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# Elucidating Microbial Community Dynamics, Co-Occurrence Networks, And Functional Potential In Corn Stover Silage: Implications For Fermentation Efficacy And Pathogenic Risk

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**Abstract:** Background: Corn stover is a globally abundant agricultural byproduct with significant potential as livestock feed. Ensiling is a crucial method for its preservation, relying on microbial fermentation. However, a deep understanding of the complex bacterial community structure, their interactions, functional roles, and associated pathogenic risks during corn stover ensiling remains limited. This study aimed to elucidate these microbial dynamics to improve fermentation efficiency and ensure feed safety.

Methods: Corn stover was ensiled in laboratory-scale silos for up to 60 days. Fermentation quality was assessed by measuring pH, organic acids, and ammonia nitrogen. The bacterial community dynamics were profiled using high-throughput 16S rRNA gene sequencing. Bioinformatic analyses were employed to determine microbial diversity and succession, construct co-occurrence networks to identify keystone taxa, and predict the functional potential of the microbiome using PICRUSt2 based on the KEGG database.

Results: The ensiling process was successful, characterized by a rapid pH drop to below 4.0 and a high concentration of lactic acid. Microbial community analysis revealed a distinct temporal succession, with initial epiphytic Proteobacteria being replaced by a dominant Firmicutes population. Specifically, Lactiplantibacillus (formerly Lactobacillus) emerged as the most abundant genus in mature silage. Co-occurrence network analysis identified several keystone taxa that likely drive community stability. Functional predictions showed a significant enrichment of pathways related to carbohydrate metabolism, consistent with vigorous lactic acid fermentation. Notably, genera containing opportunistic pathogens, such as Pseudomonas and Clostridium, were detected, though their relative abundance decreased significantly in the well-fermented silage.

Conclusion: This study provides a comprehensive ecological perspective on the corn stover silage microbiome. Integrating community structure, network interactions, and functional predictions offers a deeper understanding of the fermentation process. The findings highlight the importance of promoting LAB dominance to not only ensure preservation but also to mitigate the risks associated with potential pathogens, thereby enhancing the overall safety and quality of silage.

**Keywords:** Corn stover, Silage, Microbial community, 16S rRNA sequencing, Co-occurrence network, Functional prediction, Pathogen risk.

**Introduction:** Maize (Zea mays L.) stands as one of the world's most vital cereal crops, forming the cornerstone of global food security, animal feed production, and various industrial applications [6]. The global demand for maize continues to escalate, driven by a growing population and an expanding livestock sector [39]. In 2022, global maize production reached unprecedented levels, yet the utilization of the entire plant remains largely inefficient [6]. Following the grain harvest, vast quantities of lignocellulosic biomass, primarily in the form of corn stover (stalks, leaves, and cobs), are left in the field. This stover represents a

massive, underutilized reservoir of energy and nutrients [7]. Traditionally, a significant portion of this residue is either burned, which contributes to air pollution and greenhouse gas emissions, or incorporated back into the soil, a process with variable benefits and potential drawbacks [9, 13].

The effective utilization of corn stover is paramount for developing sustainable agricultural systems and circular bioeconomies [10]. For the livestock industry, particularly in regions facing forage scarcity due to climate change or land use pressure, corn stover presents a valuable opportunity as a roughage source [3, 4]. However, its direct use as animal feed is severely limited by inherent nutritional deficiencies. Corn stover is characterized by high concentrations of structural carbohydrates like cellulose and hemicellulose, which are encased in a recalcitrant lignin matrix. This composition results in low digestibility, poor palatability, and suboptimal nutrient availability for ruminants [8]. Therefore, processing technologies are essential to unlock the nutritional potential of this abundant biomass.

Among the various strategies for upgrading low-quality forages, ensiling is a widely adopted, cost-effective, and practical method of preservation [41]. This process involves the anaerobic fermentation of fresh forage by a consortium of microorganisms, primarily Lactic Acid Bacteria (LAB). These bacteria metabolize water-soluble carbohydrates (WSC) present in the crop into organic acids, predominantly lactic acid [43]. The resulting accumulation of acid rapidly lowers the pH of the silage mass, inhibiting the growth of spoilage microorganisms such as yeasts, molds, and undesirable bacteria like clostridia and enterobacteria [45]. A successful fermentation not only preserves the nutrients of the original crop but can also improve its hygienic quality and, in some cases, its digestibility and palatability [11, 12].

