

Article

Fermentation Regulation: Revealing Bacterial Community Structure, Symbiotic Networks to Function and Pathogenic Risk in Corn Stover Silage

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Abstract

Improving agricultural by-product utilization can alleviate tropical feed shortages. This study used corn stover (CS, *Zea mays* L.) at the maturity stage as the material, with four silage treatments: control, lactic acid bacteria (LAB, *Lactiplantibacillus plantarum*), cellulase (AC, *Acremonium cellulolyticus*), and LAB+AC. After 60 days fermentation in plastic drum silos, the silos were opened for sampling. PacBio single-molecule real-time sequencing technology was used to study bacterial community structure, symbiotic network functionality, and pathogenic risk to clarify CS fermentation regulatory mechanisms. The CS contained 59.9% neutral detergent fiber and 7.1% crude protein. Additive-treated silages showed better quality than the control: higher lactic acid (1.64–1.83% dry matter, DM), lower pH (3.62–3.82), and reduced ammonia nitrogen (0.54–0.81% DM). Before ensiling, the CS was dominated by Gram-negative *Rhizobium larrymoorei* (16.30% of the total bacterial community). Functional prediction indicated that the microbial metabolism activity in diverse environments was strong, and the proportion of potential pathogens was relatively high (14.69%). After ensiling, *Lactiplantibacillus plantarum* as Gram-positive bacteria were the dominant species in all the silages (58.39–84.34% of the total bacterial community). Microbial additives facilitated the establishment of a symbiotic microbial network, where *Lactiplantibacillus* occupied a dominant position ($p < 0.01$). In addition, functional predictions showed an increase in the activity of the starch and sucrose metabolism and a decrease in the proportion of potential pathogens (0.61–1.95%). Among them, the synergistic effect of LAB and AC inoculants optimized the silage effect of CS. This study confirmed that CS is a potential high-quality roughage resource, and the application of silage technology can provide a scientific basis for the efficient utilization of feed resources and the stable development of animal husbandry in the tropics.

Keywords: crop by-product; microbial co-occurrence network; pathogen assessment; regulation mechanisms; silage function prediction



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1. Introduction

In tropical developing countries, roughage mainly comes from grass, forage, and agricultural crop by-products [1]. In the past few years, alongside the development of global animal husbandry, the stable supply of high-quality roughage has become an increasingly pressing issue, especially during the dry season [2]. The warm and humid climate

conditions in summer and autumn provide ideal conditions for grass growth, resulting in a significant increase in biomass and nutritive value. However, this seasonal supply of forage far exceeds actual livestock demand, creating a temporary surplus. During the dry season in winter and spring, harsh climatic conditions severely restrict the growth and development of grass, leading to stunted growth or even death of the grass [3]. Hence, a seasonal imbalance has emerged, characterized by vigorous growth in summer, abundance in autumn, scarcity in winter, and near extinction in spring. If livestock cannot obtain high-quality roughage, this will result in a decline in the yield and quality of livestock products [4]. Therefore, the development of feed production technology with high storage efficiency is of great practical significance for alleviating feed shortages during the dry season and promoting the sustainable development of animal husbandry [5].

Corn (*Zea mays* L.) is widely cultivated around the world due to its high yield and adaptability [6]. As one of the world's three major food crops, its large-scale cultivation has created abundant corn stover (CS) resources [7]. It is estimated that approximately 240 billion kilograms of CS are produced annually in China [8]. In recent years, the issue of abundant CS resources but low utilization efficiency has become increasingly prominent [9]. Traditional incineration methods release greenhouse gases, causing negative impacts on the ecological environment [10]. Ensiling can convert and utilize the nutrients in CS, providing livestock with a stable supply of high-quality feed during the dry season. In this context, ensiling is considered the optimal method for processing CS. However, the low fermentation substrate and hollow physical structure of stover hinder the success of natural ensiling. It has been reported that various additives can improve fermentation parameters and silage quality, such as lactic acid bacteria (LAB) inoculants and cellulase [11,12]. These additives can effectively regulate agricultural by-products to enhance the nutritive value, palatability, and aerobic stability of silage [13].

Traditional microbial plate culture methods cannot comprehensively analyze the dynamic changes in the bacterial community in complex silage systems [14]. Although next-generation sequencing technology have been widely used to describe bacterial community in silage, the resolution is limited to the genus level [15]. PacBio's sequencing technology of single-molecule real time (SMRT) offers the advantage of longer read lengths, enabling the classification of microorganisms at the species level and significantly improving detection sensitivity and accuracy [16]. Therefore, this study aimed to gain insights into the regulation of CS silage fermentation through comprehensive assessment of fermentation quality, bacterial community structure, microbial symbiotic networks, and the predicted functional and potential pathogenic content.

2. Materials and Methods

2.1. Preparation of CS Before and After Ensiling

This experiment used corn (cv. Jiahua 4) grown at the Yangzhou Fresh Corn Introduction and Breeding Base (Yangzhou, Jiangsu, China) at the maturity stage as the material. On 10 September 2024, a harvester (Jixin 4YB-2, Hebei Jixin Agricultural Machinery Co., Ltd., Xingtai, China) was used to perform whole-plant harvesting of corn. After harvesting, the corn ears were manually removed, and the remaining CS was chopped into 1–2 cm lengths using a chopper (CX-201, Yamamoto Co., Ltd., Tendo, Japan). Subsequently, the samples were thoroughly mixed, 1 kg of CS was placed in an ice box at 4 °C, and immediately transported to the laboratory. A portion was used for chemical composition and microbial population analysis, and the remainder was frozen at −80 °C for bacterial community analysis.

This experiment evaluated four silage treatment methods: (1) control (CK, no additive), (2) LAB (*Lactiplantibacillus plantarum* strain Chikusou-1, Snow Brand Milk Products Co.,

Ltd., Sapporo, Japan), (3) cellulase (AC, *Acremonium cellulolyticus* preparation from Meiji Co., Ltd., Tokyo, Japan), and (4) LAB+AC mixture. The LAB inoculant was applied as freeze-dried powder at 5 mg/kg fresh matter (FM), yielding 1.0×10^5 colony-forming unit (CFU)/g of FM. The cellulase preparation contained carboxymethyl cellulase, pectinase, and glucanase as primary enzymatic components. The activity of total cellulase is 7350 U/g, and its application rate was 0.01% FM of CS. The LAB and AC inoculants were prepared into solutions of 2% and 1%, respectively, and applied using an electronic sprayer at a rate of 1 mL/kg of CS. At the same time, the control was sprayed with an equal volume of deionized water. Silage was prepared using uniformly sized polyethylene drum silos (10 L, Huafang Plastic Co., Ltd., Dezhou, China). For each CS silage treatment, four independent replicates were established, with each replicate assigned to a separate drum silo. In total, this setup resulted in 16 silage-containing drum silos (4 treatments $\times$ 4 replicates).

After completing the additive spraying, the materials were thoroughly mixed and homogenized. Approximately 7.5 kg of CS was taken and manually loaded into a drum silo, then the silo was sealed with a threaded cap. After the silage preparation process was complete, the drum silos were stored at room temperature (25–29 °C). Following a 60-day fermentation period, three samples were randomly selected from each treatment for silo opening and sampling; the surface material was removed, and the remaining sample was poured out from the silo. The sample was then thoroughly mixed on a clean plastic film, and a portion was taken for analysis of chemical composition, fermentation quality, and bacterial community structure.

