and BET significantly reduced dry matter loss (DM loss) and ammonia nitrogen ($\text{NH}_3\text{-N}$) ($P < 0.05$), with LBB achieving a 70.39% reduction in DM loss and a 35.18% decrease in ammonia nitrogen. The application of these treatments also enhanced the antioxidant properties of silage, as evidenced by increased total antioxidant capacity (T-AOC) and glutathione peroxidase (GSH-Px) levels ($P < 0.05$). Notably, the relative abundance of *Lactiplantibacillus* and *Pediococcus* was elevated in LBB and BET-treated silages, respectively. The bioactive components of LBB demonstrated potent antibacterial activity, with differential metabolites enriched in flavonoid and isoflavonoid biosynthesis pathways. Correlation analysis further revealed relationships between specific metabolites and microbiota. These findings highlight the potential of LBB as a valuable strategy for enhancing silage quality.

Keywords: Silage; Plant extracts; Metabolic profile; Microbial community; Fermentation quality

Abbreviations: DM, dry matter; FW, fresh weight; LA, lactic acid; AA, acetic acid; PA, Propionic acid; BA, butyric acid; WSC, water soluble carbohydrates; CP, crude protein; NH₃-N, ammonia N; NDF, neutral detergent fiber; ADF, acid detergent fiber; LAB, lactic acid bacteria; cfu, colony-forming units; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable importance in the projection; LDA, Linear Discriminant Analysis; LEfSe, Linear Discriminant Analysis Effect Size; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; CON, control group; LBP, silage with *Lycium barbarum* polysaccharide; LBB, silage with *Lycium barbarum* by-products; FLA, silage with flavonoid; BET, silage with betaine.

Background

Lycium barbarum a traditional herbal medicine originating from East Asia, is renowned for its substantial medicinal and nutritional benefits (Skenderidis et al., 2019). Its bioactive compounds, such as *Lycium barbarum* polysaccharides (LBPs), flavonoids, betaine, polyphenols, vitamins, and trace elements (Lu et al., 2019), exhibit diverse properties, including anti-tumor, anti-inflammatory, antioxidant, hypolipidemic, and hypoglycemic effects (Azami and Sun, 2019; Tian et al., 2019; Zhang et al., 2020). As modern medicine increasingly explores its pharmacological potential, the consumption of *Lycium barbarum* has steadily risen due to growing awareness of its health benefits. However, *Lycium barbarum* by-products (LBB), including fruit residues and stalks, are often discarded despite their rich nutrient contents and bioactive compounds. This inefficiency leads to resource wastage, environmental pollution, and considerable economic loss. Therefore, improving resource recycling and promoting sustainability

by utilizing LBB more effectively is critical. Previous studies have highlighted LBB's potential to enhance immune function, productivity, and product quality in livestock (Chen et al., 2020; Menchetti et al., 2020). Fermented LBB feed has been shown to improve feed intake and immunity in ruminants (Zhang et al., 2022). Furthermore, the inclusion of 5% fermented LBB improved slaughter performance in Tan sheep, enhancing meat quality by increasing total antioxidant capacity, color, tenderness, and water-holding capacity while significantly reducing malondialdehyde content (Liao et al., 2024).

Recently, plant-derived bioactive compounds have gained attention as viable alternatives to antibiotic feed additives in livestock (Wang et al., 2019a). Bioactive components extracted from LBB, such as LBPs, flavonoids, and betaine, have garnered interest due to their various pharmacological effects. LBPs, the key bioactive ingredient in LBB, promote the growth of beneficial gut bacteria, inhibit harmful pathogens, and enhance immune function and antioxidant activity, ultimately improving animal growth performance (Chen et al., 2020; Long et al., 2020). Betaine has been linked to enhanced animal performance and carcass quality (Dong et al., 2020; Pardo et al., 2023). Additionally, flavonoids exhibit antioxidant, anti-inflammatory, and anti-apoptotic properties, which contribute to increased productivity in ruminant animals (Olagaray and Bradford, 2019). Incorporating LBB and its extracts into livestock diets can improve feed utilization, animal production, and health, making them beneficial additives in livestock feed.

The rising demand for ruminant livestock products, driven by the growing global population, is being constrained by the limited availability of high-quality forage, hindering the advancement of grain-saving animal husbandry. Alfalfa silage, a high-quality feed with notable protein and digestibility potential, plays a pivotal role in

meeting this demand (Hartinger et al., 2019). As the need for silage grows, so do the requirements for enhanced silage quality, functionality, safety, and utilization efficiency. Chinese herbal medicines are increasingly being explored as potential additives for fermented feed, improving fermentation quality and flavor, promoting beneficial metabolites, and yielding positive effects on livestock (Lin et al., 2020), as well as offering potential health benefits to human consumers. Given the characteristics of LBB and their extracts, it is hypothesized that their inclusion in alfalfa silage could help reduce the abundance of undesirable bacteria. Beyond their nutritional value, these extracts may improve the overall functionality of the feed. However, further research is necessary to comprehensively understand the impact of these extracts on silage fermentation.

This study aimed to evaluate the influence of LBB and its primary bioactive components—LBPs, flavonoids, and betaine—on the fermentation quality, antioxidant properties, microbial communities, and metabolites of alfalfa silage. The objectives were to assess how LBB and its extracts affect fermentation quality and antioxidant properties and to examine the influence of LBPs, flavonoids, and betaine on fermentation parameters, microbial communities, and silage metabolites. This research provides a scientific foundation for the use of LBB and its extracts as silage additives, enhancing fermentation quality and improving the efficiency of agricultural waste resource utilization.

Methods and materials

Raw materials and silage preparation

The first-cut alfalfa (*Medicago sativa*; WL 440) was harvested at the early bloom stage from four random fields on a farm in Yinchuan, Ningxia, China. Immediately after harvesting, the alfalfa was chopped into 1-2 cm pieces using a hay cutter and

transported to the laboratory. The chopped alfalfa was then wilted to achieve a dry matter (DM) content of approximately 350 g/kg fresh weight (FW) for silage production. Fresh alfalfa from each field was frozen at -20°C for subsequent analysis, while the remaining forage was randomly divided into five small piles, each assigned to one of the following treatments: (1) no additives (control, CON); (2) *Lycium barbarum* by-products (LBB, 6% FW); (3) *Lycium barbarum* polysaccharide (LBP, 0.63% FW); (4) flavonoid (FLA, 0.24% FW); (5) betaine (BET, 0.08% FW). Based on previous studies (Zhang et al., 2022), which indicated that the addition of 4–6% of LBB in feed yields optimal effects on animal growth, a 6% of LBB concentration was used in this study. LBB consists of crushed *Lycium barbarum* fruit residues and stalks. Three primary bioactive components were extracted from LBB: LBP, FLA, and BET. The concentrations of these components in LBB are 47.1 mg/g, 30.0 mg/g, and 10.0 mg/g, respectively, with purities of 45.1%, 75.3%, and 80.4%. The amounts of bioactive components added were based on their concentrations in LBB, and treatments were adjusted with water and mixed evenly with solutions. The chemical composition of both fresh alfalfa and LBB was determined (Table 1). Approximately 500 g of forage was placed into polyethylene plastic bags (450×280 mm) and vacuum-sealed tightly using a vacuum packaging machine (Zhucheng Yizhong Machinery Ltd., Shandong, China). The ensiled 20 bags were then stored at ambient temperature ($22\text{--}25^{\circ}\text{C}$) for 90 days.

Chemical composition, microbial population and silage quality analysis

After 90 days of fermentation, samples were collected to assess chemical composition and fermentation quality. A 20 g sample of fresh silage was mixed with 180 mL distilled water and homogenized for 30 seconds in a juicer (BA-828, Mannengda Plastics Co., Ltd., Guangdong, China) (Ke et al., 2018). A portion of the filtrate was acidified with 50% H_2SO_4 and then filtered using a $0.22\text{ }\mu\text{m}$ dialyzer. The

resulting filtrate was analyzed for organic acids using high-performance liquid chromatography (KC-811, column, Shodex, Shimadzu, Japan), as previously described (Guo et al., 2019). Ammonia nitrogen (NH₃-N) content was determined following the method of Broderick and Kang (1980).

Lactic acid bacteria (LAB), yeast, and mold counts in wilted alfalfa and ensiled forage were determined using the procedure described by Reich and Kung (2010).

Briefly, 10 g of sample was homogenized in 90 mL of sterile Ringer's solution for 1 minute and serially diluted. Then, LAB was enumerated using Rogosa agar and incubated at 30°C for 48-72 hours. Yeasts and molds were counted by pour plating with serial 10-fold dilutions of water extracts on malt extract agar, followed by incubation at 32°C for 48-72 hours under aerobic condition. Fresh alfalfa and silage samples were dried at 65°C for 72 hours to measure DM content. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an ANKOM fiber analyzer (A2000i, ANKOM Technology, New York, USA) following the methods of Van Soest et al. (1991) and Robertson and Van Soest (1981), respectively. Water-soluble carbohydrates (WSC) were measured using the anthrone-concentrated sulfuric acid method (Murphy, 1958) and quantified using an ultraviolet spectrophotometer (UV-1800, Shanghai Ma Pada Instrument Co., Ltd, Shanghai, China). Crude protein (CP) content was determined using the Kjeldahl method (AOAC, 2000).

Antioxidant analysis of silage

The concentrations of total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) were quantified using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. The free radical scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was analyzed following the method

of More and Makol (2020).

DNA extraction, metagenomic sequencing, annotation, and analysis

Genomic DNA was extracted from fresh and ensiled alfalfa samples using the E.Z.N.A.[®] Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.), following the manufacturer's guidelines. DNA concentration and purity were assessed using the TBS-380 and NanoDrop 2000, respectively. DNA quality was evaluated on a 1% agarose gel. To prepare for paired-end library construction, DNA was fragmented to approximately 400 bp via sonication using a Covaris M220 (Gene Company Limited, China). A paired-end sequencing library was generated using the TruSeq[™] DNA Sample Prep Kit (Illumina, San Diego, CA, USA), with adapters containing sequencing primer sites ligated to blunt-ended DNA fragments. Sequencing was performed on the Illumina NovaSeq 6000 platform using a 150-bp paired-end strategy at Majorbio BioPharm Technology Co., Ltd (Shanghai, China). Metagenomic sequences were filtered to remove low-quality sequences and adapters, producing clean data using Sickle software (<https://github.com/najoshi/sickle>). Cleaned data was assembled using MEGAHIT software (Li et al., 2015). Representative sequences from the non-redundant gene catalog were aligned to the non-redundant (NR) database with an e-value cutoff of $1e^{-5}$ using Diamond (Buchfink et al., 2014) software (<http://www.diamondsearch.org/index.php>, version 0.8.35) for taxonomic annotations. Cluster of orthologous groups (COG) annotations for representative sequences were conducted against the eggNOG database, applying an e-value cutoff of $1e^{-5}$. The KEGG annotations were performed against the Kyoto Encyclopedia of Genes and Genomes database (<http://www.genome.jp/kegg/>) with the same e-value cutoff.

Metabolite extraction and LC-MS/MS analysis

A 50 mg solid sample was placed in a 2 mL centrifuge tube with a 6 mm grinding

bead. **To this**, 400 μ L of extraction solution (methanol:water = 4:1, v/v) containing 0.02 mg/mL of internal standard (L-2-chlorophenylalanine) was added. The samples were ground for 6 **minutes** at -10°C using a Wonbio-96c, followed by 30 **minutes** of low-temperature ultrasonic extraction at 5°C . After incubation at -20°C for 30 minutes, the samples were centrifuged for 15 **minutes at 4°C and 13,000 g**. The supernatant was transferred to an injection vial for LC-MS/MS analysis. A pooled quality control (QC) sample was prepared by mixing equal volumes of all **individual** samples. The QC samples were handled and **analyzed in the same manner as** the analytical samples and were injected at regular intervals (every 5-15 samples) to monitor **the stability of the** analysis.