The success of silage fermentation is fundamentally a microbiological process, dictated by the composition and activity of the microbial communities present on the forage at the point of ensiling (epiphytic microorganisms) and those that proliferate during the anaerobic phase [1, 5]. The initial microbial population on corn stover is typically dominated by aerobic bacteria, yeasts, and molds, which are undesirable for the ensiling process. The key to achieving high-quality silage is to facilitate a rapid shift in the microbial community towards a dominance of homofermentative LAB, such as Lactiplantibacillus plantarum (formerly Lactobacillus plantarum) [42]. These bacteria are highly efficient in converting hexoses into lactic acid, ensuring a swift and deep acidification of the silage [55]. However, the natural population of LAB on fresh corn stover can be low and variable, often leading to inconsistent fermentation outcomes [1, 40].

While the general principles of silage microbiology are well-established [41], our understanding of the intricate ecological interactions that govern the fermentation process is still evolving. Traditional culture-based methods have provided foundational knowledge but are limited in their ability to capture the full diversity of the silage microbiome, as many species are viable but not culturable under standard laboratory conditions. The advent of next-generation sequencing (NGS) and other culture-independent techniques has revolutionized microbial ecology, offering unprecedented insights into complex microbial ecosystems [15]. These technologies, such as 16S rRNA gene amplicon sequencing, allow for a comprehensive characterization of bacterial community structure, diversity, and succession with high resolution [16, 23].

Recent studies have begun to apply these powerful tools to silage, revealing complex dynamics and successional patterns that were previously unappreciated [20, 46, 50]. Beyond simply cataloging microbial taxa, advanced bioinformatic approaches now enable the inference of functional potential and the construction of microbial co-occurrence networks [14, 51]. Co-occurrence network analysis can help identify potential symbiotic or competitive relationships between microorganisms and pinpoint keystone taxa—species that exert a disproportionately large influence on the community structure and function relative to their abundance [28]. Furthermore, predictive functional profiling tools like PICRUSt2 can infer the metabolic capabilities of the microbial community from 16S rRNA gene data, providing insights into the collective metabolic pathways that drive fermentation [56, 57]. Concurrently, these methods can also be used to assess the presence and abundance of opportunistic pathogens, a critical aspect of feed safety that has significant implications for animal health [49, 53].

Despite this progress, a holistic investigation integrating bacterial community structure, symbiotic networks, functional roles, and pathogenic risk in corn stover silage is still lacking. Understanding these interconnected elements is crucial for developing targeted strategies—such as the design of more effective microbial inoculants or the optimization of ensiling conditions—to consistently produce high-quality, safe silage from corn stover. Therefore, the objectives of this study were: (1) to characterize the temporal dynamics of the bacterial community and fermentation characteristics during the ensiling of corn stover; (2) to construct microbial co-occurrence

networks to identify keystone taxa and reveal interspecies relationships; (3) to predict the functional metabolic pathways of the bacterial community throughout the fermentation process; and (4) to assess the dynamic risk posed by potential pathogenic bacteria.

## 2. METHODS

### 2.1. Silage Preparation and Experimental Design

Fresh corn stover was harvested at the dough-ripe stage from an experimental farm. The material was wilted under shade for approximately 12 hours to achieve a target dry matter (DM) content of 30-35%. The wilted stover was then chopped into 2-3 cm pieces using a forage chopper. The chopped material was thoroughly mixed to ensure uniformity.

The experiment was designed with two treatment groups: (1) a control group with no additives (CON), and (2) an inoculated group (IN) treated with a commercial silage inoculant containing Lactiplantibacillus plantarum and Lentilactobacillus buchneri at an application rate of 1.0×105 colony-forming units (CFU) per gram of fresh forage. The inoculant was diluted in distilled water and sprayed evenly onto the chopped stover, while the CON group was sprayed with an equal amount of distilled water.

For each treatment, the chopped stover was packed into laboratory-scale silos (polyethylene bags, 20 cm × 30 cm). Approximately 500 g of material was packed into each bag, which was then sealed using a vacuum sealer to establish anaerobic conditions. A total of 30 silos (2 treatments × 3 replicates × 5 sampling points) were prepared. All silos were stored in the dark at ambient temperature (approximately 25°C). Silos were destructively sampled on days 0 (fresh material), 3, 7, 30, and 60 of the ensiling period for chemical and microbiological analyses.

### 2.2. Fermentation Quality and Chemical Analysis

At each sampling point, a 20 g sample was taken from each silo and homogenized with 180 mL of sterilized distilled water. The mixture was blended for 1 minute and then filtered through four layers of cheesecloth. The pH of the filtrate was measured immediately using a calibrated glass-electrode pH meter.

The filtrate was then centrifuged at 10,000 × g for 10 minutes at 4°C. The supernatant was collected for further analysis. The concentrations of organic acids, including lactic acid, acetic acid, propionic acid, and butyric acid, were determined using a high-performance liquid chromatography (HPLC) system equipped with a UV detector, following the method described by Cai [19].