2.2. Chemical Composition, Fermentation Quality and Microbial Population

The CS material and silage were oven-dried at 65 °C, with dry matter (DM) content determined by measuring mass differences before and after drying. To ensure homogeneity, samples were thoroughly mixed and ground (T1-200 grinder; CMT Co., Ltd., Tokyo, Japan), then stored in sealed desiccators. Chemical analyses included: crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) quantified according to AOAC methods [17], and water-soluble carbohydrate (WSC) determined using the Anthrone colorimetric method [18].

Following Cai [19], the cold-water extraction method was employed to analyze the fermentation characteristics of the silage, including pH, organic acid, and ammonia nitrogen ($\text{NH}_3\text{-N}$). The sample was mixed with sterile water at a ratio of 1:10, then filtered using sterile gauze. After that, a pH meter (S-90, Mettler Toledo, Zurich, Switzerland) was used to measure the pH of the filtrate. High-performance liquid chromatography (HPLC, Agilent 1260 Infinity II, Santa Clara, CA, USA) was used to determine the organic acids of the silage, including lactic acid, acetic acid, propionic acid, and butyric acid, following the method described by Wang et al. [20]. The experimental conditions were as follows: column (7.8 mm $\times$ 300 mm; Phenomenex, Torrance, CA, USA), column temperature 60 °C, UV detection wavelength 450 nm, mobile phase 3 mmol/L HClO_4 , and flow rate 1.0 mL/min. The $\text{NH}_3\text{-N}$ content in the silage was determined using the phenol-sodium hypochlorite colorimetric method [21].

Ten grams of CS material and silage were homogenized with 90 mL of 0.85% sterile saline solution and shaken for 2 h at low temperature (TATUNG HZ-200B shaker; Shanghai Shibei Instrument Equipment Factory, Shanghai, China). The resulting suspension was serially diluted (10^{-1} to 10^{-8}) in sterile saline. Microbial population assay was performed using: MRS agar (Difco Laboratories, Detroit, MI, USA) for LAB, nutrient agar (Nissui-Seiyaku, Tokyo, Japan) for aerobic bacteria, blue light broth agar (Nissui-Seiyaku) for coliform bacteria, and potato dextrose agar (Nissui-Seiyaku) for yeast/mold [22]. Using

the spread plate technique, 60 μ L of each dilution was evenly distributed on triplicate agar plates, with results reported as lg colony-forming unit (CFU)/g of FM.

2.3. Measurement of Bacterial Community Structure

The supernatant from microbial population analysis was utilized for bacterial community genomic DNA extraction. The mother liquor was filtered through pre-sterilized four-layer gauze, and the filtrate was centrifuged at 10,000 rpm for 5 min using a high-speed refrigerated centrifuge (GL-18M; Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., Shanghai, China) to obtain microbial pellets. Genomic DNA was extracted following Du et al. [23] using the Tiangen DNA Extraction Kit (DP302-02; Beijing, China), with all procedures performed according to the manufacturer's protocols.

The full-length 16S rRNA gene sequence enable higher taxonomic resolution, thereby maximizing the capture of bacterial community diversity at the species level. To amplify the V3–V4 region of the bacterial 16S rRNA gene, forward primer 27F (sequence: AGRGTTTGATYNTGGCTCAG) and reverse primer 1492R (sequence: TASGGHTAC-CTTGTTASGACTT) were designed using Mothur software (version 1.22.2). The amplification process was performed on the PacBio Sequel II platform using the Sequel II Reagent Kit 2.0 in combination with the above primers. After amplification, equal amounts of amplified fragments were pooled, and library construction and sequencing were performed using sequencing technology of SMRT [24]. Following sequencing, the raw read data were filtered and demultiplexed using SMRT Link software (version 8.0). With parameters set to a minimum pass ≥ 5 and minimum predicted accuracy ≥ 0.9 , this process generated circular consensus sequencing (CCS) read data. The USEARCH software (version 10.0) was used to classify sequences with a similarity of $\geq 97\%$ as the same operational taxonomic unit (OTU) [25]. Based on the SILVA database (version 138.1), OTU classification and annotation were conducted employing the Naive Bayes classifier integrated in QIIME2, with a confidence threshold set at 70% [26].

For bacterial community alpha diversity, the QIIME software (version 1.9.1) was used to calculate indices such as abundance-based coverage estimator (ACE), Chao 1, Shannon, and Simpson [27]. The Mothur software (version 1.22.2) was used to construct rarefaction curves [28]. The beta diversity of bacterial community was conducted using the QIIME software (version 1.8.0) to perform principal coordinate analysis (PCoA) [29]. Matplotlib (version 1.4.3) was used to plot bar charts to present the feature number [30]. Venn diagrams of bacterial community at the OTU level were constructed using R software (version 3.1.1) [31]. Python (version 2.0) was used to visualize the relative abundance and species distribution bubble charts of bacterial community at the genus and species levels [32,33]. Circos software (version 0.66-7) was used to draw species composition circle plots based on the composition ratios of dominant species of samples at the genus level [34]. Based on the abundance and variety of species in the samples, Spearman's correlation coefficient was used to calculate the correlation between species. The R software (version 3.6.1) was used for bacterial community symbiosis network analysis due to updated packages [35].

2.4. Functional Prediction and Phenotypic Traits of the Bacterial Community

Picrust2 (version 2.3.0) was used to extract information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, calculate the abundance of each functional category and obtain impacted KEGG metabolic pathways [36]. Bugbase software (version 0.1.0) was used to predict the bacterial community phenotypes of materials and silages [37].

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure in SPSS software (version 19.0), with statistical significance defined as

$p < 0.05$, to assess the variability in chemical composition, fermentation quality, microbial population, and potential pathogens in CS material and silage [38].

3. Results

3.1. Biochemical Properties of CS Material

As shown in Table 1, the pH of CS was below 5.3, with DM and CP contents exceeding 21% and 7.1%, respectively. The material also contained high levels of NDF (>59%), ADF (>36%), and WSC (>8.7%). Microbial analysis revealed that CS material contained LAB, aerobic bacteria, and coliform bacteria at levels of 10^5 , yeast at levels of 10^6 , and mold at levels below the detection limit.

Table 1. pH, chemical composition and microbial population of corn stover material.

Item	CS
pH	5.25 ± 0.07
Chemical composition	
DM (%)	21.09 ± 0.40
CP (% DM)	7.18 ± 0.06
NDF (% DM)	59.93 ± 0.54
ADF (% DM)	36.61 ± 0.15
WSC (% DM)	8.80 ± 0.14
Microbial population (Lg CFU/g FM)	
Lactic acid bacteria	5.01 ± 1.68
Aerobic bacteria	5.74 ± 0.70
Coliform bacteria	5.46 ± 0.15
Yeast	6.21 ± 0.46
Mold	ND

Note: The data in the table are presented as mean $\pm$ standard deviation and were analyzed statistically using three independent replicate samples. ADF, acid detergent fiber; CFU, colony-forming unit; CS, corn stover; CP, crude protein; DM, dry matter; FM, fresh matter; ND, not detected; NDF, neutral detergent fiber; WSC, water-soluble carbohydrate.

