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# Co-Ensiling Whole-Plant Cassava with Corn Stalk for Excellent Silage Production: Fermentation Characteristics, Bacterial Community, Function Profile, and Microbial Ecological Network Features

**Mao Li [1,2,†], Xuejuan Zi [3,†], Rong Sun [3], Wenjun Ou [1], Songbi Chen [1], Guanyu Hou [1,2,*] and Hanlin Zhou [1,2,*]**

[1] Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou 571737, China; limao@catas.cn (M.L.); wenjunou@catas.cn (W.O.); songbichen@catas.cn (S.C.)
[2] Zhanjiang Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Zhanjiang 524000, China
[3] Key Laboratory of Ministry of Education for Genetics and Germplasm Innovation of Tropical Special Trees and Ornamental Plants, Key Laboratory of Germplasm Resources of Tropical Special Ornamental Plants of Hainan Province, School of Tropical Agriculture and Forestry, Hainan University, Danzhou 571737, China; zixuejuan@hainanu.edu.cn (X.Z.); sunrong@hainanu.edu.cn (R.S.)
* Correspondence: guanyuhou@catas.cn (G.H.); zhouhanlin@catas.cn (H.Z.)
† These authors have contributed equally to this work.

**Abstract:** The objective of this study was to explore excellent silage production through co-ensiling whole-plant cassava and corn stalk, and different ratios of whole-plant cassava (0%, 10%, 20%, 30%, 40%, and 50%, fresh-matter basis) co-ensiled with corn stalk were analyzed based on the silage bacterial community, function profile, and microbial ecological network features. The results demonstrated that co-ensiling 30% whole-plant cassava with 70% corn stalk could be considered an efficient mode of production. The mixed silage showed great quality, as reflected by the reduced pH value and concentrations of acetic acid, butyric acid, and ammonia nitrogen and the enhanced lactic acid concentration, V-score, and nutritional value compared with corn stalk ensiled alone. Meanwhile, co-ensiling restricted the undesirable bacterial *Acetobacter fabarum* of corn stalk and *Pseudomonas aeruginosa* of whole-plant cassava and raised the abundance of lactic acid bacteria (LAB) such as *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lactobacillus harbinensis*, etc. Besides that, the predicted functions of the bacterial community showed large differences in mixed silage compared with whole-plant cassava or corn stalk ensiled alone. Moreover, the analysis of co-occurrence networks showed that mixed silage affected microbial network features, module numbers, and bacterial relative abundances and weakened the complexity and stability of the networks compared with whole-plant cassava single silage. Furthermore, silage microbial community composition had a huge impact on the network properties, and undesirable *Pseudomonas aeruginosa* played a crucial role in the complexity and stability. Overall, this study revealed the characteristics of whole-plant cassava with corn stalk mixed-silage microbial communities and co-occurrence network modules, complexity, and stability and partly clarified the microbial mechanism of co-ensiling for producing high-quality silage. The findings of this study have important implications for deeply understanding the ensiling process and precisely regulating silage fermentation quality.

**Keywords:** co-ensiling; whole-plant cassava; corn stalk; fermentation quality; bacterial community; co-occurrence networks

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## 1. Introduction

The dietary structure of Chinese residents has undergone significant changes in recent years, with a considerable increase in the consumption of animal-based foods such as beef, mutton, and milk. This rising demand has led to the rapid development of "Grass-based Livestock Husbandry", which has greatly increased the total amount of ruminant livestock

being raised. However, the shortage of high-quality forage has been accentuated due to the limited production of existing grasslands [1]. Consequently, large amounts of forage must be imported each year, and the import of feed has become a key constraint in the development of domestic animal husbandry [2,3]. The tropical regions of southern China are important areas for ruminant livestock production, but they lack large-scale natural grasslands. This mismatch causes a serious deficit in forage [4], necessitating the effective use of local plant resources to meet farming needs.

Cassava (*Manihot esculenta* Crantz) is an important crop in subtropical and tropical areas globally, and it is also widely planted in southern China to produce starch, biofuels, and feed [4,5]. The whole cassava plant includes the tubers and roots underground and the stems and leaves aboveground. It has high biomass and is rich in essential nutrients (including protein, fiber, vitamins, and minerals) for animal growth [6] and can be harvested conveniently. The cassava tuber is a vital source of calories for both humans and animals in tropical areas as it contains abundant carbohydrates [7–10]. Cassava leaves are suitable feed with good digestibility for ruminants, pigs, and poultry, as they are rich in biomass and protein with low fiber content [4,11,12]. The cassava stem is also a high-quality feed that has about 30% starch and only a moderate cellulose content [6,13–15]. Cassava leaves, stems, and tubers each have been used as feed resources [4,11,16,17], and whole-plant cassava represents an ideal feed source with the potential to aid the sustainable development of local animal husbandry in tropical regions. Due to the seasonal limitations of cassava cultivation, it is necessary to preserve whole-plant cassava through silage processing, but the fermentation characteristics of the silage and the techniques of silage manipulation remain to be further examined.

Corn (*Zea mays* L.) stalk is the most widely used forage in the world and is used in ruminant livestock production from cold to tropical regions [18,19]. Naturally fermented corn stalk can typically be well preserved due to its high content of water-soluble sugar [20], but Bernardes et al. [21] indicated that in tropical regions, due to the impact of high temperatures and the presence of undesirable bacteria, the corn stalk silage may have too much butyric acid or experience alcoholic fermentation and undergo aerobic deterioration. Furthermore, the single silage of corn stalk generally does not have sufficient protein, which is not helpful in balancing the carbon and nitrogen contents in the diet of ruminants [22]. Some modulation methods improve the storage of the corn stalk by enhancing silage quality and preserving nutrition [18].

Silage fermentation can be regarded as a dynamic microbial ecology process, and microbial co-occurrence networks, network modules, and network stability are the three most important characteristics of microbial ecological systems [18,23,24]. Due to the lack of epiphytic lactic acid bacteria (LAB), low sugar content, or high buffer capacity, additives are needed to produce silage from most forage [4,12]. Treating a single raw material with additives can significantly enhance the silage quality but typically does not improve the nutritional profile. The nutritional value of the silage is decreased when some nutrients are consumed during the anaerobic fermentation [21]. In addition, previous studies have shown that the silage quality and the microbial co-occurrence network interact with each other. The raw material epiphytic microbiota, LAB inoculants, and storage temperatures can affect the microbial co-occurrence networks and modules in the silage, thereby enhancing fermentation quality [3,18,25,26].

The co-ensiling of two or more raw materials to create mixed silage improves the fermentation quality by changing the physical and chemical properties of the materials and the composition of epiphytic microorganisms. Many studies have shown that the co-ensiling of lower-nutrition forage with rich-nutrition forage readily improves the fermentation quality and the nutritional value of the silage. We previously reported that the 1:1 co-ensiling of cassava leaves with king grass strongly improved the quality and the feeding value of the silage [12]. Numerous studies have demonstrated that co-ensiling corn stalk with high-protein-content forage (e.g., soybean, alfalfa, Neolamarckia cadamba) was beneficial to silage fermentation and nutrient preservation [22,27–29]. Since whole-plant

cassava is rich in nutrients, we speculated that co-ensiling it with corn stalk may contribute to the fermentation. However, the underlying microbial principles, e.g., the correlation between the mixed silage and the complexity and stability of the bacterial networks, for the success of the co-ensiling practice is largely unknown. Analyzing the microbial ecological network in the mixed silage will help to understand the ensiling process and regulate the fermentation quality.

As stated in the above analyses, we speculate that the co-ensiling of whole-plant cassava and corn stalk may help to produce high-quality feed with good fermentation quality and excellent nutritional value. However, as far as we know, existing research has not explored the bacterial community, function, and microbial ecological network of the mixed silage of whole-plant cassava and corn stalk. The objective of this study is to optimize the production of animal feed by co-ensiling whole-plant cassava and corn stalk at different ratios, with a particular focus on silage bacterial community structure, function profile, microbial ecological network characteristics, and network complexity and stability.

## 2. Materials and Methods

### *2.1. Silage Preparation*

The present study was conducted at the Chinese Academy of Tropical Agricultural Sciences (CATAS) in Danzhou, Hainan, China (109°30′ E, 19°30′ N, 149 m ASL). Two cultivars of materials were used. The whole-plant cassava (SC7) and corn (Huamei) were planted on 15 March 2021 and harvested on 15 September 2021 from 3 randomly selected plots. The corn was harvested at half milk-line.

The fresh raw materials were shredded to about 2 cm in size using a 9Z-2.5 grass chopper (Zhengzhou Jinhongxing Jixie Co. Ltd., Zhengzhou, China) and then mixed evenly according to the formulations given in Table 1 (fresh-matter basis). For each formulation, three vacuum-sealed plastic bags (30 cm × 10 cm × 4 cm) of the blended materials (500 g) were prepared, and the incubation was carried out at room temperature. The samples were analyzed on day 60 to determine the chemical composition, fermentation quality, and microbial community.

**Table 1.** Co-ensiling formulation settings.

| Group | Corn Stalk (CS) | Whole-Plant Cassava (CF) |
|---|---|---|
| CS | 100% | 0% |
| CS10CF | 90% | 10% |
| CS20CF | 80% | 20% |
| CS30CF | 70% | 30% |
| CS40CF | 60% | 40% |
| CS50CF | 50% | 50% |
| CF | 0% | 100% |

### *2.2. Chemical Composition and Fermentation Index*

The weight of the dry matter (DM) was measured after heating the silage samples at 65 °C for 72 h. The dried materials were then ground and passed through a sieve (1 mm), and the water-soluble carbohydrates (WSC), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured according to the protocols given by the Association of Official Analytical Chemists (AOAC, 1990), and NDF was assayed with a heat-stable amylase. The starch content was determined using a total starch determination kit based on the procedures described by Bai et al. [20]. The number of lactic acid bacteria, molds, and coliform bacteria was determined with MRS agar, potato dextrose agar, and violet red bile agar, respectively [29].