LC-MS/MS analysis was **conducted** on a Thermo UHPLC-Q Exactive HF-X system, equipped with an ACQUITY HSS T3 column (100 mm $\times$ 2.1 mm i.d., 1.8 μm ; Waters, USA), at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The mobile phases **used** were **solvent A**: 0.1% formic acid in water: acetonitrile (95: 5, v/v) and **solvent B**: 0.1% formic acid in acetonitrile: isopropanol: water (47.5: 47.5, v/v). The flow rate was **set to** 0.40 mL/min, and the column temperature was maintained at 40°C . The injection volume was 3 μL . MS conditions included a Thermo UHPLC-Q Exactive Mass Spectrometer with electrospray ionization (ESI) in both positive and negative modes, with **a source temperature of 400°C** , sheath gas **flow of 40 arb**, and **auxiliary gas flow of 10 arb**. The ion-spray voltage floating (ISVF) was **set to -2800V for negative mode and 3500V for positive mode**. MS/MS collision energy was **set to 20-40-60V**, with **a full MS resolution of 70,000 and an MS/MS resolution of 17,500**. Data acquisition was **performed in** Data Dependent Acquisition (DDA) mode, covering a mass range of 70-1050 m/z.

Metabolomic data preprocessing and analysis

LC/MS **raw** data were processed using Progenesis QI (Waters Corporation ,
Milford, USA) software to identify peaks, remove noise, and **generate** a data matrix
containing retention time, mass-to-charge ratio (m/z) values, and peak intensities.
Principal component analysis (PCA) and orthogonal partial least squares discriminant
analysis (OPLS-DA) were conducted using **the** R package (Version 1.6.2). Metabolites
with the variable importance in projection (VIP) > 1.0 and **P-values from** the Student's
t-test ($P < 0.05$) were considered significantly different. Differential metabolites were
mapped to biochemical pathways **using the** Kyoto Encyclopedia of Genes and Genomes
(KEGG) database (<http://www.genome.jp/kegg/>) and **further** enriched using Python
packages (<https://docs.scipy.org/doc/scipy/>).

Statistical analysis

All data were analyzed using SPSS Statistics software (version 23.0, IBM,
Chicago, USA) to detect significant differences between treatments, where $P < 0.05$,
was considered statistically significant, and $0.05 < P < 0.10$ was **interpreted as showing**
a trend. Correlation tests were **conducted** using Pearson's correlation coefficient.

Results and Analysis

Characteristics of raw material before ensiling

The chemical composition and microbial **populations** of dry LBB and fresh alfalfa
are **presented** in Table 1. Fresh alfalfa was wilted to a DM content of 375 g/kg. Both
alfalfa and LBB **demonstrated** considerable CP content, with values of 191 g/kg DM
and 154 g/kg DM, respectively. **The fiber** contents were moderate, with **alfalfa showing**
419 g/kg DM for NDF and 275 g/kg DM for ADF, while LBB contained 138 g/kg DM
for NDF and 275 g/kg DM and 110 g/kg DM for ADF.

WSC contents were 61.10 g/kg DM **for LBB** and 70.50 g/kg DM **for alfalfa**. The
epiphytic microbial populations of wilted alfalfa **comprised** yeasts and molds at counts

of 5.64 and 4.20 log₁₀ cfu/g FW, respectively, while LAB were present at 5.48 log₁₀ cfu/g FW. In LBB, the microbial counts of LAB, yeasts, and molds were 4.79, 4.79, and 2.86 log₁₀ cfu/g FW, respectively.

Fermentation parameters, microbial population and chemical composition of silages

The fermentation parameters, microbial populations, and chemical composition of the silages after 90 days of ensiling are summarized in Table 2. The pH values differed significantly among treatments ($P < 0.01$), with the CON having the highest pH (5.09), and LBB and LBP treatments showing the lowest values (4.50 and 4.47, respectively). LA content was significantly higher ($P < 0.01$) in treated silages, whereas acetic acid (AA) content was lower ($P < 0.01$) in these silages compared to the CON. PA was not detected in LBP, FLA, and BET treatments, with the CON exhibiting the highest PA content. Butyric acid (BA) was undetected in all silages. NH₃-N concentration was significantly lower in the LBB and bioactive component-treated silages compared to the CON ($P < 0.01$).

The DM content was highest in the LBB group, followed by the LBP, FLA, and BET groups. DM loss was significantly lower in the LBB group compared to the other treatments. The contents of NDF and ADF were slightly reduced in the LBB group compared to the CON. The WSC content was significantly higher in the LBP group, followed by FLA and LBB, with the CON having the lowest WSC content.

Antioxidant properties of silages

The activities of T-AOC, SOD, CAT, and GSH-Px after 90 days of ensiling were shown in Table 3. LBB and its bioactive components significantly influenced T-AOC, CAT, and GSH-Px activities ($P < 0.05$). T-AOC activity varied significantly across treatments ($P < 0.01$), with the CON showing the lowest T-AOC value, and FLA having

the highest, followed by the LBP and LBB. SOD activity did not differ significantly across treatments. FLA exhibited the highest GSH-Px activity, followed by LBP and LBB, with the CON showing the lowest GSH-Px activity. CAT activity was highest in the CON, while LBB exhibited the lowest CAT activity, followed by LBP. Additionally, the DPPH clearance rate was significantly higher in the FLA group compared to all other treatments ($P < 0.01$). LBP, LBB, and BET also demonstrated high antioxidant activity, though significantly lower than FLA ($P < 0.01$).

Microbial community analysis in alfalfa silages

Shannon and ACE indices were employed to assess the diversity and richness of bacterial communities across all silage samples (Fig. 1A and B). A significant increase in the Shannon index was observed in the CON group compared to other treatments ($P < 0.05$). PCoA based on Bray-Curtis distance indicated clear separation between treated silages (LBB, BET) and the CON silage (Fig. 1C). Notably, LBP-treated silage was more similar to FLA-treated silage.

In the fresh matter (FM), the dominant bacterial phyla included Proteobacteria (46.73%), Streptophyta (31.35%), and Actinobacteria (15.95%). After ensiling, Firmicutes (80%) became the predominant phylum across all treatments, leading to a decline in Proteobacteria. LBB-treated silage showed the highest abundance of Proteobacteria (18.05%), followed by BET (14.41%) (Fig. 1D). The dominant genera in FM were *Pantoea* (26.13%), *Medicago* (27.20%), *Pseudomonas* (9.41%), and *Enterobacter* (9.41%) (Fig. 1E). Post-ensiling, *Lactiplantibacillus* was increased in all silages, with the highest abundance observed in LBB-treated silage (51.27%). *Pediococcus* also increased across all silages, especially in LBP, FLA, and BET groups. At the species level, *unclassified Pantoea* (32.63%) and *Enterococcus faecalis* (6.02%) dominated FM, but after ensiling, these were replaced by *Lactiplantibacillus plantarum*,

Pediococcus acidilactici, and other LAB species in treated silages. LBB and LBP treatments enhanced the abundance of *Lactiplantibacillus plantarum*, while BET increased *Pediococcus acidilactici* abundance.

LEfSe analysis and LDA confirmed significant microbial shifts at various taxonomic levels (Fig. 2). Treated silages (LBB, LBP, FLA, BET) exhibited higher relative abundances of *Lactiplantibacillus*, *Lactococcus*, *Pediococcus*, *Lactiplantibacillus plantarum*, and *Pediococcus acidilactici* (LDA > 5.0), while *Weissella* and *Levilactobacillus* were more prevalent in the CON group (LDA > 4.0). These shifts suggest that bioactive components in LBB and its extracts selectively promoted beneficial LAB while inhibiting undesirable microorganisms.

The functional annotation and KEGG pathways

Functional annotation and gene abundance data from various databases were used to categorize the pathways via the KEGG database (Fig. 3). At level 3, several pathways were identified, including metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, ABC transporters, biosynthesis of cofactors, biosynthesis of amino acids, carbon metabolism, purine metabolism, two-component systems, and the phosphotransferase system (PTS) (Fig. 3A). The top three pathways observed across the LBB, LBP, FLA, and BET groups were metabolic pathways, biosynthesis of secondary metabolites, and microbial metabolism in diverse environments. KEGG pathways differences among treatments are depicted in a cladogram generated via LEfSe (Fig. 3B). A total of 7, 16, 7, 3, and 16 significantly different KEGG pathways were identified for CON, LBB, LBP, FLA, and BET treatments, respectively ($P < 0.05$). LBB-treated silage exhibited a greater influence on the biosynthesis of amino acids, ABC transporters (LDA > 3.5), biosynthesis of secondary metabolites, cysteine and methionine metabolism, lysine biosynthesis, and

phenylalanine, tyrosine, and tryptophan biosynthesis (LDA > 3.0). LBP treatment primarily affected purine metabolism (LDA > 3.0), nucleotide metabolism, pyrimidine metabolism, fatty acid biosynthesis and taurine and hypotaurine metabolism (LDA > 2.5). FLA-treated silage significantly impacted two-component systems and quorum sensing (LDA > 3.0), while BET-treated silage influenced carbon metabolism, fatty acid metabolism, and methane metabolism (LDA > 3.5). CON-treated silage primarily affected ribosome biosynthesis, aminoacyl-tRNA biosynthesis, and nucleotide sugar biosynthesis (LDA > 2.5).

Metabolome analysis of silage

To investigate the impact of LBB and its bioactive components on alfalfa silage, metabolomics analysis was conducted using an untargeted LC-MS approach to assess metabolites across twenty silage samples. An Upset plot was generated to visualize the similarity and overlap of metabolite compositions across different groups (Fig. S1). The results revealed that the LBP group contained the highest abundance of metabolites. Each treatment group exhibited unique metabolites, indicating that different bioactive components selectively enriched specific metabolites in silage. A total of 1434 metabolites were identified and quantified across the five groups (Fig. S2). These metabolites included categories such as lipids and lipid-like molecules, organic acids and derivatives, organoheterocyclic compounds, phenylpropanoids and polyketides, benzenoids, organic oxygen compounds, and nucleosides, nucleotides, and analogues (Table S1).

The PCA score plot displayed the overall differences and visualized the trends and outliers among the metabolite data (Fig. S3), with sample distributions within the 95% Hotelling's T-squared ellipse. Samples from the LBB, bioactive components treatment groups, and CON group were distributed in distinct regions, further supporting that the

different treatments induced metabolomic variations in silages. OPLS-DA analysis was also performed to examine the metabolic changes (Fig. S4). The corresponding R^2 values for the OPLS-DA models (LBB vs. CON, LBP vs. CON, FLA vs. CON, BET vs. CON) were all > 0.80 , indicating stable and reliable models. Permutation tests yielded negative Q^2 values, confirming that the models were not overfitted. These results suggest that LBB and its bioactive components significantly altered the secondary metabolic profiles of alfalfa silages.