3.2. Fermentation of CS Silage Treated with Different Additives

As presented in Table 2, compared to the CK, the additive-treated silages exhibited significantly higher ($p < 0.001$) lactic acid content, and lower ($p = 0.02$) pH and ($p = 0.03$) $\text{NH}_3\text{-N}$ content. No significant differences ($p > 0.05$) in DM or CP content were observed among all the treatments. Compared to the CK, the AC treatments (AC and LAB+AC) significantly reduced ($p < 0.001$) the contents of NDF and ADF. Microbial counts revealed that all additives significantly increased ($p = 0.03$) LAB counts. Coliform bacteria and mold were undetectable in all silages.

Table 2. Fermentation characteristics, chemical composition, and microbial populations of CS silage with or without additives after 60 days of ensiling.

Item	CK	LAB	AC	LAB+AC	SEM	<i>p</i> -Value
Fermentation quality						
pH	4.12 ^a	3.75 ^c	3.82 ^b	3.62 ^d	0.06	0.02
Lactic acid (% DM)	1.27 ^c	1.71 ^{ab}	1.64 ^b	1.83 ^a	0.03	<0.001
Acetic acid (% DM)	0.53 ^{ab}	0.51 ^{ab}	1.83 ^b	0.66 ^a	0.06	0.02
Propionic acid (% DM)	ND	ND	ND	ND	--	--
Butyric acid (% DM)	ND	ND	ND	ND	--	--
$\text{NH}_3\text{-N}$ (% DM)	1.13 ^a	0.67 ^c	0.81 ^b	0.54 ^c	0.04	0.03

Table 2. Cont.

Item	CK	LAB	AC	LAB+AC	SEM	p-Value
Chemical composition						
DM (%)	20.42	19.66	20.01	20.31	0.31	0.35
CP (% DM)	6.99	6.95	6.98	7.01	0.05	0.82
NDF (% DM)	59.46 ^a	58.05 ^a	53.12 ^c	54.00 ^b	0.41	<0.001
ADF (% DM)	36.27 ^a	35.91 ^a	32.22 ^c	33.04 ^b	0.13	<0.001
Microbial population (Lg CFU/g FM)						
Lactic acid bacteria	5.03 ^c	6.46 ^b	6.16 ^b	6.88 ^a	0.44	0.03
Aerobic bacteria	5.04	4.77	4.63	4.35	0.53	0.18
Coliform bacteria	ND	ND	ND	ND	--	--
Yeast	5.21	5.06	4.62	4.04	0.67	0.62
Mold	ND	ND	ND	ND	--	--

Note: The data are presented as the mean values of three samples. In the same row, values marked with different superscripts (^{a–d}) denote statistically significant differences ($p < 0.05$) within each treatment. --, the value is zero; AC, *Acremonium cellulase*; ADF, acid detergent fiber; CFU, colony-forming unit; CP, crude protein; CS, corn stover; DM, dry matter; FM, fresh matter; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*; ND, not detected; NDF, neutral detergent fiber; NH₃-N, ammonia nitrogen; SEM, standard error of the mean.

3.3. Bacterial Diversity of CS Material and Silage Treated with Different Additives

As exhibited in Figure 1A, compared to the CS material, the ACE index was significantly higher ($p < 0.01$) in AC silage, while Shannon and Simpson indices were significantly lower ($p < 0.05$) in all silages. As sequencing depth increased, the rarefaction curve index plotted based on the Sobs index gradually flattened (Figure 1B). The PCoA based on the OTU level showed that samples from all silages exhibited intra-group clustering, while CS material and all silages were clearly separated, with the PC1 axis explaining 72.18% of the bacterial community difference (Figure 1C).

As shown in Figure 2A, compared to the CS material (700), the feature number increased in all silages (1011 to 1804) after ensiling. The Venn diagram analysis showed that CS and CK treatments had 245 shared OTU, and their unique OTU were 1559 and 983, respectively (Figure 2B). In comparison to the CK (816), the number of unique OTUs in all additive-treated silages (289 to 634) decreased.

3.4. Bacterial Community Structures of CS Silage Treated with Different Additives

As illustrated in Figure 3, the dominant epiphytic bacterium in CS material was *Rhizobium* (*Rhizobium larrymoorei*). After ensiling, the dominant bacterium in all silages shifted to *Lactiplantibacillus plantarum*. In contrast to the CK (58.40%), all additive-treated silages (67.38% to 84.35%) showed an increase in the relative abundance of *Lactiplantibacillus plantarum*. Notably, the LAB+AC-treated silage exhibited the highest relative abundance of this bacterium. The species compositions and distributions of the CS material and silage are further visualized in Figure 4. *Rhizobium*, marked in pink, was the dominant bacterial community in the CS material, accounting for 40% of the relative abundance; while *Lactiplantibacillus*, marked in orange, had a relative abundance of 60–85% in all silages (Figure 4A). *Rhizobium* (*Rhizobium larrymoorei*) is represented by a large circle in the CS material, but was not detected in all silages (Figure 4B,C). *Lactiplantibacillus* (*Lactiplantibacillus plantarum*) is represented by a large circle in all silages, with the largest circle in the LAB+AC-treated silage.