In addition, a mixture of the silage sample (50 g) in distilled water (200 mL) was incubated at 4 °C for 24 h and then filtered. Half of the filtrate was used for the analysis of the fermentation quality as follows [30]. First, the pH was measured with a glass electrode pH meter. The concentrations of lactic acid, acetic acid, propionic acid, and butyric acid were

determined by high-performance liquid chromatography (HPLC, SHIMADZU-10A, Kyoto, Japan). The ammonia nitrogen ($NH_3$–N) was assayed by the phenol-sodium hypochlorite method according to the protocol described by Liu et al. [31]. The V-Score was calculated as the index of fermentation quality based on the ratio of $NH_3$–N to total N, the content of acetic acid and propionic acid, and the amount of higher volatile fatty acids [32]. All of the above indexes had three replicates. The other half of the filtrate was stored at −80 °C until use in the analysis of the microbial communities.

### *2.3. Microbial Communities and Functional Profile*

The total bacteria DNA of the silage was extracted from the saved filtrate using the TGuide S96 Magnetic Soil /Stool DNA Kit from Tiangen Biotech (Beijing, China) Co., Ltd. The DNA concentration was determined using the Qubit dsDNA HS Assay Kit and a Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The universal primer set (27F: AGRGTTTGATYNTGGCTCAG, 1492R: TASGGHTACCTTGTTASGACTT) was used to amplify the full-length 16S rRNA gene from the total genomic DNA extracted from each sample. The PCR protocols (amplification, purification, quantification) followed the work of Bai et al. [18]. The high-quality PCR products were sequenced on a PacBio Sequel platform (Pacific Biosciences, Menlo Park, CA, USA) according to the standard protocols of Biomarker Technologies (Beijing, China).

The bioinformatics analysis was performed at the BMK Cloud (Biomarker Technologies Co., Ltd., Beijing, China). The raw reads generated from sequencing were filtered and demultiplexed using the SMRT Link software (version 8.0) to obtain the circular consensus sequencing (CCS) reads, with the minPasses set at ≥5 and minPredictedAccuracy at ≥0.9. The CCS reads were assigned to the corresponding samples based on their barcodes using lima (version 1.7.0). The CCS reads containing no primers or outside the length range (1200–1650 bp) were discarded using Cutadapt (version 2.7). Chimera sequences were detected and removed using UCHIME (version 8.1) to obtain the clean reads. Sequences with ≥97% similarity were clustered into the same operational taxonomic unit (OTU) using USEARCH (version 10.0), and the OTUs were filtered if their redundancy was less than 0.005%.

The taxonomy annotation of the OTUs was performed based on the Naive Bayes classifier in QIIME2 using the SILVA database (release 138) with a similarity cut-off of 70% [33]. The alpha diversity indices (Shannon index and Simpson's index) were calculated using QIIME2 and visualized using the R software v4.2.3. The beta diversity (as determined by the principal coordinate analysis, PCoA) was determined using QIIME to evaluate the degree of similarity between microbial communities from different samples. The Linear Discriminant Analysis (LDA) effect size (LEfSe) was used to test the significant taxonomic difference among groups [34]. The bacterial functions were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and by applying the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) [35]. The analyses of the silage microbial network (co-occurrence network, network modules, complexity, and stability) were performed using ggClusterNet (https://github.com/taowenmicro/ggClusterNet/, accessed on 15th May 2023) [24]. Network features include nodes, edges, network density, average degree, negative/positive edge ratios, etc. The basic elements in a network are nodes and edges, and the connections between nodes are called edges. Network density is the ratio of the actual number of edges present in a network to the upper limit of the number of edges it can accommodate. The average degree refers to the average number of edges connected to each node in a network graph. The correlation heatmaps were drawn and the canonical correlation analysis (CCA) between the network properties and microbial community was carried out to investigate the impact of the silage microbial communities on the microbial networks. Data were analyzed using the free online BMKCloud Platform (https://www.biocloud.net/). The sequencing data were deposited in the Sequence Read Archive (SRA, accession number PRJNA1011842).

### 2.4. Statistical Analysis

All statistical analyses were performed using SPSS 20 and GraphPad Prism 8.0. The results are reported as the mean of three replicates. For each treatment, the fermentation quality and the chemical composition were analyzed by one-way analysis of variance (ANOVA). The orthogonal polynomial contrasts (linear correlation) were used to evaluate the effects of the mixing ratio. The differences between the mean values were considered significant if Duncan's multiple-range test gave $p < 0.05$.

## 3. Results

### 3.1. Raw Material

Table 2 lists the chemical composition and epiphytic microbial number of the fresh whole-plant cassava and the corn stalk. For the whole-plant cassava, the DM content was 242.5 g/kg fresh matter (FM), and the CP, ADF, NDF, WSC, and starch contents were 158.6, 116.8, 158.9, 177.2, and 215.9 g/kg DM, respectively. For the corn stalk, the DM content was 305.1 g/kg FM, and the CP, ADF, NDF, WSC, and starch contents were 96.4, 226.3, 407.5, 128.4, and 104.6 g/kg DM, respectively. Both the whole-plant cassava and the corn stalk had a similar number of epiphytic lactic acid bacteria, but the whole-plant cassava had less mold and *Enterobacter*.

**Table 2.** Chemical composition of raw materials.

| Items | Abbreviation | Whole-Plant Cassava | Corn Stalk |
|---|---|---|---|
| Dry matter (g/kg FM) | DM | 242.5 | 305.1 |
| Crude protein (g/kg DM) | CP | 158.6 | 96.4 |
| Acid detergent fiber (g/kg DM) | ADF | 116.8 | 226.3 |
| Neutral detergent fiber (g/kg DM) | NDF | 158.9 | 407.5 |
| Water-soluble carbohydrates (g/kg DM) | WSC | 177.2 | 128.4 |
| Starch (g/kg DM) | | 215.9 | 104.6 |
| Lactic acid bacteria (Log cfu/g FM) | LAB | 5.76 | 5.88 |
| Mold (Log cfu/g FM) | | 2.43 | 4.52 |
| *Enterobacter* (Log cfu/g FM) | | 2.68 | 4.73 |

Note: FM, fresh matter; DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; WSC, water-soluble carbohydrates; LAB, lactic acid bacteria.

### 3.2. Fermentation Quality

The mixed silage clearly had higher fermentation quality than the single silage (Table 3). Co-ensiling effectively reduced pH, acetic acid, propionic acid, butyric acid, and ammonia nitrogen content and significantly increased lactate content and improved the V-score. Among all treatment groups, CS30CF had the best fermentation quality, with the lowest pH value and acetic acid, propionic acid, butyric acid, and $NH_3$–N concentrations ($p < 0.05$), as well as the highest lactic acid content and V-score ($p < 0.05$).

**Table 3.** Fermentation quality of mixed silage.

| Items | Treatments | | | | | | | SEM | *p*-Value | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CS | CS10CF | CS20CF | CS30CF | CS40CF | CS50CF | CF | | T | L |
| pH | 4.95 a | 4.56 b | 4.23 c | 3.97 d | 4.18 c | 4.23 c | 4.27 c | 0.12 | <0.001 | >0.05 |
| Lactic acid (g/kg DM) | 42.10 d | 51.19 b | 53.77 b | 62.39 a | 54.00 b | 48.25 c | 46.93 c | 2.43 | <0.001 | >0.05 |
| Acetic acid (g/kg DM) | 25.73 a | 17.92 b | 14.87 b | 9.29 c | 13.96 b | 16.64 b | 19.28 a | 1.92 | <0.001 | >0.05 |
| Propionic acid (g/kg DM) | 4.31 | 3.98 | 3.14 | 2.25 | 3.24 | 4.07 | 4.16 | 0.28 | >0.05 | >0.05 |
| Butyric acid (g/kg DM) | 1.08 a | 0.69 a | 0.44 b | N | 0.31 b | 0.55 b | 0.88 a | 0.11 | N | N |
| $NH_3$–N (g/kg TN) | 80.52 a | 73.84 b | 66.56 b | 51.81 c | 62.34 b | 69.45 b | 85.78 a | 4.29 | <0.001 | >0.05 |
| V-Score | 75.26 c | 79.71 b | 83.17 b | 92.30 a | 85.05 b | 81.71 b | 75.80 c | 2.21 | <0.001 | >0.05 |

Note: DM, dry matter, TN, total nitrogen. Within the same row, values with different letters denote statistically significant ($p < 0.05$) differences. SEM, standard error of the mean. N, not detected; T, treatment; L, linear.

Table 4 demonstrates the chemical compositions of the mixed silage. The contents of DM, ADF, and NDF were the highest for the single silage of corn stalk ($p < 0.05$) and gradually decreased with a rising amount of whole-plant cassava. In contrast, the contents of CP, WSC, and starch were the highest for the single silage of whole-plant cassava and gradually decreased with the addition of corn stalk. Significant differences existed in the chemical composition of mixed silage, and there was a notable linear correlation with the mixing ratio ($p < 0.01$). Both whole-plant cassava and corn stalk contained abundant nutrients, and the nutrients, especially the CP, WSC, and starch, were consumed to some extent after ensiling.