Compared to CON-treated silage, LBB treatment resulted in 180 upregulated and 353 downregulated metabolites, while the LBP treatment led to 108 upregulated and 92 downregulated metabolites (Fig. 4A, B). FLA- and BET-treated silages showed 76 and 120 upregulated metabolites, respectively (Fig. 4C, D). The significantly altered metabolites spanned various categories, including lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives, organic oxygen compounds, phenylpropanoids and polyketides, benzenoids, hydrocarbon derivatives, nucleosides, nucleotides, and analogues. The upset diagram highlighted the specific and common shared differential metabolites across different comparison pairs, with LBB exhibiting the highest number of unique metabolites (Fig. 4E). The LBB vs. CON comparison identified 533 unique differential metabolites, followed by FLA vs. CON (223 metabolites), LBP vs. CON (200 metabolites), and BET vs. CON (191 metabolites) (Fig. 4E). Additionally, nine metabolites were common across various comparison pairs, with eight showing the highest levels in the LBB group, four being elevated in both LBB and bioactive components groups, and one displaying the highest level in the CON group (Table S2). Notably, metabolites such as clavulanic acid, bisnoryangonin, and L-asparagine were abundant in silages treated with LBB and bioactive components, whereas zearalenone and zeranol were reduced in the LBB and LBP treatments.

Metabolic pathway analysis of differential metabolites

As depicted in Fig. 5A, the KEGG functional pathways of metabolites after ensiling primarily involved three categories: metabolism, genetic information processing, and environmental information processing, with a major focus on metabolism. Among these pathways, the biosynthesis of other secondary metabolites exhibited the highest metabolite abundances, followed by amino acid metabolism, lipid metabolism, carbohydrate metabolism and the metabolism of cofactors and vitamins. Differential metabolites were predominantly enriched in the following metabolic pathways: flavonoid biosynthesis, isoflavonoid biosynthesis, plant hormone signal transduction, alpha-linolenic acid metabolism, and flavone and flavonol biosynthesis (Fig. 5). The comparison between LBB and CON groups showed the strongest association with tryptophan metabolism and lysine degradation (Fig. 5C). For LBP versus CON, arginine and proline metabolism and alpha-linolenic acid metabolism were significantly enriched compared LBP to CON (Fig. 5D). BET-treated silages exhibited differential metabolites related to arginine biosynthesis and biotin metabolism. Flavonoids, such as quercetin, hesperetin, luteolin and phlorizin were significantly upregulated in silages treated with LBB and bioactive components.

Correlation analysis of the microbiome and metabolites

To explore the relationship between metabolites and bacterial communities during ensiling. Spearman correlation analysis was conducted at the genus level (Fig. 6). The abundances of the top 15 genera and the top 30 metabolites were analyzed. After fermentation, *Lactiplantibacillus* abundance was positively correlated with metabolites such as 4-hydroxysphinganine, methylmalonic acid, and styrene, while these metabolites exhibited a negative correlation with *Levilactobacillus*, *Weissella*, and *Pediococcus*. Additionally, Corchorifatty acid F and Hexadecanoic acid exhibited a

positive correlation with *Levilactobacillus*, *Weissella* and *Pediococcus*.

Discussion

Chemical compositions, microbial population, and fermentation quality of alfalfa silage with different treatments

The pH value is a critical indicator of fermentation quality, with a range of 4.20 to 5.00 considered acceptable for legume silages (Kung et al., 2018). In this study, lower pH values in silages treated with LBB and LBP enhanced the efficiency of LAB in converting WSC into organic acids. The decrease in pH was mainly due to the accumulation of organic acids, particularly LA (Kung et al., 2018). The significantly higher LA content and lower AA content in treated silages reflect improved fermentation, as LA is the primary organic acid produced during proper fermentation. Notably, the LA content in LBB-treated silages was similar to that in LBP-treated silages, despite the lower pH in LBB-treated silages. This may be due to LBP providing additional substrates for acid production, while other bioactive components in LBB contribute directly to pH reduction. It is well-established that PA content in silage is undesirable (McDonald et al., 1991). Therefore, the absence of PA content suggested that LBP, FLA and BET effectively suppressed propionic acid-producing bacteria, which are typically associated with undesirable fermentation.

NH₃-N is a key indicator of proteolysis in silage (Pahlow et al., 2003), and its reduction in treated silages supports the hypothesis that LBB and its bioactive components inhibit proteolysis by preventing the growth of proteolytic bacteria and reducing proteolytic enzymes activity. Undesirable bacteria such as *Clostridia*,

Enterobacter, and *Enterococcus*, along with the activity of proteolytic enzymes in plants, are often linked to relatively high NH₃-N levels (Kung et al., 2018; Yang et al., 2022). Both Clostridial bacteria and plant proteolytic enzymes are most active in the pH range of 5.0 to 6.0 (Wang et al., 2019b). The inhibition of these microorganisms, such as *Clostridium*, by LBB and its bioactive components aligns with the reduced PA levels, as previously discussed. These results are consistent with findings by Tan et al (2019), who reported that LBB and LBP could inhibit proteolytic enzyme activity. Additionally, the absence of mold growth in FLA and BET-treated silages suggest that these treatments help prevent spoilage by inhibiting mold growth. The LAB populations in CON silages were relatively lower than those in treated silages, possibly due to the availability of substrates that promote LAB proliferation.

In this study, DM decreased after 90 days of ensiling in all samples, except for those treated with LBB. As expected, the inclusion of dry LBB increased the DM content of the silages, which could help inhibit microbial activity and reduce DM loss. Furthermore, the lower NDF and ADF contents in LBB and LBP-treated silages suggest that these treatments may promote fiber degradation, potentially through the action of fibrinolytic enzymes produced during fermentation. WSC is an important factor in fermentation, with 60–70 g/kg DM WSC typically required for well-preserved silage (Smith, 1962). The higher WSC content in LBB, LBP, and FLA-treated silages may be attributed to the nutritional composition of these additives, providing additional substrates for fermentation.

Antioxidant properties of alfalfa silages with different treatments

444 The addition of LBB and its bioactive components significantly enhanced the
445 antioxidant properties of silages, as evidenced by increased T-AOC and GSH-Px
446 activities. T-AOC is a key indicator of the overall antioxidant status, reflecting the
447 combined effects of antioxidant enzymes and substances within an organism (Cui et al.,
448 2023). The elevated T-AOC in treated silages demonstrates the effectiveness of LBB
449 and its components in enhancing the silage's antioxidant capacity. SOD, GSH-Px, and
450 CAT act as primary antioxidants in cells, scavenging superoxide anion free radicals
451 (O_2^-) and converting hydrogen peroxide (H_2O_2) into water and oxygen, respectively
452 (Ighodaro and Akinloye, 2018). Among the bioactive components, FLA exhibited the
453 highest T-AOC and GSH-Px activities, reducing oxidative damage by inhibiting lipid
454 peroxidation and lowering malondialdehyde levels. Furthermore, FLA enhances the
455 antioxidant defense capacity of cells by activating the intracellular antioxidant enzyme
456 system, further improving the overall antioxidant status (Ismail et al., 2012). The
457 reduction in CAT activity in LBP-treated silages may be attributed to the lower pH
458 during the anaerobic fermentation process, which is known to inhibit catalase activity
459 (Garcia-Molina et al., 2005). The optimal pH range for catalase activity is typically
460 between 6.0 and 7.5, with catalase from most bacteria and plants exhibiting the highest
461 activity in this range. In acidic environments (pH below 6.0), catalase activity is
462 suppressed as the pH decreases (Yumoto et al., 2000). DPPH is commonly used to
463 assess the free radical scavenging ability of hydrogen-donating antioxidants (Zhu et al.,
464 2017), providing a measure of the antioxidant capacity of silages. The improvement in
465 DPPH clearance rates further highlights the enhanced free radical-scavenging ability of

the treated silages, with FLA demonstrating the most pronounced effect. The biological function of LBB is closely linked to its components, including LBP, FLA, and BET, all of which contribute to the observed T-AOC and GSH-Px activities. Given that oxidative stress negatively impacts animal production and product quality (Miller et al., 1993), enhancing the antioxidant capacity of silage is crucial for improving ruminant health.

Changes in microbial Community in alfalfa Silages with different treatments

The reduction in bacterial diversity and richness observed in the treated silages suggests that LBB and its extracts exhibit selective antimicrobial effects, likely due to their antioxidative and antibacterial properties (Gao et al., 2017; Pires et al., 2018). These bioactive components, including LBP, FLA, and BET, likely inhibit the growth of harmful bacteria, such as *Enterobacter* and *Salmonella*, which are known to hinder fermentation quality (Chen et al., 2020). The decreased diversity may also reflect a shift toward the dominance of beneficial microbes, such as *Lactiplantibacillus* and *Pediococcus*, which are essential for promoting efficient fermentation. PCoA analysis further highlights the influence of these treatments on the microbial community. Interestingly, LBB-treated silage exhibited greater separation from BET-treated silage, while being relatively closer to LBP and FLA-treated silages. This suggests that different bioactive components may have distinct effects on the microbial ecosystem.

The dominance of Firmicutes and Proteobacteria in the silage samples aligns with previous research (Wang et al., 2019c), as Firmicutes are well-adapted to thrive in anaerobic conditions and are known for their role in organic matter degradation (Wang et al., 2022b). The increased abundance of Firmicutes across all silages may be linked

to the reduced pH and anaerobic conditions during fermentation, which favor the growth of these bacteria (Wang et al., 2018). The shift from Proteobacteria to Firmicutes, particularly in LBP-treated silage, suggests that LBP may promote the growth of Firmicutes by increasing WSC content, thus supporting the fermentation process (Ma et al., 2018). These changes indicate that LBP not only affects bacterial diversity but also enhances silage quality by fostering microorganisms that produce lactic acid.

The increased abundance of *Lactiplantibacillus* in the LBB, LBP, and FLA-treated silages further underscores its critical role in silage fermentation. *Lactiplantibacillus* promotes lactic acid accumulation and reduces pH, creating an environment that suppresses the growth of spoilage microorganisms. This aligns with the findings that *Lactiplantibacillus* plays a key role in inhibiting harmful bacteria like *Clostridium* and improving fermentation quality (Du et al., 2022). The increase in *Pediococcus* abundance, particularly in the LBP, FLA, and BET treatments, also highlights the beneficial effects of these bioactive components on fermentation. *Pediococcus* is known for producing pediocin, an effective antilisterial bacteriocin (Porto et al., 2017), and its increased presence in LBP, FLA, and BET-treated silages suggests that these treatments not only promote desirable LAB but also improve the overall fermentation quality. The decrease in *Enterococcus* and *Enterobacter* abundance in LBP-treated silages further confirms the success of these treatments in improving fermentation conditions. *Enterobacter* is a gram-negative bacterium that competes with LAB for nutrients and can produce NH₃-N, which hinders acidification and degrades silage quality (Silva et al., 2016). Both *Enterococcus* and *Enterobacter* are known to be sensitive to low pH (Du et al., 2021; Ni et al., 2017). *Enterobacter* is more sensitive to

herbal plant extracts than LAB (Takahashi et al., 2021). The reduction in *Enterobacter* in LBP-treated silages suggests that LBP effectively creates an acidic environment that favors LAB growth while inhibiting undesirable microorganisms.

At the species level, a shift was observed from *Pantoea* and *Enterococcus faecalis* to LAB species such as unclassified *Lactiplantibacillus*, *Lactiplantibacillus plantarum*, and *Pediococcus acidilactici* in all silages. *Lactiplantibacillus plantarum*, in particular, is well-known for its ability to ferment sugars and produce lactic acid, which helps lower the pH and suppress the growth of harmful microorganisms (Cai et al., 2020). The increased abundance of *Lactiplantibacillus plantarum* in LBB and LBP-treated silages supports the idea that LBP can enhance the relative abundance of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, and Firmicutes (Feng et al., 2022).