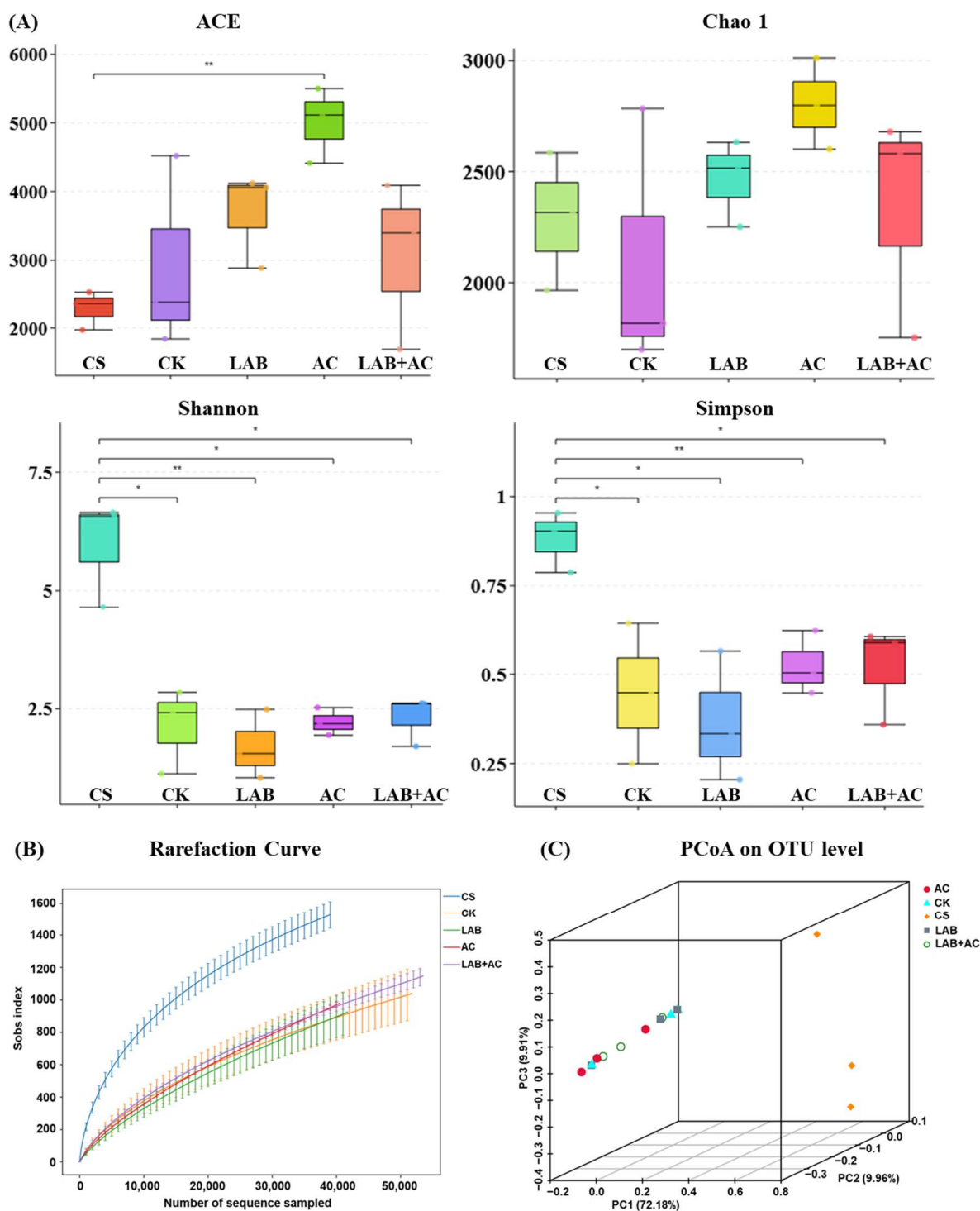


Figure 1. Bacterial community diversity of CS material and silage. **(A)** The ACE, Chao1, Shannon, and Simpson indices were used to characterize the alpha diversity of the bacterial community. **(B)** The rarefaction curve index was constructed based on Sobs index. **(C)** Based on the OTU level, the PCoA was used to calculate the beta diversity of the bacterial community. *, $0.01 < p \leq 0.05$; **, $0.001 < p \leq 0.01$; AC, *Acremonium cellulase*; ACE, abundance-based coverage estimator; CS, corn stover; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*; OTU, operational taxonomic unit; PCoA, principal coordinate analysis.

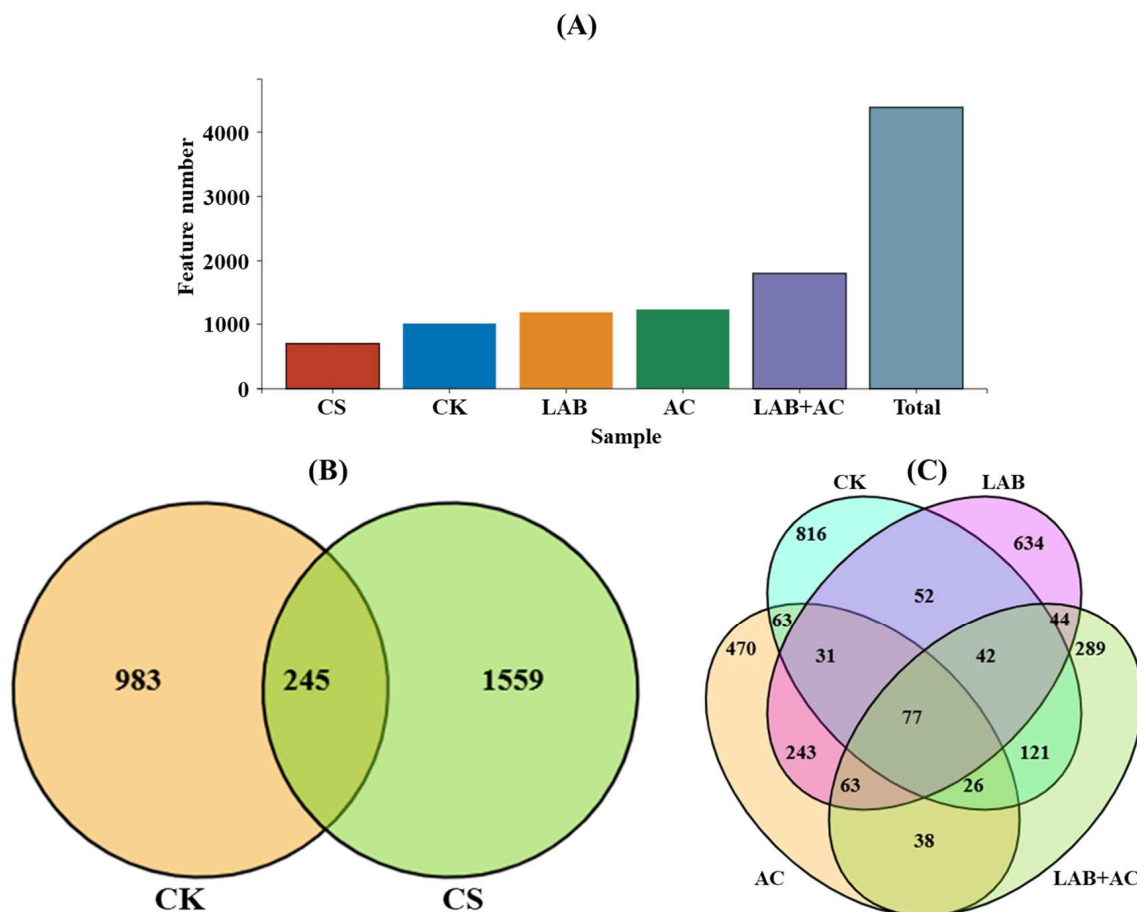


Figure 2. Feature number visualization and Venn analysis of OTU overlap characteristics between CS material and silage. **(A)** Feature number in CS material and silages. **(B)** The Venn diagram of the OTU in fresh and ensiled CS. **(C)** The OTU overlap analysis of CS silages from the CK, LAB, AC, and LAB+AC treatments. AC, *Acremonium cellulase*; CS, corn stover; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*; OTU, operational taxonomic unit.

3.5. Bacterial Symbiotic Networks, Metabolic Pathway, and Phenotypic Analysis of CS Material and Silage

As shown in Figure 5A, in the CS material, *Rhizobium* was positively correlated with *Pseudomonas*. In the CK, *Lactiplantibacillus* exhibited a negative correlation with *Levilactobacillus* (Figure 5B). In the LAB+AC-treated silage, *Lactiplantibacillus* exhibited a negative correlation with *Lactobacillus* (Figure 5C).

As shown in Figure 6A, compared with CS material, CK silage had a lower ($p < 0.001$) proportion of microbial metabolism in diverse environments. Compared with the CK, the LAB+AC-treated silage had a higher proportion of starch and sucrose metabolism (Figure 6B).