**Table 4.** Chemical composition of mixed silage.

| Items | Treatments | | | | | | | SEM | *p*-Value | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CS | CS10CF | CS20CF | CS30CF | CS40CF | CS50CF | CF | | T | L |
| DM (g/kg FM) | 282.5 a | 276.2 b | 269.9 b | 263.5 bc | 257.2 c | 250.9 c | 219.3 d | 7.89 | <0.001 | <0.001 |
| CP (g/kg DM) | 83.4 d | 89.3 cd | 95.2 cd | 101.1 c | 107.0 b | 113.0 b | 142.5 a | 7.38 | <0.001 | <0.001 |
| ADF (g/kg DM) | 198.6 a | 188.2 b | 177.7 c | 167.3 d | 156.8 de | 146.4 e | 94.1 f | 13.05 | <0.001 | <0.001 |
| NDF (g/kg DM) | 379.7 a | 355.0 b | 330.2 c | 305.5 cd | 280.8 d | 256.1 e | 132.4 f | 30.88 | <0.001 | <0.001 |
| WSC (g/kg DM) | 56.1 c | 59.7 c | 63.3 bc | 66.9 bc | 70.5 b | 74.1 b | 92.0 a | 4.48 | <0.001 | <0.001 |
| Starch (g/kg DM) | 70.7 d | 79.2 dc | 87.6 c | 96.1 bc | 104.6 b | 113.1 a | 155.4 | 10.58 | <0.001 | <0.001 |

Note: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; WSC, water-soluble carbohydrates. Within the same row, values with different letters denote statistically significant ($p < 0.05$) differences. SEM, standard error of the mean. T, treatment; L, linear.

### *3.3. Bacterial Communities*

#### 3.3.1. Diversity, Compositions, Structure, and Predicted Functions

Figure 1 plots the alpha and beta diversities of the bacterial communities in the silage. The Shannon diversity and Simpson's diversity indices were the lowest for the single silage of corn stalk (Group CS, $p < 0.05$) and increased by the co-ensiling of corn stalk and whole-plant cassava. The silage of all seven groups had 5928 OTUs in total and shared 25 OTUs in common (Figure 1C), and the unique OTU number was the lowest for CS (507) and the highest for CS10CF (1069). The structure of the microbial communities shifted in response to the mixing ratio, as the microbial communities in the silage varied significantly among the seven groups (Figure 1D).

The compositions of the bacterial communities in the silage were identified at the genus level and compared with each other (Figure 2A). The single silage of corn stalk (Group CS) had predominantly *Acetobacter* (36.67%) and *Levilactobacillus* (12.20%), and the abundance of *Acetobacter* decreased significantly after co-ensiling with whole-plant cassava ($p < 0.05$), reaching zero for CS50CF and CF. Meanwhile, the single silage of whole-plant cassava (Group CF) was dominated by *Lactiplantibacillus* (14.37%), *Levilactobacillus* (11.90%), and the undesirable *Pseudomonas* (5.79%). For all mixed silage, *Levilactobacillus*, *Schleiferilactobacillus*, and *Limosilactobacillus* were the top three genera, and their total abundance was the highest in CS30CF. At the species level, *Acetobacter fabarum* was the most abundant in CS, while *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lactobacillus harbinensis*, and *Pseudomonas aeruginosa* were the top bacterial species in other groups (Figure 2B).

The differences in the bacterial communities among silage were analyzed by LEfSe to identify the microbial taxa specific to each group (Figure 2C, Supplementary Materials Table S1). The bacteria that were enriched significantly in a treatment group were deemed indicator bacteria. The indicator bacteria were *Clostridia*, *Oscillospirales*, *Lactiplantibacillus plantarum*, and *Companilactobacillus nuruki* for CF; *Acetobacter*, *Acetobacter fabarum*, *Lacticaseibacillus*, *Gluconobacter*, and *Gluconobacter japonicus* for CS; *Blautia hansenii* for CS20CF; *Schleiferilactobacillus*, *Levilactobacillus*, *Lentilactobacillus*, *Lactobacillus harbinensis*_DSM_16991, *Levilactobacillus brevis*, *Lentilactobacillus parabuchner*, *Lentilactobacillus buchneri*, and *Companilactobacillus paralimentarius* for CS30CF; and *Pseudomonas*, *Pseudomonas aeruginosa*, and *Lactobacillus* for CS50CF. While the indicator bacteria of CS and CS50CF were undesirable for the silage, CS30CF had many healthy lactic acid bacteria as its indicator bacteria.

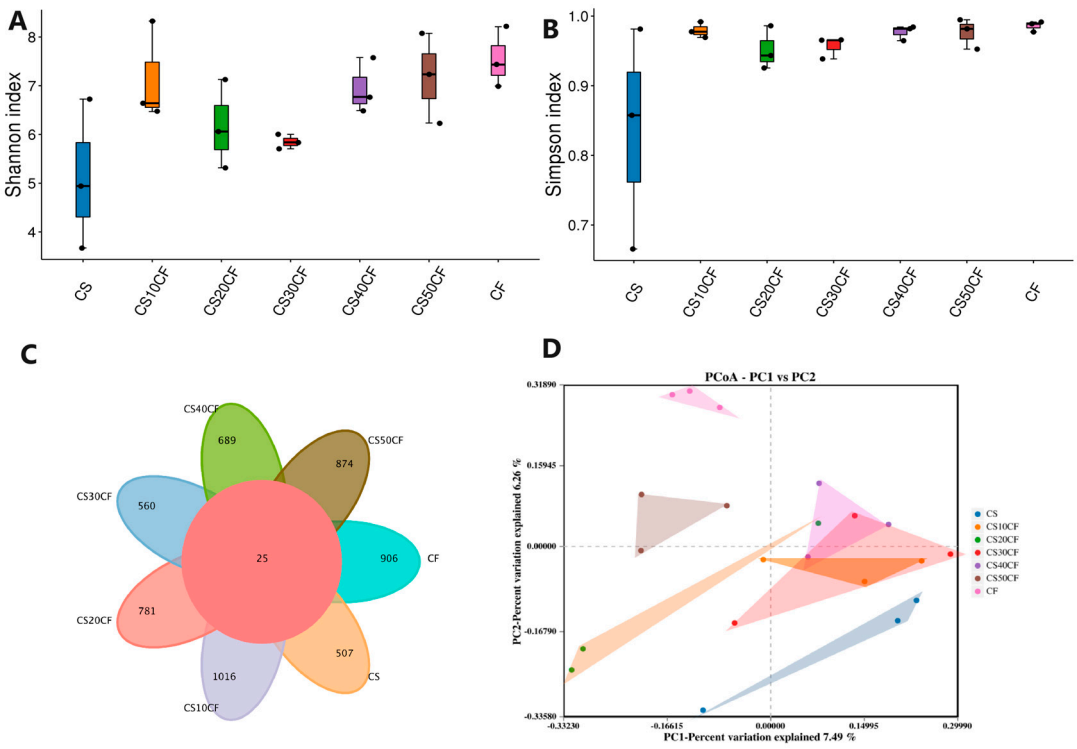



**Figure 1.** (**A**) The Shannon index and (**B**) the Simpson index of the bacterial communities in the silage. The number on the horizontal line represents the *p*-value. (**C**) Venn diagram of the bacterial OTUs. (**D**) Principal Coordinates Analysis (PCoA) and the beta diversity of the bacterial communities in the silage. Notations are as defined in Table 1.

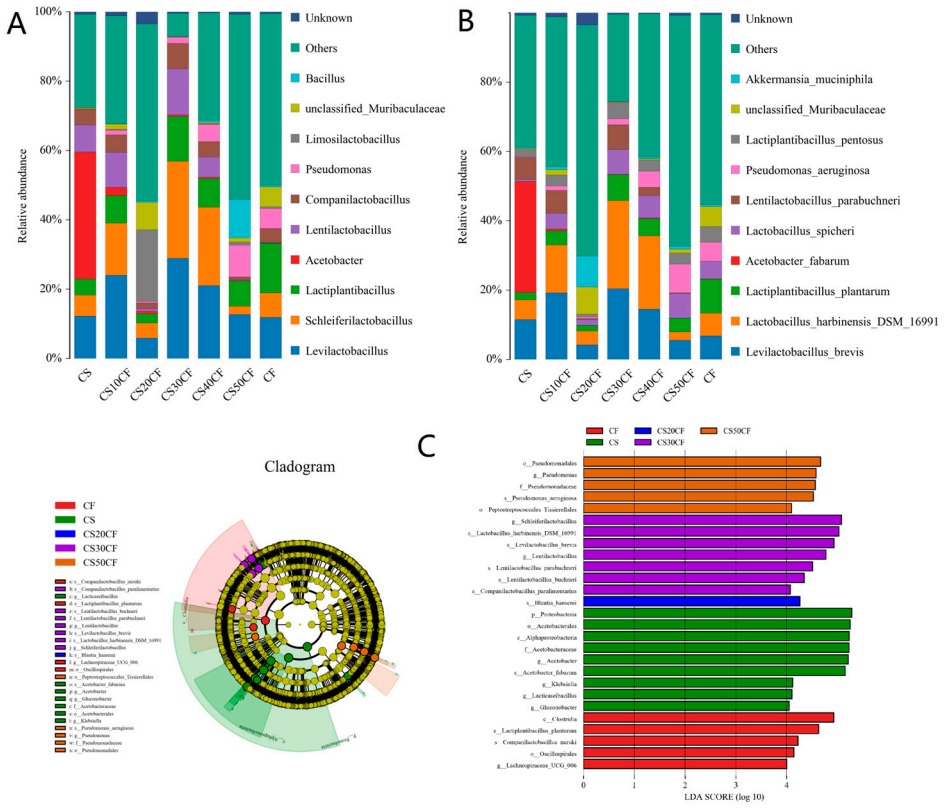



**Figure 2.** Relative abundance of bacteria at the (**A**) genus and (**B**) species levels in the silage. (**C**) Comparison of microbial variations and the identification of indicator bacteria using LEfSe.