The significant changes in microbial composition, revealed through LEfSe and LDA analyses, further confirm that LBP, FLA, and BET stimulate the growth of beneficial LAB while inhibiting the growth of undesirable microorganisms. These results suggest that bioactive components such as polysaccharides, flavonoids, and alkaloids may function as prebiotic agents, modulating microbial community structure and enhancing silage fermentation (Cueva et al., 2020).

Functional annotation and KEGG pathway analysis revealed distinct differences in metabolic pathways across the treatments, reflecting the unique microbial communities present under each treatment. These results highlight the substantial influence of LBB and its extracts on microbial metabolism in silage, as reflected in the observed pathways. LBB-treated silage exhibited significant effects on the biosynthesis of amino acids, ABC transporters, and secondary metabolites. Rich in carbohydrates, LBB likely provided essential nutrients that supported LAB growth, thereby influencing amino acid synthesis (Park et al., 2022; Wang et al., 2022a). LBP-treated

silage primarily affected purine metabolism, nucleotide metabolism, and fatty acid biosynthesis. Purine and pyrimidine nucleotides, which are crucial for energy transfer in cellular processes, are also vital for activating precursors in polysaccharide synthesis (Kilstrup et al., 2005). These findings suggest that LBP stimulates nucleotide metabolism, which may enhance polysaccharide utilization during fermentation, further improving silage quality. Overall, these findings suggest that LBB, LBP, FLA, and BET each influence distinct metabolic pathways in silage fermentation, leading to the production of unique metabolic compounds. The differences in these pathways underscore the role of bioactive components in shaping the microbial communities and metabolic activities during silage fermentation, potentially improving fermentation efficiency and silage quality.

Metabolome and metabolic pathway analysis of alfalfa Silages

The metabolomic analysis revealed that LBB and its bioactive components significantly altered the metabolic profile of alfalfa silage, enriching it with unique metabolites. These changes indicate that bioactive compounds such as LBP, FLA, and BET influence both the metabolite composition and functional characteristics of silage. Notably, treated silages exhibited the highest number of unique metabolites, including antimicrobial compounds like clavulanic acid, bisnoryangonin, and L-asparagine. The presence of clavulanic acid in treated silage suggests its role as a potent inhibitor of β -lactamase enzymes, which prevent the growth of pathogenic bacteria resistant to β -lactam antibiotics (Santos et al., 2009). This compound has proven effective against pathogenic bacteria, regardless of β -lactamase production, and may be used in the treatment of inflammation (Finlay et al., 2003). L-asparagine is a vital amino acid involved in regulating cellular functions in nerve and brain tissues, as well as serving as a nitrogen storage source for many plants (Jeyapappa and Selvarajan, 2023).

Bisnoryangonin has demonstrated broad-spectrum antibacterial activity, particularly against Gram-positive bacteria, making it an effective inhibitor of *Enterococcus faecalis* and *Staphylococcus aureus* (Go et al., 2015). The bioactive components of LBP, FLA, and BET also contributed to the antimicrobial and antifungal properties of treated silages, with chrysophanein standing out as particularly noteworthy. Chrysophanein is known for its potent antibacterial and antifungal activity, likely playing a key role in inhibiting the growth of undesirable microorganisms in silage (Abd-Alla et al., 2009). The combination of these compounds enhances both antioxidant and antimicrobial activities, leading to synergistic effects. These findings underscore the potent antimicrobial and anti-inflammatory properties of LBP, FLA, and BET in silages. It has been demonstrated that the antimicrobial and anti-inflammatory status of ruminants can be improved by dietary supplementation of LBB-treated silage. Additionally, the significant reduction of zearalenone and zeranone in LBB and LBP-treated silages is a crucial finding. Zearalenone, a toxic metabolite produced by fungi, poses significant risks to animal health and reproduction. Zeranone, a metabolite of the mycotoxin zearalenone, has been banned in livestock farming within the European Union since 1981 (Thevis et al., 2011). The metabolomic analysis reinforces the potential of LBB and LBP as effective additives for enhancing silage quality, emphasizing their role in improving both the nutritional and safety profiles of silage for ruminants.

KEGG pathway analysis revealed that LBB and its bioactive components modulated various metabolic pathways in silage, with a predominant focus on metabolism. The highest abundance of metabolites was observed in the biosynthesis of secondary metabolites, amino acid metabolism, lipid metabolism, and carbohydrate metabolism, aligning with previous findings (Xu et al., 2019). Among these, amino acid and carbohydrate metabolism were most significantly affected, likely due to protein

hydrolysis and sugar degradation during silage fermentation. These results support the role of amino acids and carbohydrates in microbial metabolism, influencing both the flavor and nutritional quality of silage (Du et al., 2021). Flavonoids, as a major class of plant secondary metabolites, are known for their potent antioxidant and anti-inflammatory properties and are effective in treating various diseases (Williamson et al., 2018). Compounds such as quercetin, which were elevated in these treatments, may provide additional health benefits for ruminants (Cheng et al., 2021). The presence of alpha-linolenic acid, an important omega-3 fatty acid, further suggests that the bioactive components in these treatments may contribute to animal health by enhancing metabolic pathways essential for cellular function. Indole acetic acid, a microbial tryptophan metabolite, has been shown to improve intestinal permeability and host immunity and exhibit higher concentrations in the LBB group (Chen et al., 2022). Additionally, the increased levels of biocytin in BET-treated silages highlight the role of BET in supporting metabolic processes critical for gluconeogenesis, lipid metabolism, and amino acid catabolism (Perry and Butterick, 2024).

Relationship between bacterial composition and metabolites

Spearman correlation analysis revealed significant interactions between bacterial communities and metabolites during silage fermentation, particularly when LBB and its extracts were included. *Lactiplantibacillus*, a dominant LAB in the silage, exhibited positive correlations with specific metabolites such as 4-hydroxysphinganine, methylmalonic acid, and styrene. These metabolites, in turn, were negatively correlated with other bacterial species, including *Levilactobacillus*, *Weissella*, and *Pediococcus*, suggesting a competitive relationship among these species. This indicates that microbial community dynamics in silage are influenced by metabolic products, resulting in either competitive or synergistic interactions. The positive correlations between Corchorifatty

acid F and Hexadecanoic acid with *Levilactobacillus*, *Weissella*, and *Pediococcus* are particularly noteworthy. Corchorifatty acid F, a phenolic acid, exhibits antibacterial and antioxidant activities, while Hexadecanoic acid has demonstrated potential as an antibacterial agent against bacteria and fungi, particularly in mustard rot (Idris, 2022). Additionally, Eriodictyol, known for its antioxidant and anti-inflammatory properties (Deng et al., 2020), showed positive correlations with multiple bacterial species. The growing interest in plant-derived bioactive compounds as potential alternatives to conventional antibiotics in livestock for enhancing growth and antibacterial properties underscores the relevance of our findings. This study emphasizes the role of LBB in producing antimicrobial silage for livestock, highlighting its potential in fostering functional LAB strains that produce beneficial biological substances during ensiling.

Conclusions

In conclusion, this study demonstrates the potential of incorporating LBB, LBP, FLA, and BET to enhance the fermentation quality of alfalfa silage. The addition of these bioactive components promoted LA production, preserved WSC, and increased T-AOC and GSH-Px activities. Moreover, LBB and its bioactive components positively influenced the microbial community, notably increasing the abundance of beneficial bacteria such as *Lactiplantibacillus*, *Pediococcus*, *Levilactobacillus* and *Weissella*. Metabolomic analysis revealed that these bioactive components enriched pathways related to flavonoid and isoflavonoid biosynthesis, suggesting their contribution to the functional properties of silage.

This innovative approach not only transforms agricultural waste into high-value resources but also promotes resource conservation, improves feed quality, and supports environmental sustainability. However, animal trials are essential to evaluate the practical impact of LBB-treated silage on animal health and performance. Future

research should validate these findings in practical farming environments to assess the scalability and economic feasibility of using LBB as a silage additive.

Data Availability Statement

The datasets presented in this study can be found in online repositories. We uploaded the sequences data in the NCBI and got an accession number PRJNA1223334. We confirm that no AI tools were used during the preparation of this work.

Author Contributions

Y. J. Zhang: Formal analysis, Data curation, Methodology, Writing—original draft. J. L. Liao: Conceptualization, Investigation. Y. X. Pan: Methodology, Investigation. Q. Zhang: Writing—review & editing. Q. Lu: Resources, Supervision. X. D. Chen: Methodology, Validation. W. C. Ke: Supervision, Writing—review & editing, Funding acquisition. G. J. Zhang: Supervision, Project administration, Funding acquisition.

Funding

This study was supported by the National Key Research and Development Program of China (grant number 2022YFD1300905), the Key Research and Development Program of Ningxia Hui Autonomous Region (grant number 2021BEF02020), the Top Discipline Construction Project of Pratacultural Science (grant number NXYLXK2017A01), and the Natural Science Foundaton of Ningxia (grant number 2023AAC05018).

Declaration of Competing Interest

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 agree to submit their articles to BMC Microbiology.

Ethics approval and consent to participate: not applicable

662 **Clinical trial number: not applicable**

663 **Appendix A. Supplementary data**

664 Supplementary data to this article can be found online.

665

References:

- Abd-Alla, H.I., Shaaban, M., Shaaban, K.A., Abu-Gabal, N.S., Shalaby, N.M.M.,
Laatsch, H., 2009. New bioactive compounds from *Aloe hijazensis*. *Nat. Prod. Res.*
23(11), 1035-1049.
- AOAC, 2000. *Official Methods of Analysis*. 17th Edition, The Association of Official
Analytical Chemists. Int. Arlingt.
- Azami, N.L.B., Sun, M., 2019. Zeaxanthin dipalmitate in the treatment of liver
disease. *Evidence-based Complement. Altern. Med.* 1, 1-14.
- Broderick, G.A., Kang, J.H., 1980. Automated simultaneous determination of
ammonia and total amino acids in ruminal fluid and in Vitro media. *J. Dairy Sci.*
63(1), 64-75.
- Buchfink, B., Xie, C., Huson, D.H., 2014. Fast and sensitive protein alignment using
DIAMOND. *Nat. Methods.* 12(1), 59-60.
- Cai, Y., Du, Z., Yamasaki, S., Ngulube, D., Tinga, B., Macome, F., Oya, T., 2020.
Community of natural lactic acid bacteria and silage fermentation of corn stover
and sugarcane tops in Africa. *Asian-Australasian J. Anim. Sci.* 33(8), 1252-1264.
- Chen, J., Long, L., Jiang, Q., Kang, B., Li, Y., Yin, J., 2020. Effects of dietary
supplementation of *Lycium barbarum* polysaccharides on growth performance,
immune status, antioxidant capacity and selected microbial populations of
weaned piglets. *J. Anim. Physiol. Anim. Nutr. (Berl).* 104(4), 1106-1115.
- Chen, Y., Tian, P., Wang, Z., Pan, R., Shang, K., Wang, G., Zhao, J., Chen, W., 2022.
Indole Acetic Acid Exerts Anti-Depressive Effects on an Animal Model of

688 Chronic Mild Stress. *Nutrients*. 14(23), 5019.

689 Cheng, Y., Liu, Y., Chen, D., Zhou, Y., Yu, S., Lin, Huajian, Liao, C.K., Lin, Haili,
690 Xu, P., Huang, M., 2021. Dual effects of quercetin on protein digestion and
691 absorption in the digestive tract. *Food Chem.* 358, 129891.