The phenotypic prediction results showed that the bacterial community in the CS material was dominated by a high proportion of Gram-negative bacteria, while the bacterial community in the silages was dominated by a high proportion of Gram-positive bacteria (Figure 7A). Meanwhile, the proportion of potential pathogens in the CS material was higher ($p < 0.001$) than that in silage (Figure 7B). Compared with the CK, additive-treated silages had a lower ($p < 0.001$) proportion of potential pathogens.

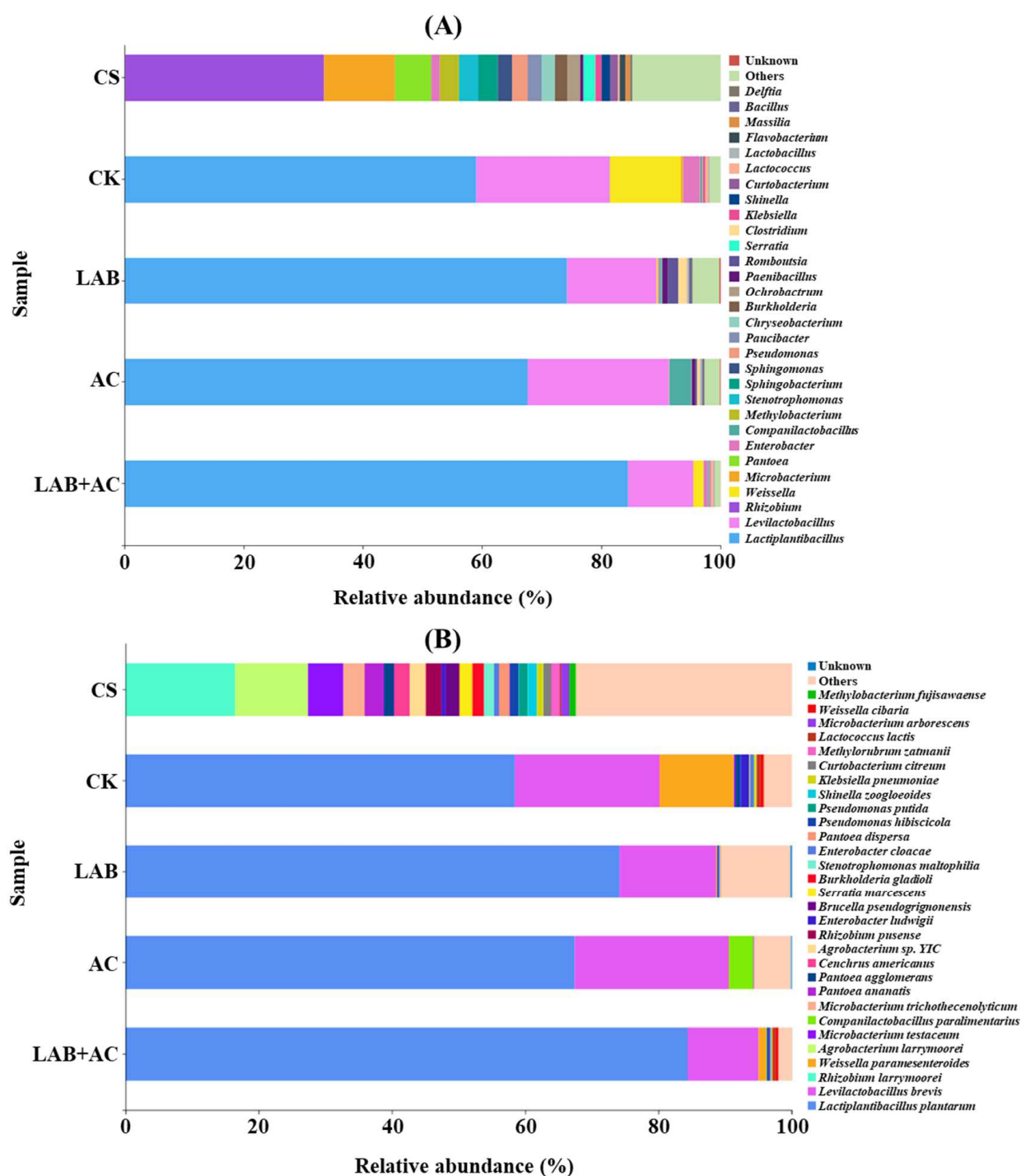


Figure 3. Relative abundance of the top 30 dominant bacterial community at the genus (A) and species (B) levels in CS material and silage. AC, *Acremonium cellulase*; CS, corn stover; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*.