In the silage of the seven treatment groups, the LAB included *Levilactobacillus*, *Schleiferilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Companilactobacillus*, and *Limosilactobacillus* and their corresponding species-level microorganisms (Figure 3A,C), and the undesirable bacteria included *Acetobacter*, *Pseudomonas*, and unclassified_*Muribaculaceae* and their corresponding species-level microorganisms (Figure 3B,D). We then calculated the total abundance of the LAB and the undesirable bacteria for each group (Figure 3). Co-ensiling clearly increased the lactic acid bacteria and reduced the undesirable bacteria compared to the single silage of whole-plant cassava or corn stalk, and CS30CF had the highest abundance of LAB and the lowest abundance of undesirable bacteria ($p < 0.05$), which is probably why the fermentation quality of CS30CF was the best.

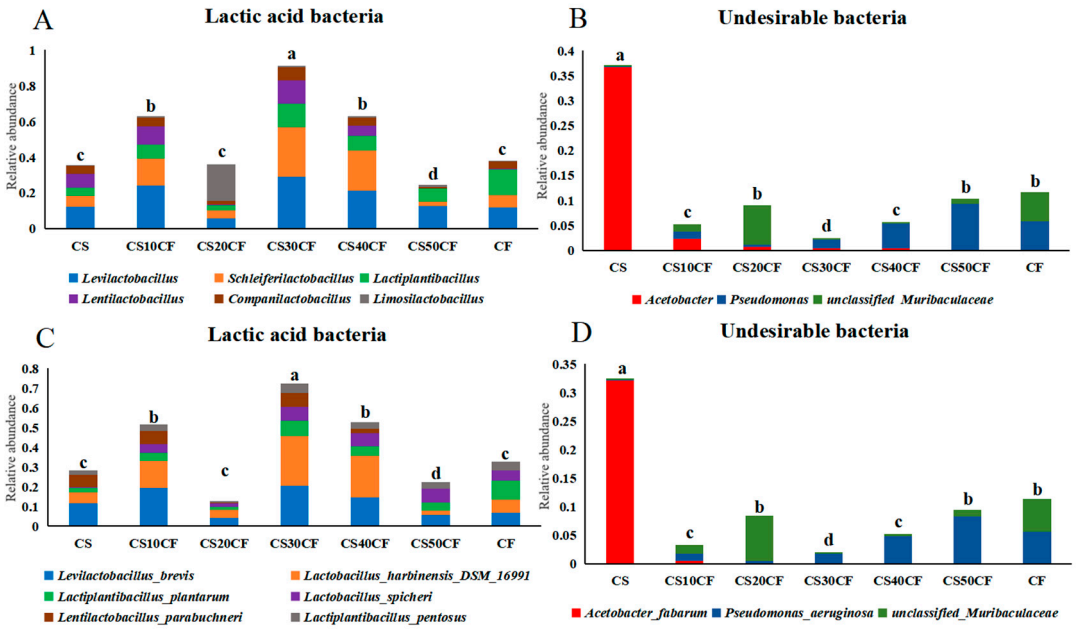



**Figure 3.** Abundance of lactic acid bacteria at the (**A**) genus and (**C**) species levels in the silage. Abundance of undesirable bacteria at the (**B**) genus and (**D**) species level in the silage. Different letters on bar chart denote statistically significant ($p < 0.05$) difference.

The potential functions of the bacterial communities were predicted using PICRUSt2 (Figure 4). The treatment groups CS, CF, and CS30CF were selected for functional prediction based on the specificity of raw materials, fermentation quality, and microbial composition. Figure 4A shows that there were 16 significantly different KEGG pathways between CF and CS30CF ($p < 0.05$). The four upregulated pathways in CF were (1) global and overview maps, (2) the metabolism of terpenoids and polyketides, (3) amino acid metabolism, and (4) the signal transduction and biosynthesis of other secondary metabolites. The twelve upregulated pathways in CS30CF were (1) carbohydrate metabolism, (2) nucleotide metabolism, (3) lipid metabolism, (4) the metabolism of other amino acids, (5) xenobiotics biodegradation and metabolism, (6) glycan biosynthesis and metabolism, (7) the metabolism of terpenoids and polyketides, (8) translation, (9) replication and repair, (10) folding, sorting, and degradation, (11) antimicrobial drug resistance, and (12) the endocrine system. Figure 4B shows that the two significantly ($p < 0.05$) downregulated KEGG pathways in Group CS compared to Group CS30CF were (1) replication and repair and (2) the metabolism of other amino acids. Moreover, the signal transduction pathways were enriched significantly ($p < 0.05$) in CF compared to CS (Figure 4C). Compared to single silage, co-ensiling significantly changed the microbial community structure, which rendered the obvious diversity in the predicted metabolic functions. The impact of co-ensiling on the predicted functions was mainly related to the metabolism of nutrients, e.g., amino acids, carbohydrates, and lipids.

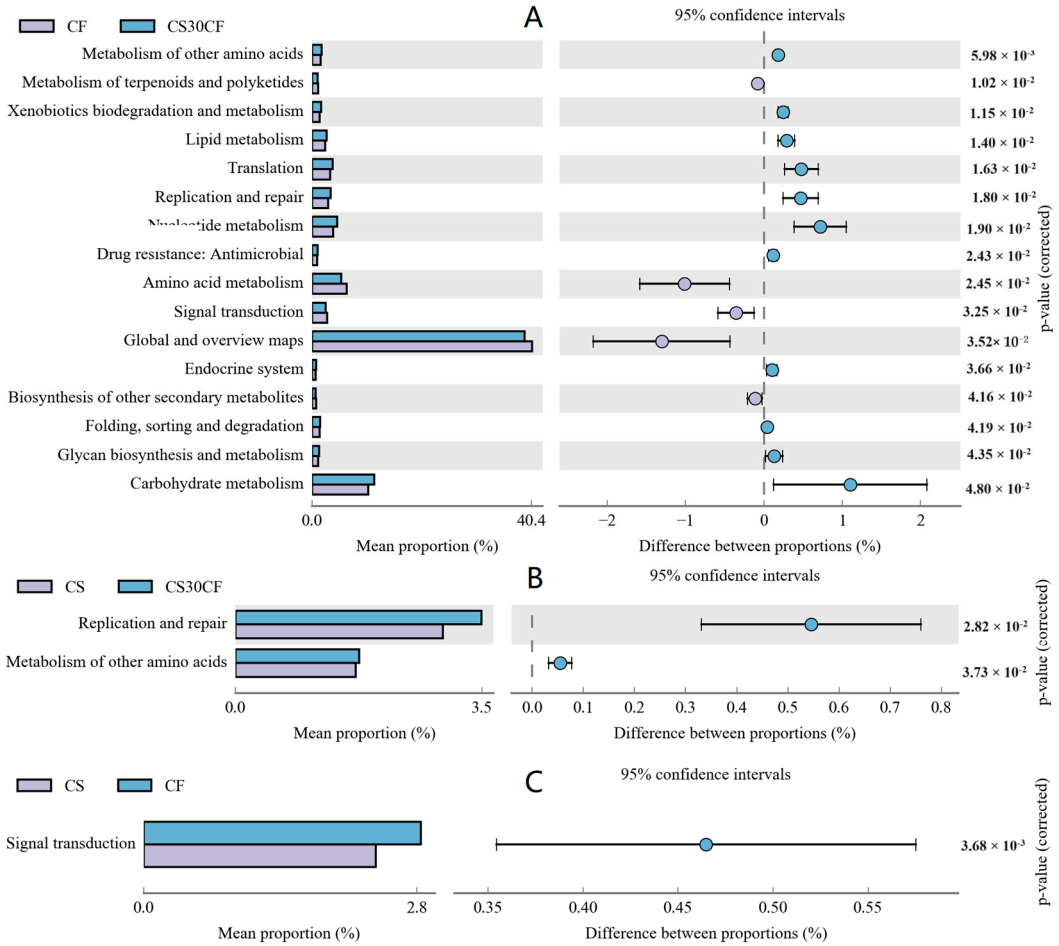



**Figure 4.** Comparison of microbial function pathways: detected pathway enrichment of (**A**) CF vs. CS30CF, (**B**) CS vs. CS30CF, and (**C**) CS vs. CF.

### 3.3.2. Co-Occurrence Network, Network Modules, and Stability

The co-occurrence networks of the bacterial communities varied with the silage. Figure 5 shows that the networks of Group CF had the greatest edges, network density, and average degree. The higher the number of edges, average degree, and network density, the tighter the network connection. That is, Group CF had more complicated microbial community structures and interactions than Group CS and other mixed silage groups. In addition, Group CF also had a significantly higher ($p < 0.05$) ratio of negative to positive interactions than all other groups. Because the microbial networks are more stable when the ratio of negative to positive edge interactions is higher, for the seven groups, the bacterial networks were less stable when the silage treatment used corn stalk.

Figure 6 shows the modules of the co-occurrence networks, and the details of the bacterial compositions of modules in different treatments are listed in Supplementary Materials Table S2–S8. The networks in Group CF had only one, but in the largest module (module_1), the dominant bacteria were all LAB, including *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Lactobacillus harbinensis*, *Lactobacillus spicheri*, *Lactiplantibacillus pentosus*, and *Companilactobacillus nuruki*. There were also some undesirable bacteria in module_1 with lower abundance, including unclassified_*Muribaculaceae*, *Pseudomonas aeruginosa*, and *Mycoplasma wenyonii*. In contrast, there were six modules in the bacterial networks of Group CS, and module_3 and module_5 were dominant. *Acetobacter fabarum* was the most dominant bacteria in module_3, but module_5 mainly contained three bacteria with high abundance, i.e., *Lentilactobacillus parabuchneri*, *Paucilactobacillus vaccinostercus*, and *Lactobacillus harbinensis*. The modules in the other five mixed silage groups had much more diverse compositions.