692 Cueva, C., Silva, M., Pinillos, I., Bartolomé, B., Moreno-Arribas, M.V., 2020.
693 Interplay between dietary polyphenols and oral and gut microbiota in the
694 development of colorectal cancer. *Nutrients*. 12(3), 1-19.

695 Cui, Z., He, F., Li, X., Li, Y., Huo, C., Wang, H., Qi, Y., Tian, G., Zong, W., Liu, R.,
696 2023. Response pathways of superoxide dismutase and catalase under the
697 regulation of triclocarban-triggered oxidative stress in *Eisenia foetida*:
698 Comprehensive mechanism analysis based on cytotoxicity and binding model.
699 *Sci. Total Environ.* 854, 158821.

700 Deng, Z., Hassan, S., Rafiq, M., Li, H., He, Y., Cai, Y., Kang, X., Liu, Z., Yan, T.,
701 2020. Pharmacological Activity of Eriodictyol: The Major Natural Polyphenolic
702 Flavanone. *Evidence-based Complement. Altern. Med.* 1, 1-11.

703 Dong, L., Zhong, Z.X., Cui, H.H., Wang, S.N., Luo, Y., Yu, L.H., Loo, J.J., Wang,
704 H.R., 2020. Effects of rumen-protected betaine supplementation on meat quality
705 and the composition of fatty and amino acids in growing lambs. *Animal*. 14(2),
706 435-444.

707 Du, Z., Sun, L., Lin, Y., Chen, C., Yang, F., Cai, Y., 2022. Use of Napier grass and
708 rice straw hay as exogenous additive improves microbial community and
709 fermentation quality of paper mulberry silage. *Anim. Feed Sci. Technol.* 285,

710 115219.

711 Du, Z., Sun, L., Lin, Y., Yang, F., Cai, Y., 2021. The use of PacBio SMRT
712 technology to explore the microbial network and fermentation characteristics of
713 woody silage prepared with exogenous carbohydrate additives. J. Appl.
714 Microbiol. 131(5), 2193-2211.

715 Feng, Y., Song, Y., Zhou, J., Duan, Y., Kong, T., Ma, H., Zhang, H., 2022. Recent
716 progress of *Lycium barbarum* polysaccharides on intestinal microbiota, microbial
717 metabolites and health: a review. Crit. Rev. Food Sci. Nutr. 64(10), 2917-2940.

718 Finlay, J., Miller, L., Poupard, J.A., 2003. A review of the antimicrobial activity of
719 clavulanate. J. Antimicrob. Chemother. 52(1), 18-23.

720 Gao, Y., Wei, Y., Wang, Y., Gao, F., Chen, Z., 2017. *Lycium barbarum*: A traditional
721 Chinese herb and a promising anti-aging agent. Aging Dis. 8(6), 778-791.

722 Garcia-Molina, F., Hiner, A.N.P., Fenoll, L.G., Rodriguez-Lopez, J.N., Garcia-Ruiz,
723 P.A., Garcia-Canovas, F., Tudela, J., 2005. Mushroom tyrosinase: catalase
724 activity, inhibition, and suicide inactivation. J. Agric. Food Chem. 53, 3702-
725 3709.

726 Go, M.K., Wongsantichon, J., Cheung, V.W.N., Chow, J.Y., Robinson, R.C., Yew,
727 W.S., 2015. Synthetic Polyketide Enzymology: Platform for Biosynthesis of
728 Antimicrobial Polyketides. ACS Catal. 5(7), 4033-4042.

729 Guo, J., Xie, Y., Yu, Z., Meng, G., Wu, Z., 2019. Effect of *Lactobacillus plantarum*
730 expressing multifunctional glycoside hydrolases on the characteristics of alfalfa
731 silage. Appl. Microbiol. Biotechnol. 103(19), 7983-7995.

732 Hartinger, T., Gresner, N., Südekum, K.H., 2019. *In vitro* ruminal fermentation
 733 characteristics of alfalfa silages in response to different pre-ensiling treatments.
 734 Anim. Feed Sci. Technol. 258, 114306.

735 Idris, N., 2022. Potential of Hexadecanoic Acid as Antimicrobials in Bacteria and
 736 Fungi that Cause Decay in Mustard Greens Brassica juncea L. Int. J. Appl. Biol.
 737 6(2), 36-42.

738 Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide
 739 dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their
 740 fundamental role in the entire antioxidant defence grid. Alex. J. Med. 54, 287-
 741 293.

742 Ismail, T., Sestili, P., Akhtar, S., 2012. Pomegranate peel and fruit extracts: A review
 743 of potential anti-inflammatory and anti-infective effects. J. Ethnopharmacol.
 744 143(2), 397-405.

745 Jeyapappa, K., Selvarajan, P., 2023. Studies on thermal, dielectric and nonlinear
 746 optical properties of iodic acid doped L-asparagine L-tartaric acid crystal. Mater.
 747 Today Proc.

748 Ke, W.C., Ding, W.R., Ding, L.M., Xu, D.M., Zhang, P., Li, F.H., Guo, X.S., 2018.
 749 Influences of malic acid isomers and their application levels on fermentation
 750 quality and biochemical characteristics of alfalfa silage. Anim. Feed Sci.
 751 Technol. 245, 1-9.

752 Kilstrup, M., Hammer, K., Jensen, P.R., Martinussen, J., 2005. Nucleotide
 753 metabolism and its control in lactic acid bacteria. FEMS Microbiol. Rev. 29(3),

754 555-590.

755 Kung, L., Shaver, R.D., Grant, R.J., Schmidt, R.J., 2018. Silage review: Interpretation
756 of chemical, microbial, and organoleptic components of silages. J. Dairy Sci.
757 101(5), 4020-4033.

758 Liao, J., Ke, W., Wang, B., Du, M., Lu, Q., Zhang, Y., & Zhang, G., 2025.
759 Transcriptomics and non-targeted metabolomics provide mechanistic insights
760 into the improvement of the growth performance and meat quality of lambs
761 supplemented with fermented *Lycium barbarum* residues. Animal Nutrition, 21,
762 11-24.

763 Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: An ultra-fast
764 single-node solution for large and complex metagenomics assembly via succinct
765 de Bruijn graph. Bioinformatics. 31(10), 1674-1676.

766 Lin, Z. ning, Ye, L., Li, Z. wu, Huang, X. sheng, Lu, Z., Yang, Y. quan, Xing, H. wei,
767 Bai, J. ying, Ying, Z. yang, 2020. Chinese herb feed additives improved the
768 growth performance, meat quality, and nutrient digestibility parameters of pigs.
769 Anim. Model. Exp. Med. 3(1), 47-54.

770 Long, L.N., Kang, B.J., Jiang, Q., Chen, J.S., 2020. Effects of dietary *Lycium*
771 *barbarum* polysaccharides on growth performance, digestive enzyme activities,
772 antioxidant status, and immunity of broiler chickens. Poult. Sci. 99(2), 744-751.

773 Lu, Y., Guo, S., Zhang, F., Yan, H., Qian, D.W., Wang, H.Q., Jin, L., Duan, J.A.,
774 2019. Comparison of functional components and antioxidant activity of *lycium*
775 *barbarum* L. Fruits from different regions in China. Molecules 24(12), 2228. 8

776 Ma, S., Fang, C., Sun, X., Han, L., He, X., Huang, G., 2018. Bacterial community
 777 succession during pig manure and wheat straw aerobic composting covered with
 778 a semi-permeable membrane under slight positive pressure. *Bioresour. Technol.*
 779 259, 221-227.

780 McDonald, P., Henderson, N., Heron, S., 1991. The biochemistry of silage.
 781 Chalcombe Publications, Marlow.

782 Menchetti, L., Curone, G., Andoni, E., Barbato, O., Troisi, A., Fioretti, B., Polisca,
 783 A., Codini, M., Canali, C., Vigo, D., Brecchia, G., 2020. Impact of goji berries
 784 (*Lycium barbarum*) supplementation on the energy homeostasis of rabbit does:
 785 Uni- and multivariate approach. *Animals*, 10(11), 1-19.

786 Miller, J.K., Brzezinska-Slebodzinska, E., Madsen, F.C., 1993. Oxidative stress,
 787 antioxidants, and animal function. *J. Dairy Sci.* 76, 2812-2823.

788 More, G.K., Makola, R.T., 2020. In-vitro analysis of free radical scavenging activities
 789 and suppression of LPS-induced ROS production in macrophage cells by
 790 *Solanum sisymbriifolium* extracts. *Sci. Rep.* 10(1), 6493.

791 Murphy, R.P., 1958. A method for the extraction of plant samples and the
 792 determination of total soluble carbohydrates. *J. Sci. Food Agric.* 9, 714-717.

793 Ni, K., Wang, F., Zhu, B., Yang, J., Zhou, G., Pan, Y., Tao, Y., Zhong, J., 2017.
 794 Effects of lactic acid bacteria and molasses additives on the microbial
 795 community and fermentation quality of soybean silage. *Bioresour. Technol.* 238.
 796 706-715.

797 Olagaray, K.E., Bradford, B.J., 2019. Plant flavonoids to improve productivity of

798 ruminants – A review. Anim. Feed Sci. Technol. 251, 21-36.

799 Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 2003.

800 Microbiology of ensiling. In: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.),

801 Silage Science and Technology. American Society of Agronomy, Madison, pp.

802 31-93.

803 Pardo, Z., Lara, L., Nieto, R., Fernández-Fígares, I., Seiquer, I., 2023. Muscle quality

804 traits and oxidative status of Iberian pigs supplemented with zinc and betaine

805 under heat stress. Meat Sci. 198, 109119.

806 Park, M.K., Lee, S., Kim, Y.S., 2022. Effects of pH and Osmotic Changes on the

807 Metabolic Expressions of *Bacillus subtilis* Strain 168 in Metabolite Pathways

808 including Leucine Metabolism. Metabolites. 12(2), 112.

809 Perry, C.A., Butterick, T.A., 2024. Biotin. Adv. Nutr. 15, 10-12.

810 Pires, T.C.S.P., Dias, M.I., Barros, L., Calhelha, R.C., Alves, M.J., Santos-Buelga, C.,

811 Ferreira, I.C.F.R., 2018. Phenolic compounds profile, nutritional compounds and

812 bioactive properties of *Lycium barbarum* L.: A comparative study with stems

813 and fruits. Ind. Crops Prod. 122, 574-581.

814 Porto, M.C.W., Kuniyoshi, T.M., Azevedo, P.O.S., Vitolo, M., Oliveira, R.P.S., 2017.

815 *Pediococcus spp.*: An important genus of lactic acid bacteria and pediocin

816 producers. Biotechnol. Adv. 35(3), 361-374.

817 Reich, L.J., Kung, L., 2010. Effects of combining *Lactobacillus buchneri* 40788 with

818 various lactic acid bacteria on the fermentation and aerobic stability of corn

819 silage. Anim. Feed Sci. Technol. 159, 105-109.

820 Robertson, J.B., Van Soest, P.J., 1981. The detergent system of analysis and its
821 application to human foods. Anal. Diet. fiber food. 123.

822 Santos, V.C., Pereira, J.F.B., Haga, R.B., Rangel-Yagui, C.O., Teixeira, J.A.C.,
823 Converti, A., Pessoa, A., 2009. Stability of clavulanic acid under variable pH,
824 ionic strength and temperature conditions. A new kinetic approach. Biochem.
825 Eng. J. 45(2), 89-93.