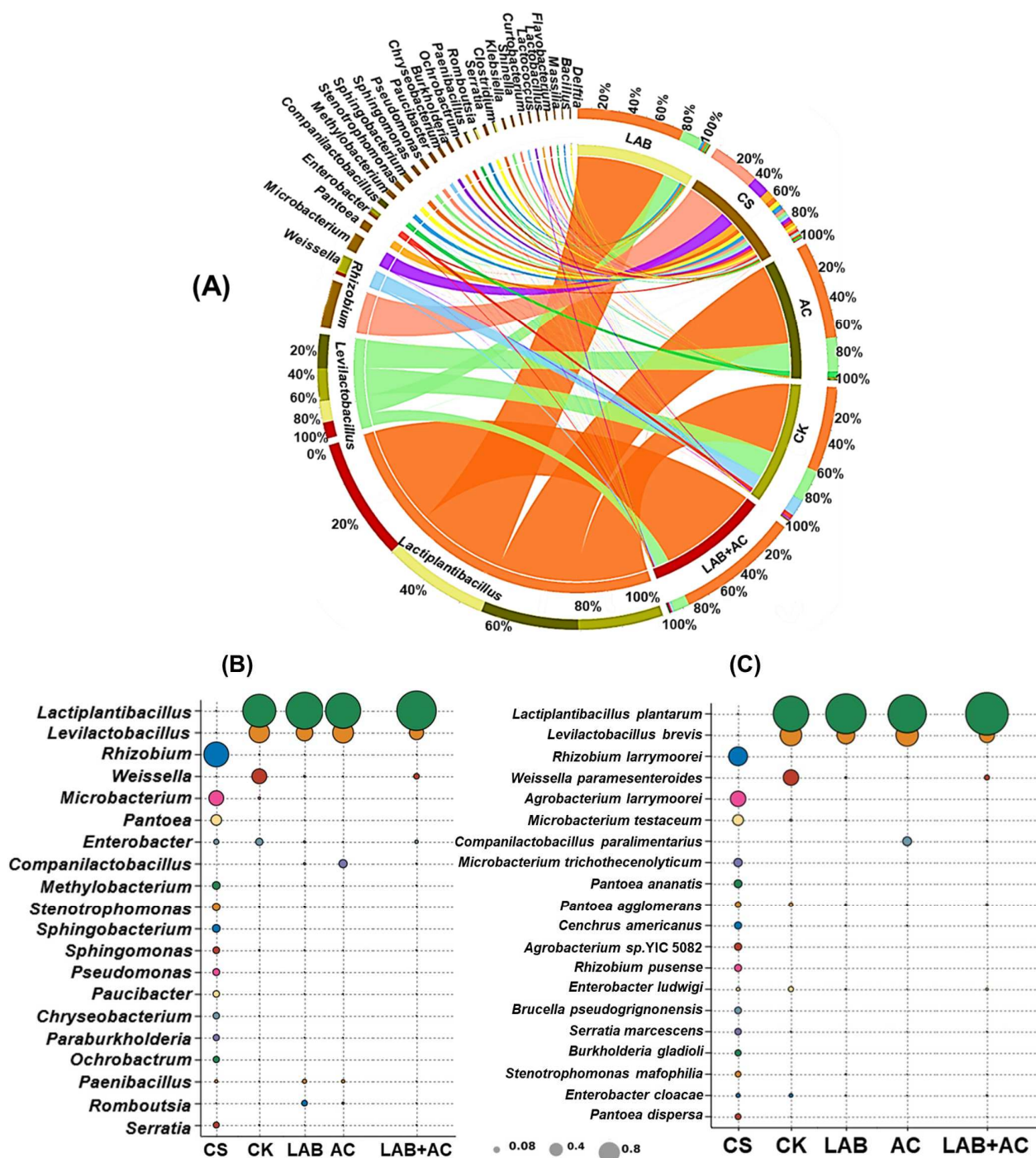


Figure 4. Visualization analysis of microbial species composition and distribution characteristics of CS material and silage. (A) Species composition circle plots of CS material and silage at the genus level. The colored band indicates the presence of a species in a sample. The width of the colored bands is related to the relative abundance of the species in the sample. The thicker the band, the more abundant the species. (B) Species distribution bubble charts of bacterial community of CS material and silage at the genus level. (C) Species distribution bubble charts of bacterial community of CS material and silage at the species level. The color of the bubble represents the species at the genus or species level, and the size of the bubble indicates the proportion of the species in the sample. AC, *Acremonium cellulase*; CS, corn stover; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*.

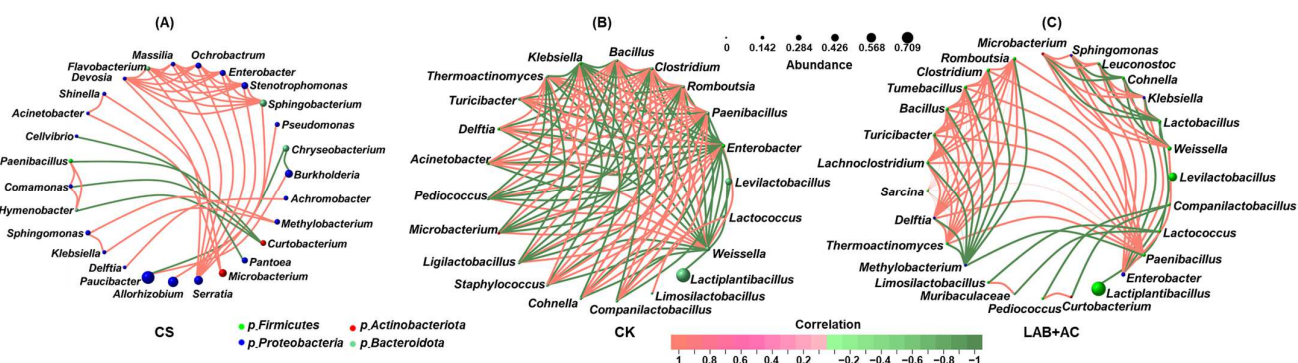


Figure 5. Species-level bacterial co-occurrence network in material (A), CK (B) and LAB+AC-treated silage (C) of CS. The circles represent bacterial species, with color corresponding to bacterial phyla and sizes reflecting bacterial abundance. CS, corn stover; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*.

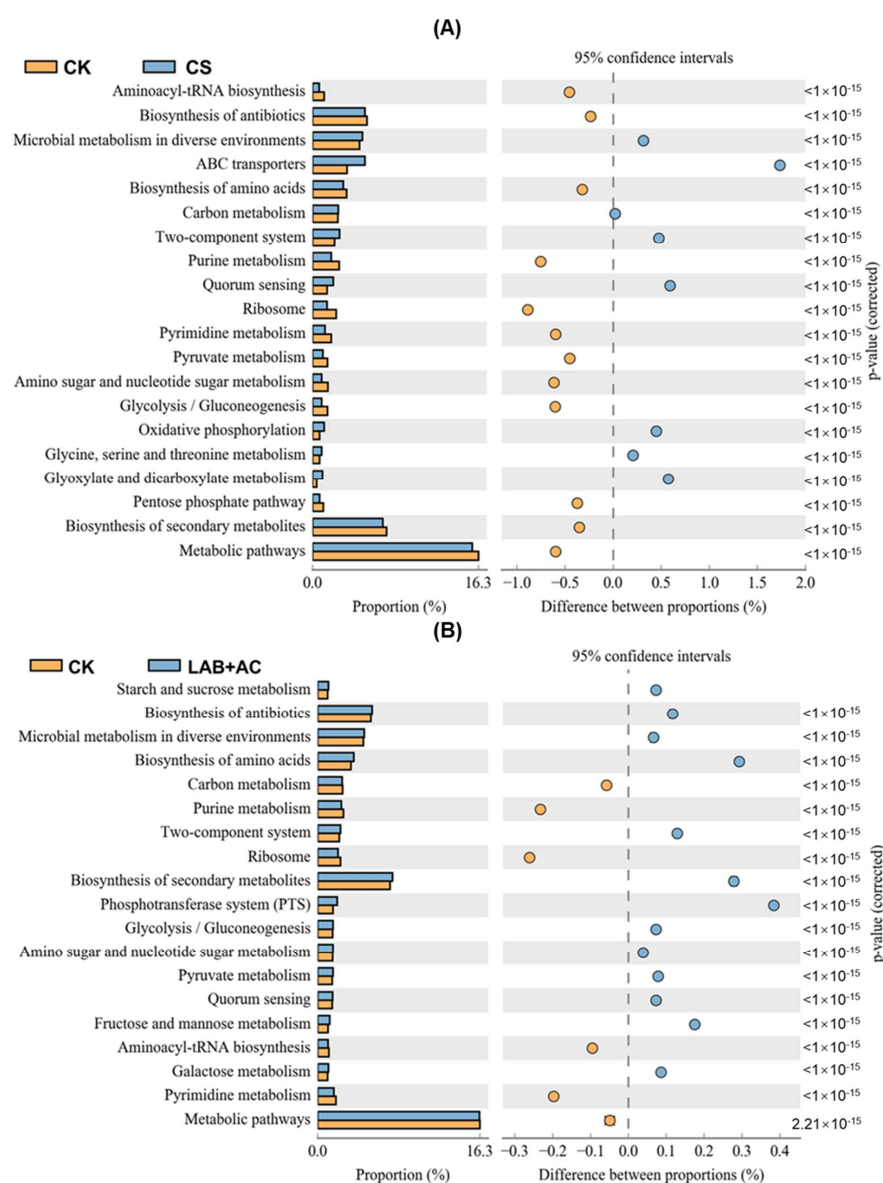


Figure 6. The KEGG metabolic pathway on the second level in CS before and after ensiling (A) and CK and LAB+AC-treated silage (B). CS, corn stover; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*; KEGG, Kyoto Encyclopedia of Genes and Genomes.