Interestingly, in the bacterial networks of Group CS50CF, *Pseudomonas aeruginosa* was the major bacteria in module_1, which was one of the dominant modules. Correspondingly, Group CS50CF had a lower abundance of lactic acid bacteria and a higher abundance of undesirable bacteria compared to other mixed silage groups, hence indicating that the module dominated by *Pseudomonas aeruginosa* affected the bacterial communities in the silage. Overall, co-ensiling significantly changed the numbers, pattern size, distributions, and relative abundances of the modules in the bacterial networks, and the diversity of the microbial network modules depended heavily on the mixing ratio of corn stalk to whole-plant cassava.

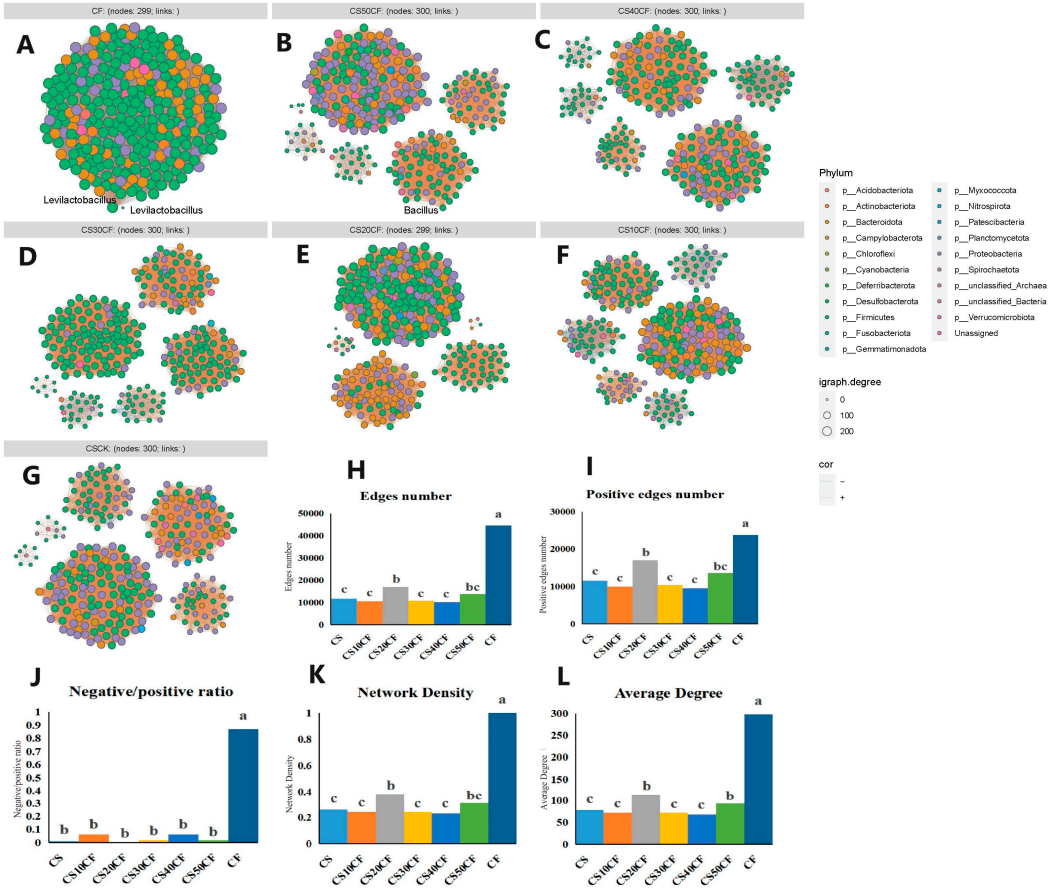



**Figure 5.** The bacterial community co-occurrence networks of (**A**) CF, (**B**) CS50CF, (**C**) CS40CF, (**D**) CS30CF, (**E**) CS20CF, (**F**) CS10CF, and (**G**) CS. (**H**) Numbers of edges, (**I**) numbers of positive edges, (**J**) negative/positive edge ratios, (**K**) network density, and (**L**) average degree of bacterial community co-occurrence networks.Different letters on bar chart denote statistically significant ($p < 0.05$) difference.

The diversity of the microbial network modules may impact the function and stability of the silage micro-ecosystem. We calculated multiple stability indices (robustness, natural connectivity, and negative correlation ratio) to assess how the microbial network stability in the silage was affected by the mixing ratio of corn stalk to whole-plant cassava (Figure 7). On the basis of either the random or targeted removal of module hubs, the silage of Group CF had noticeably higher robustness than that of all other groups (Figure 7A,B). The bacterial network appeared to have the greatest natural connectivity and thus the strongest resilience in Group CF (Figure 7C). Group CF also had a remarkably higher negative correlation ratio than all other groups (Figure 7D). In general, co-ensiling whole-plant cassava and corn stalk reduced the stability of the silage microbial network compared to

ensiling whole-plant cassava alone, probably due to the decreased network complexity associated with the connectivity and relative modularity.

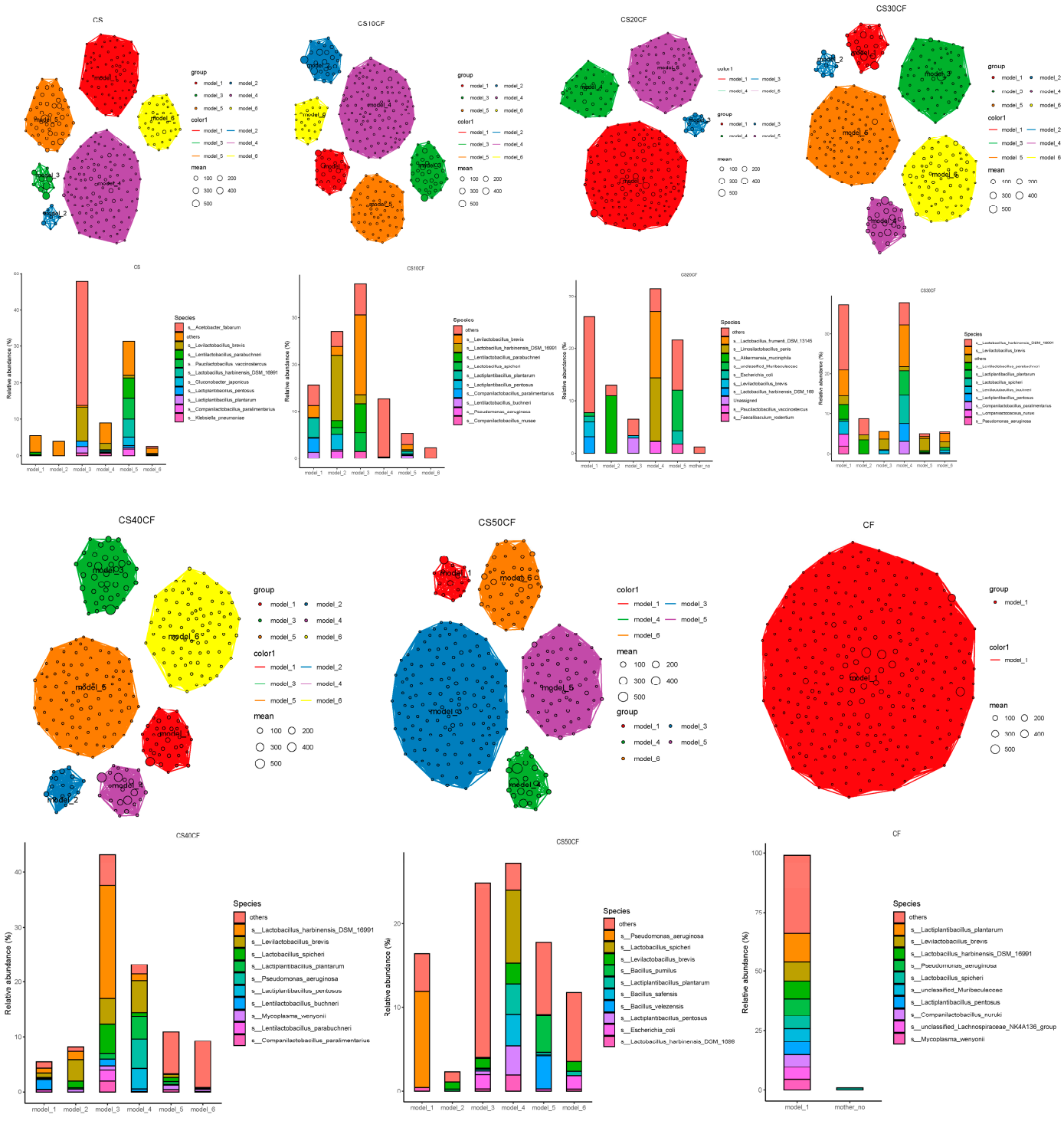



**Figure 6.** Modules in the bacterial community co-occurrence networks.

Figure 8 plots the correlation heatmap and the canonical correlation analysis (CCA) between the network properties and microbial communities to examine the impact of the microbial community composition on the microbial networks. In the correlation heatmap (Figure 8A), the edges, positive edges, edge density, and average degree had significant negative correlations with *Lactobacillus harbinensis*_DSM_16991, *Levilactobacillus brevis*, and *Lentilactobacillus parabuchneri* ($p < 0.05$). In addition, the negative edges and the negative/positive ratio had positive correlations with *Pseudomonas aeruginosa*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, and *Lactobacillus spicheri* ($p < 0.05$). According to the canonical correlation analysis (Figure 8B), the positive edges, negative/positive ratio, density, and centralization degree were affected by the microbial communities in the silage. Positive edges and the negative/positive ratio had the strongest and weakest impact, respectively. The negative/positive ratio was closely related to *Pseudomonas aeruginosa* and *Lactobacillus spicheri*, and the centralization degree was closely related to *unclassified_Muribaculaceae*.