826 Silva, V.P., Pereira, O.G., Leandro, E.S., Da Silva, T.C., Ribeiro, K.G., Mantovani,
827 H.C., Santos, S.A., 2016. Effects of lactic acid bacteria with bacteriocinogenic
828 potential on the fermentation profile and chemical composition of alfalfa silage
829 in tropical conditions. J. Dairy Sci. 99(3), 1895-1902.

830 Skenderidis, P., Mitsagga, C., Giavasis, I., Petrotos, K., Lampakis, D., Leontopoulos,
831 S., Hadjichristodoulou, C., Tsakalof, A., 2019. The in vitro antimicrobial activity
832 assessment of ultrasound assisted *Lycium barbarum* fruit extracts and
833 pomegranate fruit peels. J. Food Meas. Charact. 13(3), 2017-2031.

834 Smith, L.H., 1962. Theoretical Carbohydrates Requirement for Alfalfa Silage
835 Production 1 . Agron. J. 54(4), 291-293.

836 Takahashi, H., Nakamura, A., Fujino, N., Sawaguchi, Y., Sato, M., Kuda, T., Kimura,
837 B., 2021. Evaluation of the antibacterial activity of allyl isothiocyanate, clove oil,
838 eugenol and carvacrol against spoilage lactic acid bacteria. LWT- Food Science
839 And Technology. 145, 111263.

840 Tan, X., Sun, Z., Ye, C., 2019. Dietary *Lycium barbarum* extract administration
841 improved growth, meat quality and lipid metabolism in hybrid grouper

842 (Epinephelus lanceolatus ♂ × E. fuscoguttatus ♀) fed high lipid diets.

843 Aquaculture. 504, 190-198.

844 Thevis, M., Fußhöller, G., Schänzer, W., 2011. Zeranol: Doping offence or

845 mycotoxin? A case-related study. Drug Test. Anal. 3(11-12), 777-783.

846 Tian, X., Liang, T., Liu, Y., Ding, G., Zhang, F., Ma, Z., 2019. Extraction, structural

847 characterization, and biological functions of *lycium barbarum* polysaccharides:

848 A review. Biomolecules. 9(9), 389.

849 Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for Dietary Fiber,

850 Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal

851 Nutrition. J. Dairy Sci. 74 (10), 3583-3597.

852 Wang, B., Ma, M.P., Diao, Q.Y., Tu, Y., 2019a. Saponin-induced shifts in the rumen

853 microbiome and metabolome of young cattle. Front. Microbiol. 10, 00356.

854 Wang, C., He, L., Xing, Y., Zhou, W., Yang, F., Chen, X., Zhang, Q., 2019b. Effects

855 of mixing Neolamarckia cadamba leaves on fermentation quality, microbial

856 community of high moisture alfalfa and stylo silage. Microb. Biotechnol. 12(5),

857 869-878.

858 Wang, S., Li, J., Zhao, J., Dong, Z., Dong, D., Shao, T., 2022a. Dynamics of the

859 bacterial communities and predicted functional profiles in wilted alfalfa silage. J.

860 Appl. Microbiol. 132(4), 2613-2624.

861 Wang, S., Shao, T., Li, J., Zhao, J., Dong, Z., 2022b. Fermentation Profiles, Bacterial

862 Community Compositions, and Their Predicted Functional Characteristics of

863 Grass Silage in Response to Epiphytic Microbiota on Legume Forages. Front.

864 Microbiol. 13, 830888.

865 Wang, Y., Chen, X., Wang, C., He, L., Zhou, W., Yang, F., Zhang, Q., 2019c. The
866 bacterial community and fermentation quality of mulberry (*Morus alba*) leaf
867 silage with or without *Lactobacillus casei* and sucrose. Bioresour. Technol. 293,
868 122059.

869 Wang, Y., Wang, C., Zhou, W., Yang, F.Y., Chen, X.Y., Zhang, Q., 2018. Effects of
870 wilting and *Lactobacillus plantarum* Addition on the Fermentation quality and
871 microbial community of moringa oleifera Leaf silage. Front. Microbiol. 9, 1817.

872 Williamson, G., Kay, C.D., Crozier, A., 2018. The Bioavailability, Transport, and
873 Bioactivity of Dietary Flavonoids: A Review from a Historical Perspective.
874 Compr. Rev. Food Sci. Food Saf. 17(5), 1054-1112.

875 Xu, D., Ding, W., Ke, W., Li, F., Zhang, P., Guo, X., 2019. Modulation of
876 metabolome and bacterial community in whole crop corn silage by inoculating
877 homofermentative *lactobacillus plantarum* and heterofermentative *lactobacillus*
878 *buchneri*. Front. Microbiol. 9, 3299.

879 Yang, F., Wang, Y., Zhao, S., Feng, C., Fan, X., 2022. Dynamics of the Fermentation
880 Products, Residual Non-structural Carbohydrates, and Bacterial Communities of
881 Wilted and Non-wilted Alfalfa Silage With and Without *Lactobacillus plantarum*
882 Inoculation. Front. Microbiol. 12, 824229.

883 Yumoto, I., Ichihashi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., &
884 Kawasaki, K., 2000. Purification and characterization of a catalase from the
885 facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1T exhibiting high

886 catalase activity. Journal of Bacteriology, 182(7), 1903-1909.

887 Zhang, W.J., Wang, S., Kang, C.Z., Lv, C.G., Zhou, L., Huang, L.Q., Guo, L.P.,
888 2020. Pharmacodynamic material basis of traditional Chinese medicine based on
889 biomacromolecules: A review. Plant Methods. 16(1), 26.

890 Zhang, Y., Guo, Y., Luo, Y., Du, M., Yin, X., Xu, X., Zhang, G., 2022. Integrated
891 Metabolomics and Transcriptome Revealed the Effect of Fermented *Lycium*
892 *barbarum* Residue Promoting Ovis aries Immunity. Front. Immunol. 13, 889436.

893 Zhu, J., Li, X., Qi, H., Gu, Z., Song, S., Yang, X., ... Li, C., 2017. Oxidative and anti-
894 oxidative status in muscle of young rats in response to six protein diets. Sci. Rep.
895 7(1), 13184.

896

Table 1. Chemical composition and microbial population of wilted alfalfa and *Lycium barbarum* by-products ($\pm$ SD, n = 3).

Items	alfalfa	<i>Lycium barbarum</i> by-products
Chemical composition		
DM, g/kg FW	375 $\pm$ 1.14	928 $\pm$ 9.50
CP, g/kg DM	191 $\pm$ 1.00	154 $\pm$ 6.24
aNDF, g/kg DM	419 $\pm$ 9.07	138 $\pm$ 2.65
ADF, g/kg DM	275 $\pm$ 1.06	110 $\pm$ 1.56
WSC, g/kg DM	61.1 $\pm$ 2.50	70.5 $\pm$ 2.40
Microbial population		
LAB, log ₁₀ cfu/g FW	5.48 $\pm$ 0.10	4.79 $\pm$ 0.10
Yeasts, log ₁₀ cfu/g FW	5.64 $\pm$ 0.03	4.79 $\pm$ 0.19
Molds, log ₁₀ cfu/g FW	4.20 $\pm$ 0.17	2.86 $\pm$ 0.10

FW, fresh weight; DM, dry matter; CP, crude protein; aNDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrates; LAB, lactic acid bacteria; cfu, colony-forming unit.

Table 2. Fermentation parameters, microbial population and chemical composition of alfalfa silage ensiled for 90 d.

Items	Experimental treatments					SEM	P-value
	CON	LBB	LBP	FLA	BET		
Fermentation parameters							
pH	5.09 ^a	4.50 ^c	4.47 ^c	4.67 ^b	4.67 ^b	0.05	<0.001
LA, g/kg DM	45.4 ^b	71.3 ^a	74.7 ^a	65.0 ^a	63.4 ^a	2.64	<0.001
AA, g/kg DM	24.8 ^a	14.3 ^c	18.8 ^b	17.8 ^b	19.5 ^b	0.81	<0.001
PA, g/kg DM	9.60 ^a	2.02 ^b	0.00 ^b	0.00 ^b	0.00 ^b	0.88	<0.001
LA/AA	1.84 ^c	5.02 ^a	3.98 ^b	3.67 ^b	3.25 ^b	0.25	<0.001
NH ₃ -N, g/kg TN	137 ^a	88.8 ^c	86.9 ^c	94.6 ^b	96.3 ^b	4.24	<0.001
Microbial population							
LAB, log ₁₀ cfu/g FW	7.41 ^d	7.91 ^a	7.72 ^b	7.45 ^{cd}	7.54 ^c	0.04	<0.001
Yeasts, log ₁₀ cfu/g FW	2.62 ^a	0.38 ^a	1.48 ^a	1.43 ^a	2.21 ^a	0.31	0.178
Molds, log ₁₀ cfu/g FW	1.08 ^a	0.50 ^a	0.50 ^a	0.00 ^a	0.00 ^a	0.19	0.385
Chemical composition							
DM, g/kg	350 ^c	403 ^a	367 ^b	364 ^b	366 ^b	4.20	<0.001
DM loss, %	10.2 ^a	3.02 ^c	4.02 ^c	5.76 ^b	6.42 ^b	0.06	<0.001
CP, g/kg DM	187 ^a	197 ^a	196 ^a	190 ^a	188 ^a	1.40	0.024
WSC, g/kg DM	49.9 ^c	63.1 ^b	85.0 ^a	69.1 ^b	53.3 ^c	0.60	<0.001
aNDF, g/Kg DM	385 ^a	376 ^{ab}	370 ^b	376 ^{ab}	384 ^a	1.67	0.006
ADF, g/Kg DM	290 ^a	276 ^c	279 ^{bc}	279 ^{bc}	288 ^{ab}	1.52	0.004

CON, control silage; LBB, *Lycium barbarum* by-products; LBP, *Lycium barbarum*

polysaccharide; FLA, flavonoid; BET, betaine; DM, dry matter; LA, lactic acid; AA,

908 acetic acid; PA, propionic acid; ND, not detected; LAB, lactic acid bacteria; cfu,
909 colony-forming unit; FW, fresh weight; CP, crude protein; NH₃-N, ammonia nitrogen;
910 TN, total nitrogen; aNDF, neutral detergent fiber; ADF, acid detergent fiber; WSC,
911 water-soluble carbohydrates. SEM, standard error of means. Different lowercase
912 letters indicate significant differences between different treatments at 0.05 level.
913

914 Table 3. The antioxidant properties of alfalfa silage ensiled for 90 d.

Items	Experimental treatments					SEM	P-value
	CON	LBB	LBP	FLA	BET		
T-AOC (U/g)	198.75 ^d	268.25 ^b	278.25 ^b	311.25 ^a	231.25 ^c	9.06	<0.001
SOD (U/g)	413.25 ^a	419.75 ^a	423.50 ^a	427.00 ^a	417.10 ^a	1.82	0.118
GSH-Px (U/g)	419.00 ^d	555.00 ^b	571.25 ^b	661.25 ^a	523.25 ^c	18.03	<0.001
CAT (U/g)	30.93 ^a	15.62 ^c	13.10 ^d	20.00 ^b	21.78 ^b	1.42	<0.001
DPPH (% inhibition)	52.00 ^c	70.23 ^b	70.45 ^b	74.03 ^a	69.50 ^b	1.79	<0.001

915 CON, control silage; LBB, *Lycium barbarum* by-products; LBP, *Lycium barbarum*
916 polysaccharide; FLA, flavonoid; BET, betaine; T-AOC, total antioxidant capacity;
917 SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; DPPH,
918 Diphenyl bitter acyl radical. SEM, standard error of means. Different lowercase letters
919 indicate significant differences between different treatments at 0.05 level.