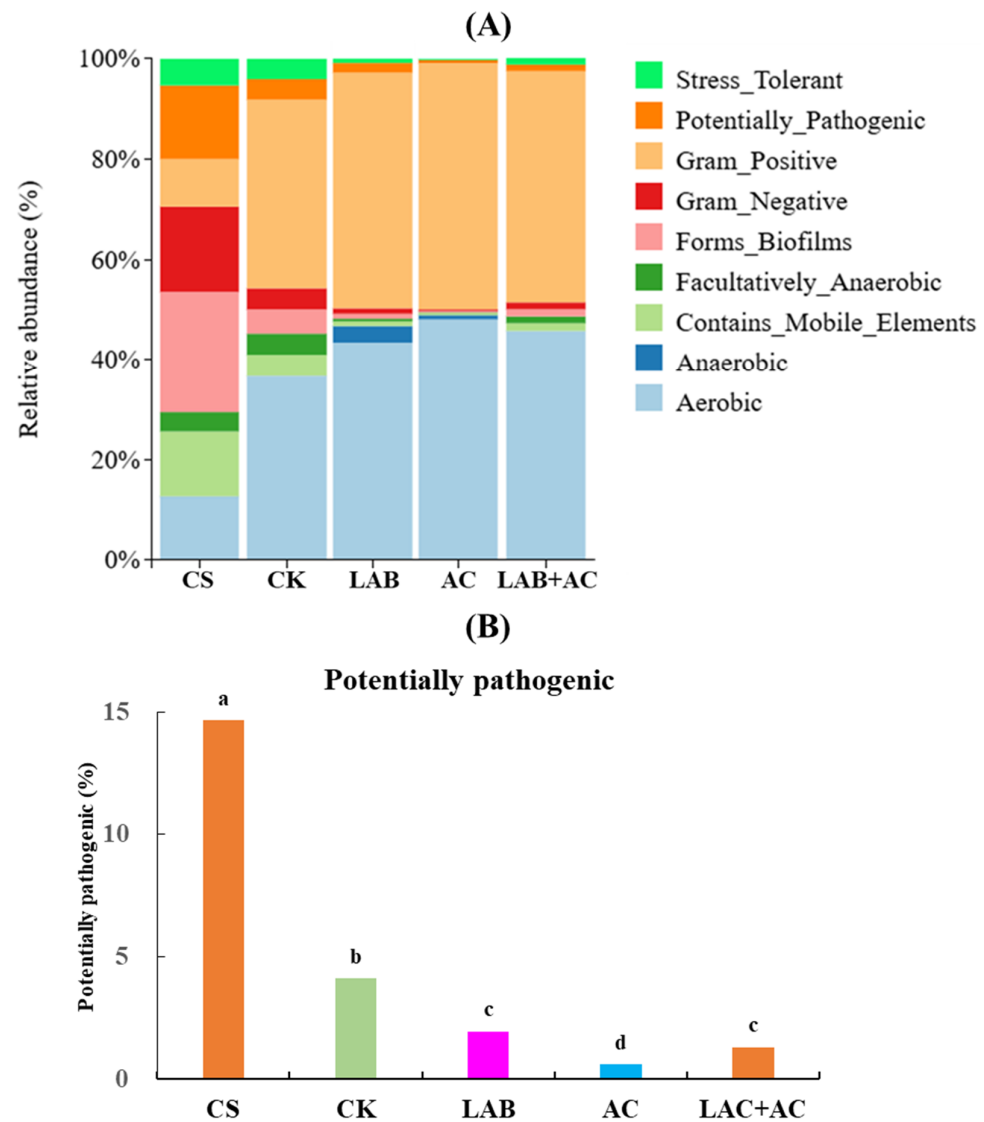


Figure 7. BugBase phenotype prediction analysis (A) and potential pathogen comparison (B) of bacterial community of CS material and silage. AC, *Acremonium cellulase*; CS, corn stover; LAB, *Lactiplantibacillus plantarum*; LAB+AC, combination of *Lactiplantibacillus plantarum* and *Acremonium cellulase*. Data are presented as the mean of three samples. Different letters (a–d) indicate statistically significant differences within each treatment ($p < 0.05$).

4. Discussion

4.1. Microbial Population and Fermentation Characteristics

As the scale of modern livestock husbandry accelerates, the contradiction between supply and demand for high-quality roughage has become increasingly prominent [39]. As a by-product of agricultural production with a large yield, CS is a high-quality roughage resource with great development potential. The use of silage technology to process CS can alleviate feed shortages and improve resource utilization efficiency, making it an important research direction within the industry [40]. The high CP ($>7.1\%$) content and fiber component ($\text{NDF} > 59\%$ and $\text{ADF} > 36\%$) of CS material meet the nutritional requirements of livestock for feed. In addition, the WSC and LAB jointly act as key biochemical factors determining the fermentation fate. Theoretically, silage fermentation is more favorable when the material contains over 5% WSC and LAB counts exceed 10^5 CFU/g of FM [41]. Notably, CS material met these ideal fermentation criteria. Therefore, CS can be used as a feed resource to prepare high-quality silage.

In this study, additives improved fermentation quality, as evidenced by decreased pH and $\text{NH}_3\text{-N}$ content, as well as increased lactic acid content and LAB counts. Among them, the LAB+AC-treated silage exhibited the best fermentation quality. This was because *Lactiplantibacillus plantarum*, a homofermentative LAB, possesses the characteristics of rapid reproduction and strong acid resistance [42]. When added to the CS silage system, it quickly colonized and proliferated, thereby dominating the bacterial community and inhibiting the growth of spoilage organisms, including coliforms and molds. On the other hand, AC can specifically break down cellulose and hemicellulose, converting them into WSC such as glucose and xylose, providing ample substrate for LAB proliferation, accelerating lactic acid production, and driving a rapid decrease in the pH of the silage [43]. As such, the addition of either LAB or AC alone effectively improved fermentation quality. More importantly, the combined addition of LAB and AC created a precise synergy between enzymatic substrate supply and microbial fermentation regulation. Through a bidirectional promotional effect, this combination enhanced silage quality of nutrient retention and fermentation efficiency, with results significantly superior to those of the single additive treatment. Additionally, compared to other silages, CS silage treated with AC alone or in combination with LAB showed significantly reduced NDF and ADF content. This was attributed to the use of AC in this study, whose main components are glucanase and pectinase. These enzymes acted independently or synergistically with other additives to efficiently break down the cellulose in the material [1].