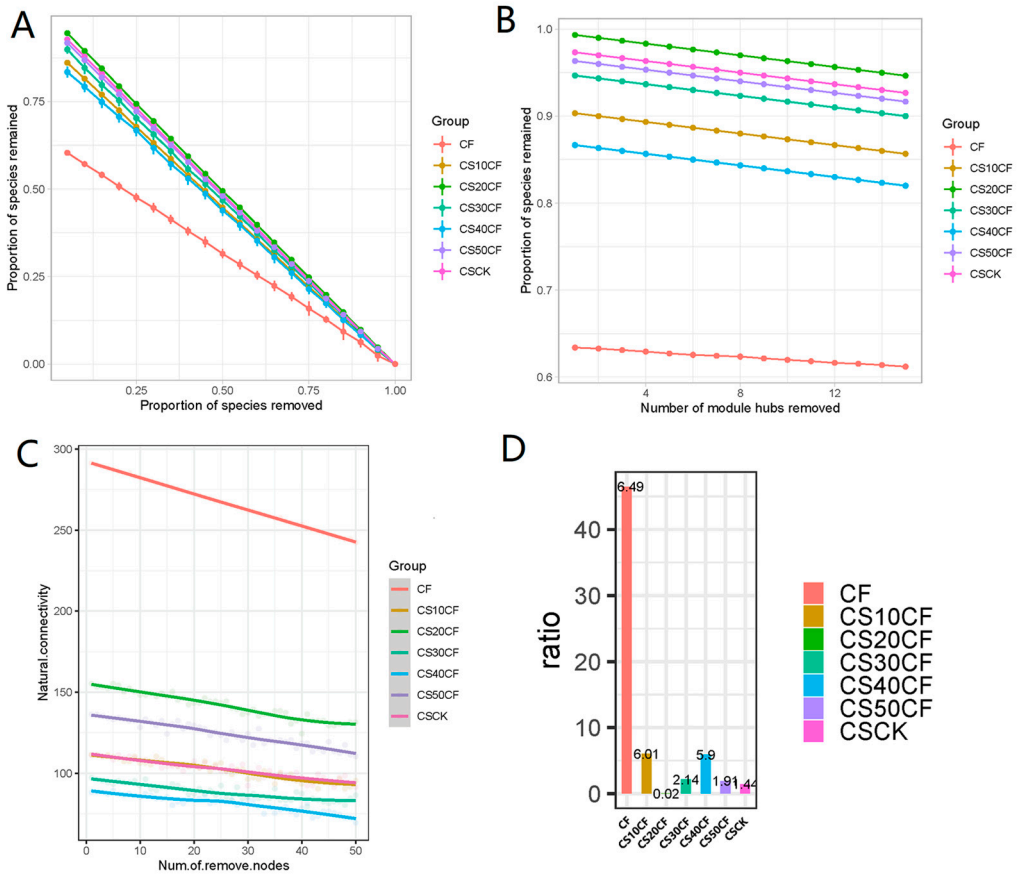



**Figure 7.** Robustness of microbial networks upon the (**A**) random and (**B**) targeted removal of module hubs. (**C**) Natural connectivity and (**D**) negative correlation ratio of the microbial networks.

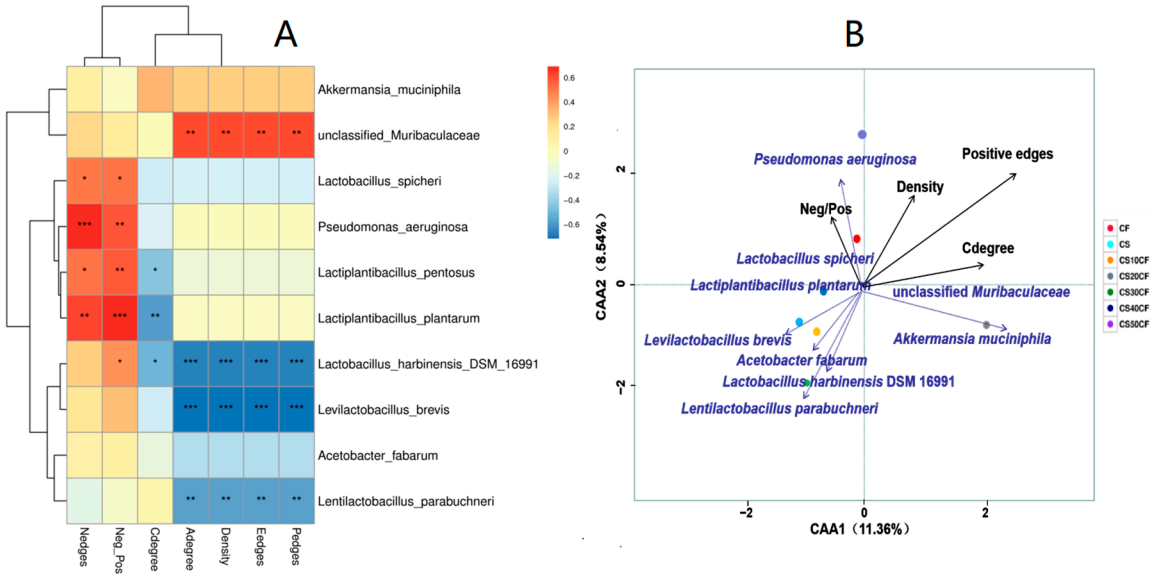



**Figure 8.** (**A**) Correlation heatmap of network properties and microbial communities. Positive and negative correlations are shown in red and blue, respectively. The color intensity is proportional to the correlation values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**B**) Canonical correlation analysis of network properties and microbial communities.

## 4. Discussion

Whole-plant cassava has more balanced nutrition for animals than its leaves, stem, or tubers [10,11,15], but the feeding value of whole-plant cassava has not been examined. The data of the corn stalk agreed with those reported by Wang et al. [28] and Bai et al. [20], confirming that corn was an ideal feed for livestock with high DM, WSC, and starch content. We found that the whole-plant cassava had abundant CP, WSC, and starch with low fiber content. In addition, both whole-plant cassava and corn stalk had abundant epiphytic LAB and few undesirable bacteria, which is beneficial to creating high-quality silage through co-ensiling.

The pH value of the silage is a key parameter of the fermentation quality. The single silage of corn stalk gave the highest pH value. The single silage of whole-plant cassava gave an obviously lower pH compared to the single silage of cassava foliage, indicating that whole-plant cassava is a better silage material [4]. The addition of whole-plant cassava to corn stalk significantly reduced the pH value for the mixed silage, and all mixed silage except CS10CF had a pH value close to or lower than 4.2, which is the pH value threshold of well-preserved silage. Wang et al. [3] and Zeng et al. [29] reported similar pH values and trends for the mixed silages of corn with soybean or alfalfa. In addition, in our results, all silage groups had a relatively high content of lactic acid, likely because the low pH fermentation environment contributed to homofermentation to produce large amounts of lactic acid. Wang et al. [3] also noted an obvious increase in lactate in the mixed silage of *Sesbania cannabina* and sweet sorghum. Furthermore, the changes in butyric acid and ammonia nitrogen, both of which are harmful to the preservation of forage nutrients, were consistent with the variation in the pH value. Butyric acid and ammonia nitrogen are the product of protein decomposition by undesirable bacteria such as *Clostridium*, aerobic bacteria, and *Enterobacteriaceae*, all of which typically grow better at a higher pH. Both corn stalk and whole-plant cassava contain abundant nutrients that may also benefit the growth of undesirable bacteria, hence potentially increasing the contents of butyric acid and ammonia nitrogen. Some previous studies also reported that the mixed silage could effectively reduce undesirable microbial fermentation and inhibit the production of butyric acid and ammonia nitrogen [3,28,29]. In summary, co-ensiling whole-plant cassava and corn stalk improved the silage quality, which was characterized by low pH value, higher lactic acid content, and significantly reduced butyric acid and ammonia nitrogen content.

The ammonia nitrogen content can reflect the decomposition of proteins or amino acids [36]. Like the mixed silage system of corn stalk and forage soybean, the mixed silage of corn stalk and whole-plant cassava also had a significantly lower content of ammonia nitrogen, indicating the stronger preservation of CP [27]. On the other hand, the sharp reduction in WSC and starch contents revealed the efficient uptake of the fermentation substrates by the lactic acid bacteria to generate organic acids in high yields. Zeng et al. [29] and Wang et al. [3] also reported similar phenomena in the mixed silage of materials with different nutritional characteristics. In this study, the change in the nutritional compositions of the mixed silage was mainly due to the change in the mixing ratio of the two raw materials, and a more balanced nutrient composition was more conducive to silage fermentation.

Typically, the LAB occupy a dominant position as the fermentation proceeds during silage production, which simplifies the structure and reduces the alpha diversity of the bacterial communities in well-preserved silage [26,29]. Among all the mixed silage, Group CS30CF had the lowest alpha diversity and the best fermentation quality. Interestingly, lower alpha diversity was also found for the single silage of corn stalk (Group CS), likely due to the high abundance of the undesirable *Acetobacter* (36.67%). The mixed silage also had an obvious impact on the beta diversity of the bacterial communities, which were clearly separated based on their silage group. The results agreed well with the findings from the mixed silages of corn stalk and alfalfa or soybean [28,29].