920

Figure captions

Fig. 1. Microbial community analysis in alfalfa silage. (A) Shannon index of Alpha diversity analysis; (B) Ace index of Alpha diversity analysis; (C) principal coordinates analysis (PCoA) based on Bray-Curtis distance; (D) Relative abundances of bacteria at phylum level; (E) Relative abundances of bacteria at genus levels; (F) Relative abundances of bacteria at species levels. CON, control group. LBB, *Lycium barbarum* by-products group. LBP, *Lycium barbarum* polysaccharide group. FLA, flavonoid group. BET, betaine group. The significant difference is indicated by $P < 0.05$.

Fig. 2. Linear discriminant analysis effect size (LEfse) analysis of differential microorganisms among all the silages. (A) Cladogram displayed the significantly different bacteria from phylum to species level. (B) The linear discriminant analysis (LDA) bar displayed the effect of the abundance of each species whose LDA score was greater than the set value 4 among all the groups. CON, control group. LBB, *Lycium barbarum* by-products group. LBP, *Lycium barbarum* polysaccharide group. FLA, flavonoid group. BET, betaine group.

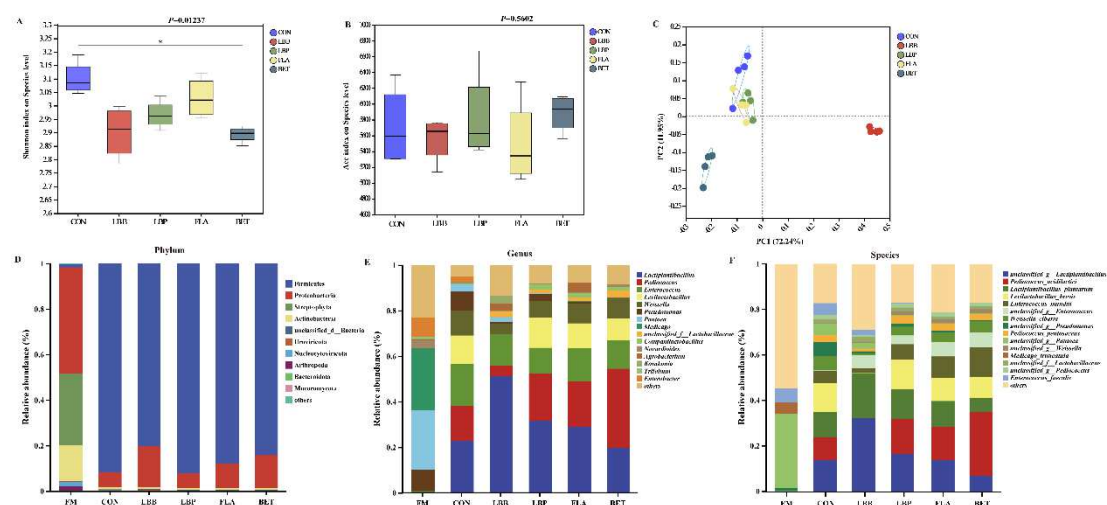
Fig. 3. The relative abundances of functionally annotated classified based on the KEGG database in silages. (A) Circos plot of function and corresponding microbes at level 3 with different treatments. (B) The linear discriminant analysis (LDA) bar showing the KEGG pathways at level 3 had significant differences among all the groups. CON, control group. LBB, *Lycium barbarum* by-products group. LBP, *Lycium barbarum* polysaccharide group. FLA, flavonoid group. BET, betaine group. The significant difference is indicated by $P < 0.05$. $LDA > 2.5$.

Fig. 4. Identification of differential metabolites. (A) The volcano plot of differential metabolites between LBB and CON. (B) between LBP and CON. (C) between FLA and CON. (D) between BET and CON; (E) The upset diagram indicated the specific and common shared differential metabolites in different comparison pairs. CON, control group. LBB, *Lycium barbarum* by-products group. LBP, *Lycium barbarum* polysaccharide group. FLA, flavonoid group. BET, betaine group. The upregulated and downregulated metabolites were shown in red and blue, respectively, whereas gray dots represented the metabolites without significance in each group.

Fig. 5. Metabolomics pathway analysis of silage. (A) KEGG functional pathways of metabolites after ensiling. (B) Differential metabolites KEGG pathway enrichment analysis of all samples. (C) KEGG pathway enrichment analysis between CON and LBB. (D) KEGG pathway enrichment analysis between CON and LBP. (E) KEGG pathway enrichment analysis between CON and FLA. (F) KEGG pathway enrichment analysis between CON and BET. (G) Major metabolic pathways involving differential metabolites. LBB, *Lycium barbarum* by-products group. LBP, *Lycium barbarum* polysaccharide group. FLA, flavonoid group. BET, betaine group. The y axis indicates the name and classification of the pathway; the x axis represents the rich factor, the higher the value, the greater the degree of enrichment. Color is used to distinguish the enrichment significance (*P* value). The darker the color, the more significantly the metabolic pathway is enriched. The color gradient on the right represents the value of *P*. Differential metabolites and *P* values are labeled around each dot.

Fig. 6. Correlation analysis of high abundance genus-level bacteria and top 30 metabolites in silage. The corresponding values in the heat map are the Spearman correlation coefficients, r , which range between -1 and 1 ; $r < 0$ indicates a negative correlation (blue), $r > 0$ indicates a positive correlation (red), and ‘*’, ‘**’ and ‘***’ represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Figure 1



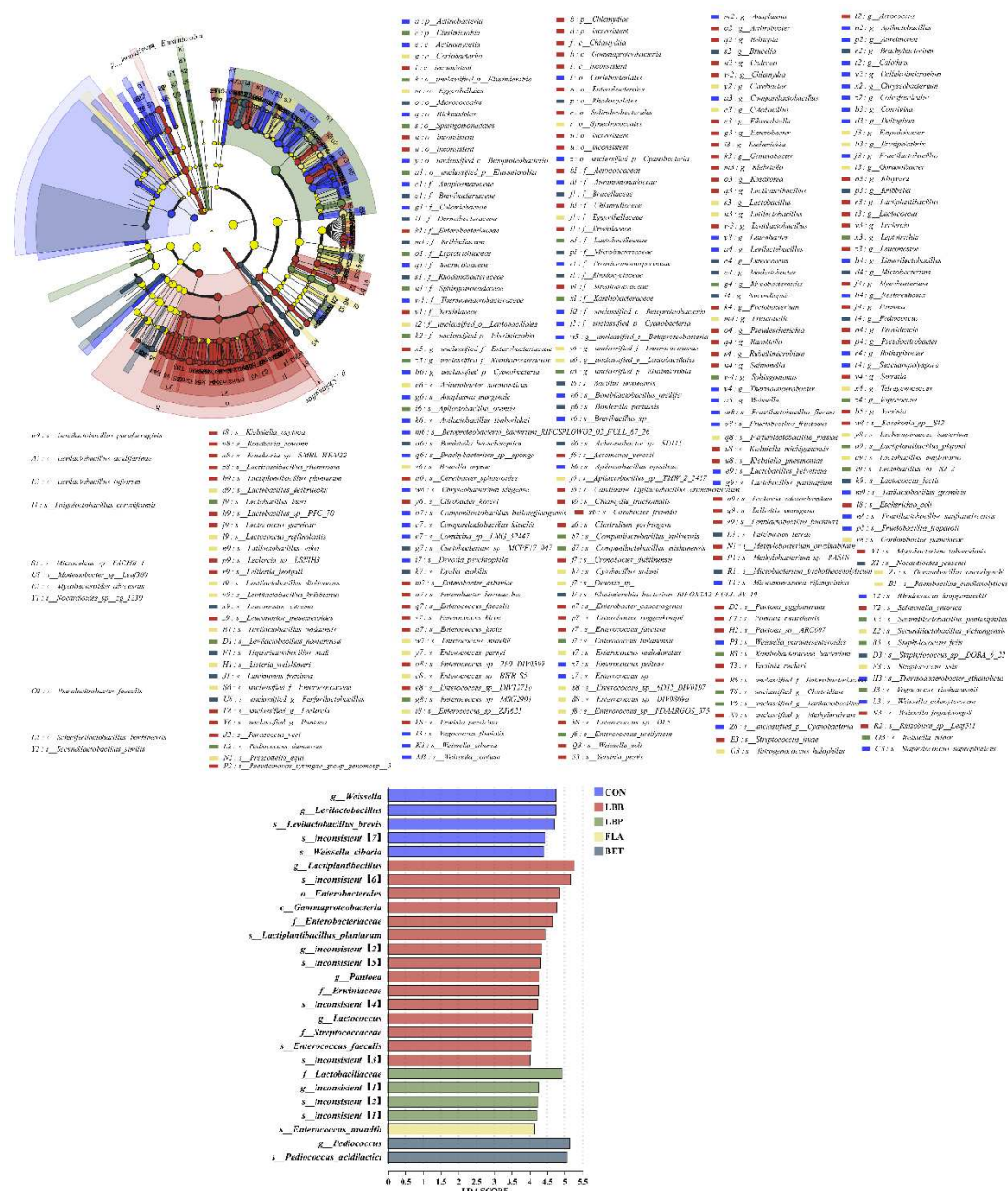
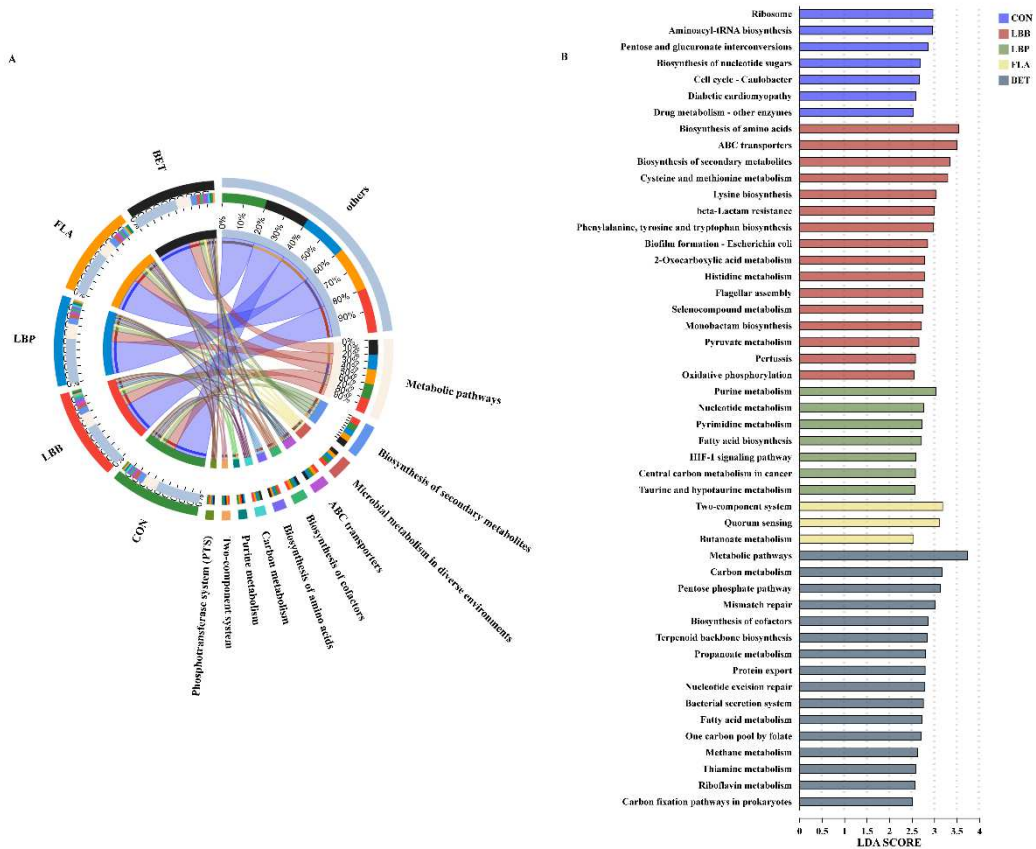
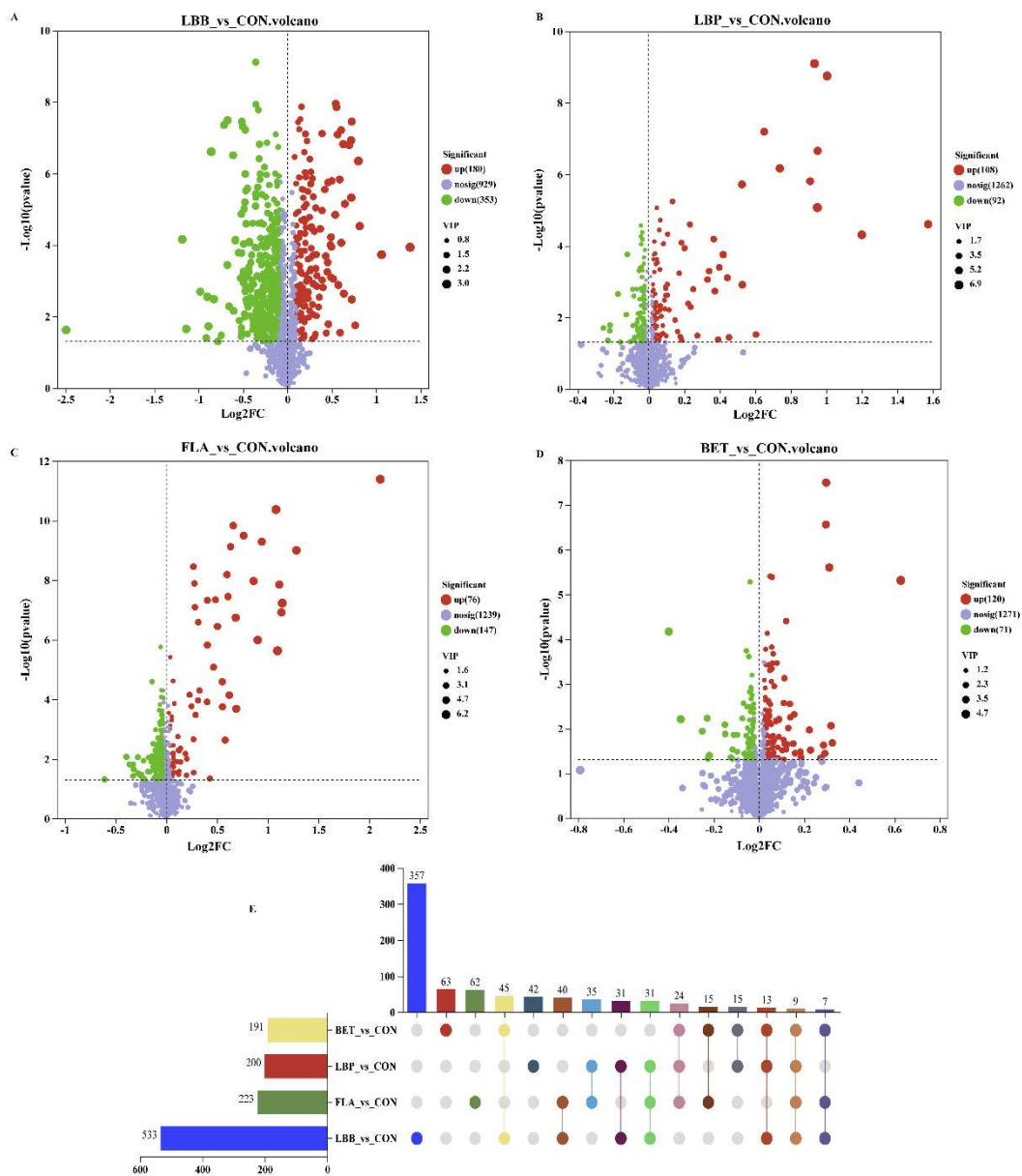