4.2. Bacterial Community Structure

In microbiomics, alpha diversity is characterized by species richness and diversity via indicators like ACE, Chao1, Shannon, and Simpson [44]. The dominant microorganisms in CS material were aerobic or facultative anaerobic ones. After ensiling, the anaerobic environment suppressed aerobic bacteria while facilitating the growth of acid-resistant bacteria, which lowered the pH to below 4.2. Therefore, the ensiling environment exhibited an increase in species richness alongside a decrease in species diversity. The rarefaction curves indicated that the current sequencing depth was sufficient to cover the vast majority of bacterial species in all the samples, and even if the amount of sequencing data is further increased, the number of newly discovered species will not increase significantly. The distinctly separated clusters between CS material and silages indicated that there were significant differences in the bacterial community composition. In particular, the observed difference was attributed to the suppression of epiphytic microbes (especially aerobes) and the subsequent emergence of newly dominant bacteria during ensiling. In contrast, all silages clustered closely, suggesting a high degree of similarity in their bacterial community composition. The higher number of OTUs in the CS material reflected the presence of a broad range of aerobic and facultative microorganisms that thrive under natural aerobic conditions. However, after ensiling, the rapid establishment of an anaerobic and acidic environment imposes dual stress on the bacterial community. This phenomenon aligned with our previous study [14]. The application of additives facilitated a more pronounced acidification process in the silage system. Intensified acidic conditions further inhibited the proliferation of undesirable acid-sensitive microorganisms such as spoilage bacteria and yeast [45]. Ultimately, this led to a reduction in the number of unique OTUs in the LAB, AC, and LAB+AC-treated silages.

In microbiome sequencing studies, the relative abundance of bacterial communities serves as a key indicator linking bacterial community structure to feed functionality. It plays a crucial role in assessing silage fermentation quality, ensuring safety, optimizing nutritive value, and regulating animal health [46]. *Rhizobium larrymoorei*, a species belonging to the *Rhizobium* genus, is the dominant bacterial strain in the CS material. This strain is

a Gram-negative heterotrophic bacterium that typically grows in aerobic environments. As a member of the *Rhizobium* genus, it promotes plant root development, enhances root absorption of water and nutrients, and thereby improves overall plant growth and stress resistance [47]. During ensiling, *Lactiplantibacillus plantarum*, a facultative anaerobic bacterium with strong acid-producing capability, played a crucial role in driving fermentation. It efficiently utilized various carbohydrates in the CS material, such as glucose, fructose, sucrose, maltose, and some hemicellulose decomposition products, through the homofermentative pathway to proliferate and produce lactic acid, rapidly lowering the pH of the silage system [48]. The accelerated acidification not only stabilized the fermentation process but also created unfavorable conditions for undesirable microbes, thereby improving silage quality. In addition, the inherent acid tolerance of *Lactiplantibacillus plantarum* was a key factor supporting their persistence in the low-pH environment established during ensiling [49].

The addition of exogenous LAB directly increased its initial microbial population in CS silage, accelerating the rapid decrease in pH and creating an acidic environment conducive to the survival of *Lactiplantibacillus plantarum*. The addition of AC facilitated the degradation of cellulose in the CS material into smaller sugar molecules, such as glucose and cellobiose. This enhanced the availability of carbon sources for *Lactiplantibacillus plantarum*, thereby promoting its growth and proliferation [50]. Furthermore, the combination of LAB and AC exerted a synergistic effect, in which the rapid acid production by LAB inhibited competing microorganisms, while AC continuously degraded cellulose to release fermentable sugars. This interaction provided a favorable environment and sufficient substrates for *Lactiplantibacillus plantarum*. Overall, the additives optimized the fermentation conditions of CS, resulting in a higher relative abundance of *Lactiplantibacillus plantarum* in additive-treated silages compared to the CK.

4.3. Microbial Symbiotic Network

The microbial symbiotic network in silage is a dynamic system in which different bacterial communities form complex ecological relationships through metabolic interactions, resource competition, or synergistic effects [51]. *Rhizobium* and *Pseudomonas* are both Gram-negative aerobic bacteria [52,53]. They exhibit differences in their preferences for carbon and nitrogen sources. These differences drove them to regulate the environment and inhibit common competitors, thereby synergistically enhancing their survival advantages, resulting in a positive correlation in abundance within the community. During ensiling, *Lactiplantibacillus* rapidly produced large amounts of lactic acid through glycolysis, causing the pH of the silage to drop quickly to 4.0. This rapid acidification characteristic effectively inhibited the growth of harmful bacteria, reduced the loss of protein and energy in the CS material, and made it a dominant bacterial species in the silages. Additionally, *Lactiplantibacillus* formed an adaptive competitive relationship with other LAB such as *Levilactobacillus* and *Lactobacillus*, optimizing fermentation efficiency. Ultimately, it improved the fermentation quality of CS silage.

4.4. KEGG Metabolic Pathway in the Bacterial Community

KEGG metabolic pathways are a systematic integration of various chemical reaction networks within living organisms, encompassing a series of processes such as substance synthesis, decomposition, and energy conversion. These processes are interconnected, ultimately forming a complex metabolic network [54]. The surface of the CS material was colonized by a complex community of microorganisms, primarily aerobic bacteria and fungi. These microorganisms maintained their survival through symbiotic, competitive, and metabolic exchange interactions, thereby promoting the expression of microbial

metabolism in diverse environments. In the anaerobic environment of silage, *Lactiplantibacillus plantarum* dominated as the primary bacterial community. By lowering the pH value through acid production, *Lactiplantibacillus plantarum* inhibited the growth of most environmental microorganisms. Therefore, compared with CS material, CK had a lower proportion of microbial metabolism in diverse environments. LAB, as facultative anaerobic bacteria, have higher metabolic activity in anaerobic environments [55]. Exogenous LAB enhanced fermentation efficiency, enabling the breakdown of starch and sucrose to occur under conditions favorable for efficient LAB metabolism. This avoided substrate loss during the aerobic phase and further intensified sugar metabolism. Consequently, compared to the CK, the LAB+AC-treated silage exhibited higher starch and sucrose metabolic pathways.

4.5. Phenotypic Prediction of Bacterial Community

Bugbase can predict various phenotypic traits of bacteria, including oxygen tolerance, Gram staining characteristics, and potential pathogens, helping researchers gain a deeper understanding of microbial physiological characteristics, metabolic pathways, and potential functions, thereby achieving a more comprehensive understanding of the bacterial community [56]. In the silage field, phenotypic prediction analysis can be used to assess the hygienic quality of silage and optimize fermentation production strategies [57]. Exposure of CS material to natural aerobic conditions favored the survival of Gram-negative bacteria, some of which are epiphytic on plant surfaces and potential pathogens. Silage processing effectively directed the bacterial succession from Gram-negative to Gram-positive bacteria, while concurrently reducing potential pathogens of the bacterial community. This differed from the result that the natural ensiling of oats did not effectively reduce the inherent pathogenic potential of the raw material [58]. The primary reason for this discrepancy was that natural ensiling of high-moisture oats failed to achieve an optimal fermentation outcome, resulting in the insufficient suppression of pathogenic Gram-negative bacteria. These findings highlighted that successful ensiling was a prerequisite for effectively reducing potential pathogens. Hence, the additive-mediated further reduction in potential pathogenicity in CS silage warrants significant consideration.

5. Conclusions

This study demonstrated ensiling was an effective method for preserving CS, which facilitated the succession of microbial communities from Gram-negative to Gram-positive bacteria, established a symbiotic network dominated by LAB, and enhanced starch and sucrose metabolism, thereby reducing the potential pathogenic risk to CS silage. Moreover, LAB and AC further enhanced fermentation quality and reduced potential pathogenic risk, proving to be suitable additives for CS silages. These findings provide valuable insights for addressing feed shortages through improved silage processing. The recommended application rates for practical production are 0.5% FM for LAB (10^5 CFU/g FM) and 0.01% FM for AC (7350 U/g).

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