Previous research suggested that corn stalk silage is generally well-preserved and dominated by LAB [18,20,28,29]. In our case, the corn stalk silage was dominated by *Acetobacter* (36.67%), which reduced the fermentation quality, possibly due to the secondary fermentation of the raw materials. In contrast, *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* were dominant in the single silage of whole-plant cassava, and they contributed to the fermentation quality by promoting lactic acid fermentation. Some previous studies reported low fermentation quality for the silage of cassava foliage not treated with additives, in which *Pseudomonas*, *Bacillus*, *Paenibacillus*, and *Clostridium* were dominant [4,37]. We found that co-ensiling corn stalk and whole-plant cassava optimized the silage microbial community structure by increasing the abundance of lactic acid bacteria (up to 80%) and reducing undesirable bacteria (down to 0.05%). Similar benefits have been observed in other co-ensiling systems. For example, Wang et al. [3] found that in the mixed silage of *Sesbania cannabina* and sweet sorghum, the abundance of *Lentilactobacillus hilgardii* and *L. buchneri* increased to over 90% while the undesirable *Bacillus* and *Enterococcus* decreased to zero. Wang et al. [28] reported that the microbial communities in the mixed silage of alfalfa and corn were dominated by *L. buchneri* and *L. plantarum*, with a combined abundance of approximately 50%. Zeng et al. [29] reported that the abundance of *Lactobacillus* was approximately 70% in the mixed silage of forage soybean and corn. Interestingly, the well-preserved silage in the current study contained many more dominant bacteria, including *Levilactobacillus*, *Schleiferilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Companilactobacillus*, and *Limosilactobacillus*. Further research is needed to account for the flourishing of dominant bacteria in the mixed silage of corn stalk and whole-plant cassava.

Ensiling is a complex micro-ecological system process in which diverse microbial communities affect metabolic products to regulate the fermentation quality. The functional prediction of the microbial communities can to some extent reflect the role of the microbes in the fermentation systems [25]. In this study, the most abundant pathways were "global and overview maps", and they were upregulated in Group CF, which was consistent with the previous report by Du et al. [38] and indicated the critical role of these pathways in microbial metabolism. Amino acid metabolism was an important metabolic pathway related to the degradation of nitrogen and amino acids. Group CF had the highest CP and ammonia nitrogen content, along with remarkably upregulated amino acid metabolism pathways. Similar observations have been reported by Bai et al. [20] and Li et al. [39]. Carbohydrate metabolism is mainly affected by the abundance of lactic acid bacteria in the microbial communities. Group CS30CF had the most abundant lactic acid bacteria, which presumably led to the upregulation of the carbohydrate metabolism pathways, similar

to the reports by Wang et al. [3] and Li et al. [39]. In addition, some pathways regarding genetic functions were also enriched in different treatment groups, possibly because the microbial communities were adapting to the acidic environments during ensiling [20]. The diverse microorganisms in the silage fermentation system enabled different metabolic pathways and functions, hence regulating the fermentation quality.

From an ecological perspective, each individual package of silage is a microbial ecosystem [3,25]. Numerous research articles suggest that the changes in the network structure of an ecosystem can affect the functionality and stability at a macroscopic scale, and the relationships between network complexity and stability have been clarified to some extent [40,41]. Yuan et al. [23] analyzed the complexity and stability of the molecular ecological networks of soil microbial communities. Recent analyses of the microbial community networks in silage revealed that environmental factors, modulation methods, the duration of fermentation, and silage materials all have a significant impact on the microbial networks. Dong et al. [26] demonstrated that the epiphytic microbiota of a sorghum–sudangrass hybrid harvested at various times of the day affected the complexity of the bacterial networks in the silage, and the AM silage was more stable. Bai et al. [18] studied the bacterial network properties of whole-plant corn silage and found that the storage temperature had a greater influence on the network complexity than treatment with added lactic acid bacteria. Wang et al. [3] showed that the bacterial network stability of the mixed silage increased as the duration of the fermentation was extended. However, the impact of co-ensiling on the features of bacterial network modules, complexity, and stability is still largely unknown. Our results revealed that compared to the single silage of whole-plant cassava, the mixed silage had microbial networks with less complexity, lower stability, higher module numbers, and different bacterial compositions. There were also strong interactions between the dominant bacterial species in the silage and the microbial network characteristics. As for the microbial networks, the complexity was influenced by the dominant lactic acid bacteria, and the stability was largely affected by the undesirable bacteria *Pseudomonas aeruginosa*. The current results suggested that the lower abundance of the undesirable bacteria in the silage played a crucial role in the complexity and stability of microbial ecological networks, and it appeared that the tested co-ensiling had a negative impact on the complexity and stability of the bacterial networks.

## 5. Conclusions

Co-ensiling whole-plant cassava with corn stalk is an efficient mode of silage production. The analysis of several kinds of ratio mixtures of corn stalk and whole-plant cassava revealed that the best silage was produced from 30% cassava and 70% corn, as reflected by excellent fermentation quality and nutritional value. The co-ensiling reduced the abundance of undesirable bacteria and increased the abundance of LAB. The analysis of the microbial co-occurrence networks revealed that co-ensiling affected microbial network features, module numbers, and bacterial relative abundances and weakened the complexity and stability of the networks. The composition of the microbial communities had a heavy impact on the network properties, and the undesirable *Pseudomonas aeruginosa* played a crucial role in the complexity and stability. The findings helped to understand the co-ensiling process by partly clarifying the microbial mechanisms of co-ensiling in producing high-quality silage.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030501/s1, Table S1: Species lefse LDA4 less strict, Table S2: CSCKplot tax module data, Table S3: CS10CFplot tax module data, Table S4: CS20CFplot tax module data, Table S5: CS30CFplot tax module data, Table S6: CS40CFplot tax module data, Table S7: CS50CFplot tax module data, Table S8: CFplot tax module data.

**Author Contributions:** M.L., X.Z., R.S., W.O., S.C., G.H. and H.Z. carried out the experimental design work. M.L., X.Z. and R.S. conducted the experiments. M.L., X.Z., R.S., W.O., S.C., G.H. and H.Z.

analyzed the data. M.L., X.Z. and R.S. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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## References

1. Fang, J.; Jing, H.; Zhang, W.; Gao, S.; Duan, Z.; Wang, H.; Zhong, J.; Pan, Q.; Zhao, K.; Bai, W.; et al. The concept of "Grass-based Livestock Husbandry" and its practice in Hulun Buir, Inner Mongolia. *Chin. Sci. Bull.* **2018**, *63*, 1619–1631. [CrossRef]
2. Tang, S.X.; He, Y.; Zhang, P.H.; Jiao, J.Z.; Han, X.F.; Yan, Q.X.; Tan, Z.L.; Wang, H.R.; Wu, D.Q.; Yu, L.H.; et al. Nutrient digestion, rumen fermentation and performance as ramie (*Boehmeria nivea*) is increased in the diets of goats. *Anim. Feed. Sci. Technol.* **2019**, *247*, 15–22. [CrossRef]
3. Wang, T.; Zhang, J.; Shi, W.; Sun, J.; Xia, T.; Huang, F.; Liu, Y.; Li, H.; Teng, K.; Zhong, J. Dynamic Changes in Fermentation Quality and Structure and Function of the Microbiome during Mixed Silage of Sesbania cannabina and Sweet Sorghum Grown on Saline-Alkaline Land. *Microbiol. Spectr.* **2022**, *10*, e0248322. [CrossRef] [PubMed]
4. Li, M.; Zi, X.; Zhou, H.; Lv, R.; Tang, J.; Cai, Y. Effect of lactic acid bacteria, molasses, and their combination on the fermentation quality and bacterial community of cassava foliage silage. *Anim. Sci. J.* **2021**, *92*, e13635. [CrossRef]
5. Malik, A.I.; Kongsil, P.; Nguyễn, V.A.; Ou, W.; Srean, P.; Sheela, M.N.; Becerra López-Lavalle, L.A.; Utsumi, Y.; Lu, C.; Kittipadakul, P.; et al. Cassava breeding and agronomy in Asia: 50 years of history and future directions. *Breed. Sci.* **2020**, *70*, 145–166. [CrossRef] [PubMed]
6. Lyu, H.; Yang, S.; Zhang, J.; Feng, Y.; Geng, Z. Impacts of utilization patterns of cellulosic C5 sugar from cassava straw on bioethanol production through life cycle assessment. *Bioresour. Technol.* **2021**, *323*, 124586. [CrossRef] [PubMed]
7. Isamah, G.K.; Asagba, S.O.; Ekakitie, A.O. Lipid peroxidation, activities of superoxide dismutase and catalase during post-harvest deterioration of cassava (*Manihot esculenta* Crantz) root tubers. *Int. Biodeterior. Biodegrad.* **2003**, *52*, 129–133. [CrossRef]
8. Kayode, B.I.; Kayode, R.M.O.; Salami, K.O.; Obilana, A.O.; George, T.T.; Dudu, O.E.; Adebo, O.A.; Njobeh, P.B.; Diarra, S.S.; Oyeyinka, S.A. Morphology and physicochemical properties of starch isolated from frozen cassava root. *LWT* **2021**, *147*, 111546. [CrossRef]
9. Bogale, S.; Haile, A.; Berhanu, B.; Beshir, H.M. Cassava production practices in Ethiopia and its use as Ingredient for injera making. *Future Foods* **2022**, *6*, 100204. [CrossRef]
10. Ono, L.T.; Silva, J.J.; Soto, T.S.; Doná, S.; Iamanaka, B.T.; Fungaro, M.H.P.; Taniwaki, M.H. Fungal communities in Brazilian cassava tubers and food products. *Int. J. Food Microbiol.* **2023**, *384*, 109909. [CrossRef]
11. Anyanwu, C.N.; Ibeto, C.N.; Ezeoha, S.L.; Ogbuagu, N.J. Sustainability of cassava (*Manihot esculenta* Crantz) as industrial feedstock, energy and food crop in Nigeria. *Renew. Energ.* **2015**, *81*, 745–752. [CrossRef]
12. Li, M.; Zi, X.; Tang, J.; Zhou, H.; Cai, Y. Silage fermentation, chemical composition and ruminal degradation of king grass, cassava foliage and their mixture. *Grassl. Sci.* **2019**, *65*, 210–215. [CrossRef]
13. Zhu, W.; Lestander, T.A.; Örberg, H.; Wei, M.; Hedman, B.; Ren, J.; Xie, G.; Xiong, S. Cassava stems: A new resource to increase food and fuel production. *GCB Bioenergy* **2015**, *7*, 72–83. [CrossRef]
14. Ojo, I.; Apiamu, A.; Egbune, E.O.; Tonukari, N.J. Biochemical Characterization of Solid-State Fermented Cassava Stem (*Manihot esculenta* Crantz-MEC) and Its Application in Poultry Feed Formulation. *Appl. Biochem. Biotechnol.* **2022**, *194*, 2620–2631. [CrossRef]
15. Fanelli, N.S.; Torres-Mendoza, L.J.; Abelilla, J.J.; Stein, H.H. Chemical composition of cassava-based feed ingredients from South-East Asia. *Anim. Biosci.* **2023**, *36*, 908–919. [CrossRef]
16. Wanapat, M.; Kang, S. Cassava chip (*Manihot esculenta* Crantz) as an energy source for ruminant feeding. *Anim. Nutr.* **2015**, *1*, 266–270. [CrossRef] [PubMed]
17. Azad, M.A.K.; Jiang, H.; Ni, H.; Liu, Y.; Huang, P.; Fang, J.; Kong, X. Diets Partially Replaced With Cassava Residue Modulate Antioxidant Capacity, Lipid Metabolism, and Gut Barrier Function of Huanjiang Mini-Pigs. *Front. Vet. Sci.* **2022**, *9*, 902328. [CrossRef]
18. Bai, J.; Ding, Z.; Su, R.; Wang, M.; Cheng, M.; Xie, D.; Guo, X. Storage Temperature Is More Effective Than Lactic Acid Bacteria Inoculations in Manipulating Fermentation and Bacterial Community Diversity, Co-Occurrence and Functionality of the Whole-Plant Corn Silage. *Microbiol. Spectr.* **2022**, *10*, e0010122. [CrossRef]