Figure 3

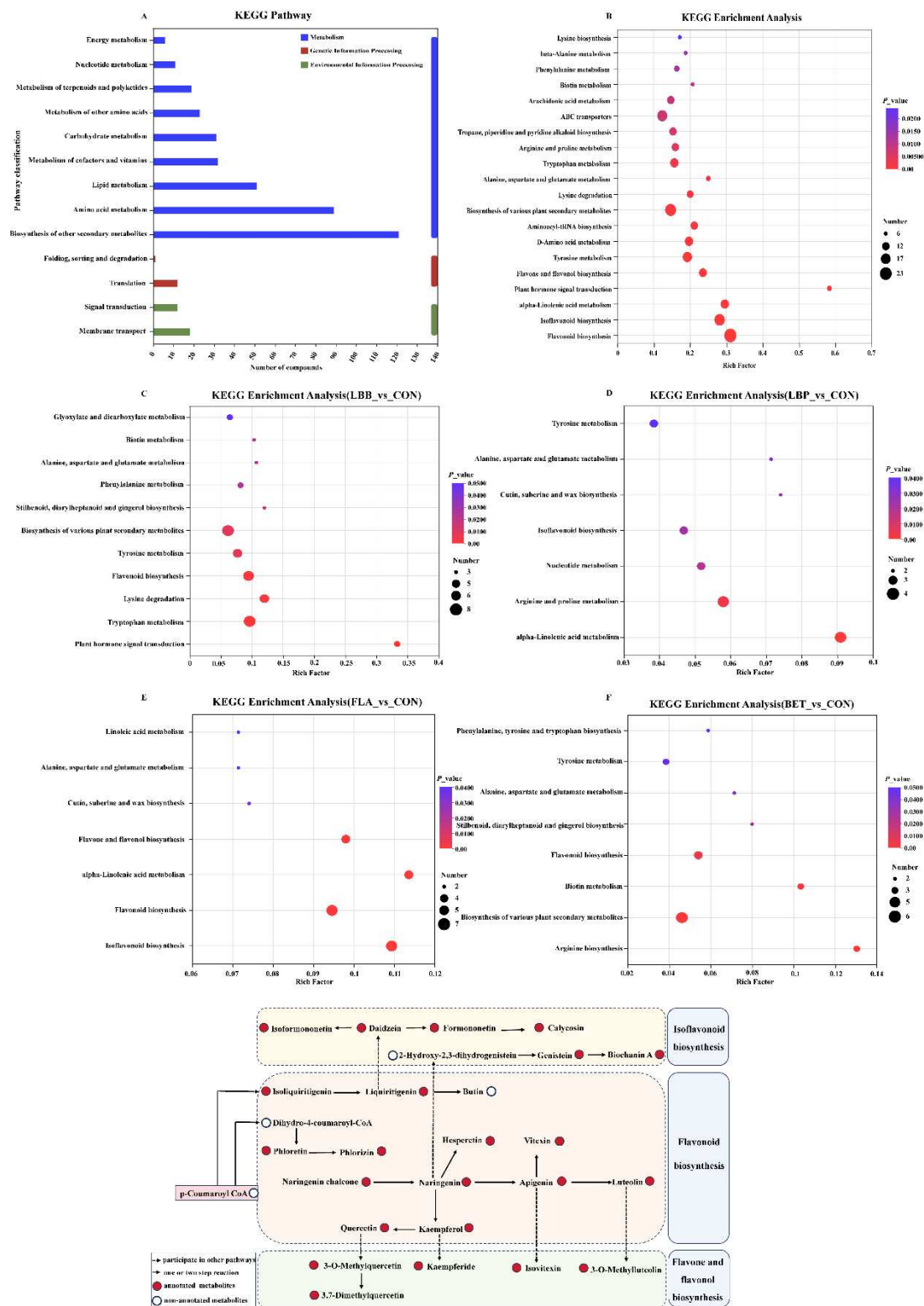


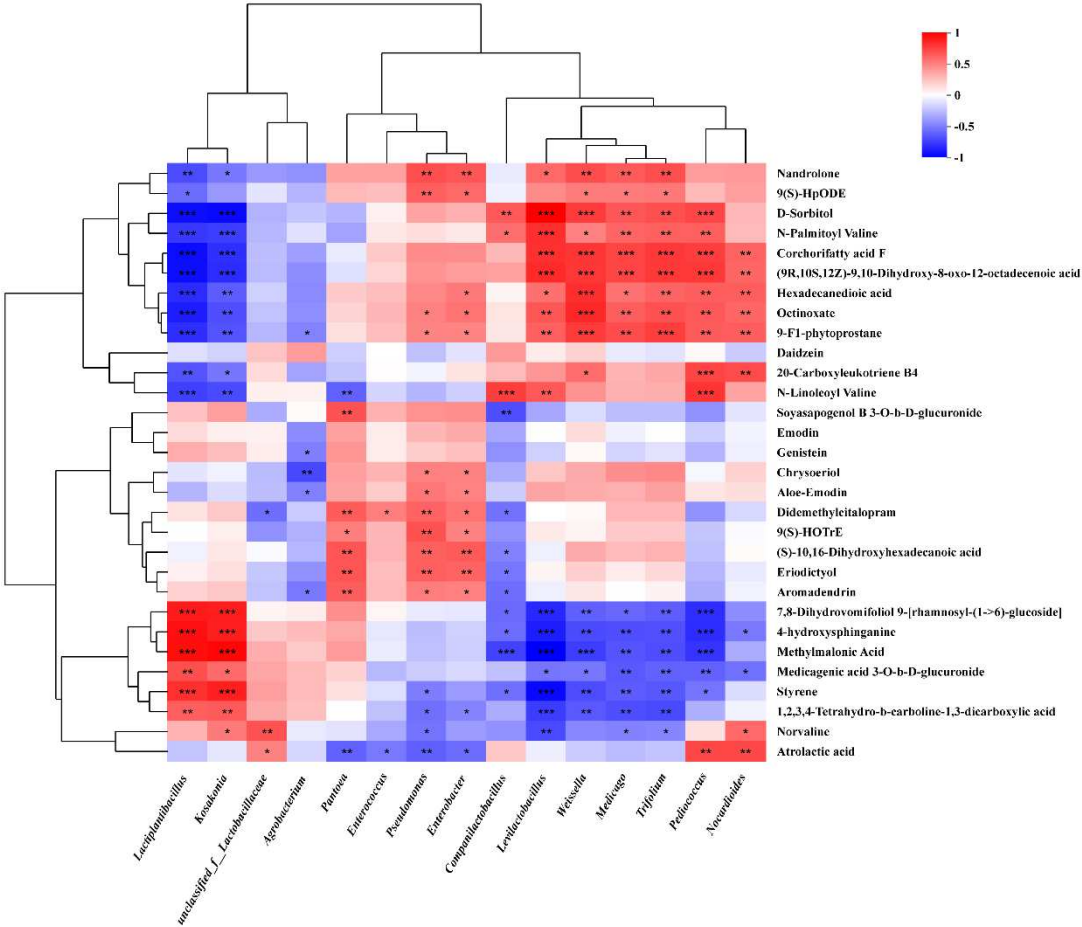
985 **Figure 4**



986

987





Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.docx](#)
- [graphicabstract.tif](#)