19. Cui, Y.; Liu, H.; Gao, Z.; Xu, J.; Liu, B.; Guo, M.; Yang, X.; Niu, J.; Zhu, X.; Ma, S.; et al. Whole-plant corn silage improves rumen fermentation and growth performance of beef cattle by altering rumen microbiota. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 4187–4198. [CrossRef]
20. Bai, J.; Franco, M.; Ding, Z.; Hao, L.; Ke, W.; Wang, M.; Xie, D.; Li, Z.; Zhang, Y.; Ai, L.; et al. Effect of Bacillus amyloliquefaciens and Bacillus subtilis on fermentation, dynamics of bacterial community and their functional shifts of whole-plant corn silage. *J. Anim. Sci. Biotechnol.* **2022**, *13*, 7. [CrossRef]
21. Bernardes, T.F.; Daniel, J.L.P.; Adesogan, A.T.; McAllister, T.A.; Drouin, P.; Nussio, L.G.; Huhtanen, P.; Tremblay, G.F.; Bélanger, G.; Cai, Y. Silage review: Unique challenges of silages made in hot and cold regions. *J. Dairy. Sci.* **2018**, *101*, 4001–4019. [CrossRef]
22. Zhou, W.; Pian, R.; Yang, F.; Chen, X.; Zhang, Q. The sustainable mitigation of ruminal methane and carbon dioxide emissions by co-ensiling corn stalk with Neolamarckia cadamba leaves for cleaner livestock production. *J. Clean. Prod.* **2021**, *311*, 127680. [CrossRef]
23. Yuan, M.M.; Guo, X.; Wu, L.; Zhang, Y.; Xiao, N.; Ning, D.; Shi, Z.; Zhou, X.; Wu, L.; Yang, Y.; et al. Climate warming enhances microbial network complexity and stability. *Nat. Clim. Chang.* **2021**, *11*, 343–348. [CrossRef]
24. Wen, T.; Xie, P.; Yang, S.; Niu, G.; Liu, X.; Ding, Z.; Xue, C.; Liu, Y.-X.; Shen, Q.; Yuan, J. ggClusterNet: An R package for microbiome network analysis and modularity-based multiple network layouts. *iMeta* **2022**, *1*, e32. [CrossRef]
25. Xu, D.; Wang, N.; Rinne, M.; Ke, W.; Weinberg, Z.G.; Da, M.; Bai, J.; Zhang, Y.; Li, F.; Guo, X. The bacterial community and metabolome dynamics and their interactions modulate fermentation process of whole crop corn silage prepared with or without inoculants. *Microb. Biotechnol.* **2021**, *14*, 561–576. [CrossRef]
26. Dong, Z.; Li, J.; Wang, S.; Dong, D.; Shao, T. Diurnal Variation of Epiphytic Microbiota: An Unignorable Factor Affecting the Anaerobic Fermentation Characteristics of Sorghum-Sudangrass Hybrid Silage. *Microbiol. Spectr.* **2023**, *11*, e0340422. [CrossRef] [PubMed]
27. Ni, K.; Zhao, J.; Zhu, B.; Su, R.; Pan, Y.; Ma, J.; Zhou, G.; Tao, Y.; Liu, X.; Zhong, J. Assessing the fermentation quality and microbial community of the mixed silage of forage soybean with crop corn or sorghum. *Bioresour. Technol.* **2018**, *265*, 563–567. [CrossRef] [PubMed]
28. Wang, M.; Franco, M.; Cai, Y.; Yu, Z. Dynamics of fermentation profile and bacterial community of silage prepared with alfalfa, whole-plant corn and their mixture. *Anim. Feed. Sci. Technol.* **2020**, *270*, 114702. [CrossRef]
29. Zeng, T.; Li, X.; Guan, H.; Yang, W.; Liu, W.; Liu, J.; Du, Z.; Li, X.; Xiao, Q.; Wang, X.; et al. Dynamic microbial diversity and fermentation quality of the mixed silage of corn and soybean grown in strip intercropping system. *Bioresour. Technol.* **2020**, *313*, 123655. [CrossRef] [PubMed]
30. Li, M.; Lv, R.; Zhou, H.; Zi, X. Dynamics and correlations of chlorophyll and phytol content with silage bacterial of different growth heights Pennisetum sinese. *Front. Plant Sci.* **2022**, *13*, 996970. [CrossRef] [PubMed]
31. Liu, Q.; Chen, M.; Zhang, J.; Shi, S.; Cai, Y. Characteristics of isolated lactic acid bacteria and their effectiveness to improve stylo (*Stylosanthes guianensis* Sw.) silage quality at various temperatures. *Anim. Sci. J.* **2012**, *83*, 128–135. [CrossRef]
32. Masaki, S. *Silage-no-Hinshitsu-Hyouka (V-SCORE); Nihon-Sochichikusan-Shushi-Kyokai*; CiNii: Tokyo, Japan, 2001; pp. 93–94.
33. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef]
34. Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60. [CrossRef] [PubMed]
35. Langille, M.G.; Zaneveld, J.; Caporaso, J.G.; McDonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Vega Thurber, R.L.; Knight, R.; et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **2013**, *31*, 814–821. [CrossRef] [PubMed]
36. Ni, K.; Wang, F.; Zhu, B.; Yang, J.; Zhou, G.; Pan, Y.; Tao, Y.; Zhong, J. Effects of lactic acid bacteria and molasses additives on the microbial community and fermentation quality of soybean silage. *Bioresour. Technol.* **2017**, *238*, 706–715. [CrossRef]
37. Li, M.; Zhang, L.; Zhang, Q.; Zi, X.; Lv, R.; Tang, J.; Zhou, H. Impacts of Citric Acid and Malic Acid on Fermentation Quality and Bacterial Community of Cassava Foliage Silage. *Front. Microbiol.* **2020**, *11*, 595622. [CrossRef]
38. Du, Z.; Sun, L.; Lin, Y.; Chen, C.; Yang, F.; Cai, Y. Use of Napier grass and rice straw hay as exogenous additive improves microbial community and fermentation quality of paper mulberry silage. *Anim. Feed. Sci. Technol.* **2022**, *285*, 115219. [CrossRef]
39. Li, X.; Chen, F.; Wang, X.; Xiong, Y.; Liu, Z.; Lin, Y.; Ni, K.; Yang, F. Innovative utilization of herbal residues: Exploring the diversity of mechanisms beneficial to regulate anaerobic fermentation of alfalfa. *Bioresour. Technol.* **2022**, *360*, 127429. [CrossRef] [PubMed]
40. Thébault, E.; Fontaine, C. Stability of ecological communities and the architecture of mutualistic and trophic networks. *Science* **2010**, *329*, 853–856. [CrossRef]
41. Toju, H.; Yamamichi, M.; Guimarães, P.R., Jr.; Olesen, J.M.; Mougi, A.; Yoshida, T.; Thompson, J.N. Species-rich networks and eco-evolutionary synthesis at the metacommunity level. *Nat. Ecol. Evol.* **2017**, *1*, 24. [CrossRef]


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