The concentration of ammonia nitrogen (NH3-N) was determined by the colorimetric phenol-hypochlorite method described by Broderick and Kang [21]. The water-soluble carbohydrate (WSC) content was measured using the anthrone-sulfuric acid method as detailed by Murphy [18].

The dry matter (DM) content of the fresh and ensiled samples was determined by oven-drying a 100 g sample at 65°C for 48 hours. The content of crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were determined according to the standard procedures of the Association of Official Agricultural Chemists [17]. All chemical analyses were performed in triplicate.

### 2.3. DNA Extraction and 16S rRNA Gene Sequencing

Total microbial genomic DNA was extracted from 10 g of each silage sample using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The quality and concentration of the extracted DNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and by 1% agarose gel electrophoresis.

The hypervariable V3-V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), which were tagged with sample-specific barcodes. The PCR amplification was performed in a 20 µL reaction volume containing 4 µL of 5× FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. The thermal cycling conditions were: initial denaturation at 95°C for 3 minutes, followed by 27 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes.

The resulting PCR amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using a Quantus™ Fluorometer (Promega, USA). The purified amplicons were pooled in equimolar concentrations and prepared for sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) to generate 2 × 300 bp paired-end reads. This sequencing approach is consistent with methodologies used in recent silage microbiome studies [23, 49].

### 2.4. Bioinformatic and Statistical Analysis

The raw paired-end reads were demultiplexed, quality-filtered, and merged using FLASH (v1.2.11). High-quality sequences were clustered into Amplicon Sequence Variants (ASVs) at a 99% similarity threshold using UPARSE (v7.1). Chimeric sequences were identified and removed. The taxonomic classification of

each ASV was assigned by aligning against the SILVA ribosomal RNA gene database (Release 138) [26] using the RDP classifier algorithm with a confidence threshold of 0.7.

Alpha diversity metrics, including the Chao1 estimator for richness and the Shannon index for diversity, were calculated in QIIME2. Rarefaction curves were generated to assess sequencing depth. Beta diversity was evaluated using Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances, which accounts for both the presence/absence and abundance of taxa, as well as their phylogenetic relationships [25]. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences in bacterial community structure between groups.

To explore the interrelationships among bacterial genera, co-occurrence networks were constructed for the initial (day 0) and late (day 60) stages of fermentation. Genera with a mean relative abundance of >0.1% across all samples were included. Spearman's rank correlation coefficients were calculated for all pairwise comparisons of these genera. A valid interaction was considered a statistically significant correlation with a Spearman's coefficient ($\rho$) > 0.6 and a p-value < 0.01. The resulting networks were visualized using the Gephi (v0.9.2) software platform. Topological properties of the networks, such as the number of nodes and edges, modularity, and average path length, were calculated to characterize the network structure. Keystone taxa were identified based on high degrees of connectivity and low betweenness centrality, as these nodes are considered critical for network stability [32, 36].

The functional potential of the bacterial communities was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) [56]. The ASV abundance table was used to predict the functional profiles based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology database [54]. The predicted functional abundances were organized into KEGG pathways at Level 2 and Level 3, and STAMP software was used to identify pathways that were significantly different between fermentation stages.

All statistical analyses of fermentation parameters and microbial diversity indices were conducted using SPSS Statistics (v25.0). A one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to determine significant differences, with $p < 0.05$ considered statistically significant. The principles of biometrical statistics were followed for all analyses [38].

## 3. RESULTS

### 3.1. Fermentation Quality and Chemical Composition of Corn Stover Silage

The chemical composition of the fresh corn stover at the start of the experiment (day 0) was as follows: DM content of 32.8%, WSC content of 75.4 g/kg DM, CP of 6.2% DM, NDF of 68.3% DM, and ADF of 41.5% DM. The initial pH of the fresh material was 6.6. The dynamics of fermentation parameters over the 60-day ensiling period are presented in Table 1.

**Table 1: Fermentation characteristics of corn stover silage during ensiling.**

| Ensiling Day | Treatment | pH | Lactic Acid (g/kg DM) | Acetic Acid (g/kg DM) | Propionic Acid (g/kg DM) | Butyric Acid (g/kg DM) | WSC[1] (g/kg DM) | $NH_3$-N[2] (% TN) |
|---|---|---|---|---|---|---|---|---|
| **0** | - | $6.60^{Ax}$ | $1.5^{c}$ | $0.8^{d}$ | $0.1^{c}$ | ND[3] | $75.4^{Ax}$ | $2.1^{Dx}$ |
| **3** | **CON[4]** | $5.10^{Ba}$ | $15.2^{b}$ | $5.5^{c}$ | $0.2^{bc}$ | ND | $58.1^{Ba}$ | $4.5^{ca}$ |
| | **IN[5]** | $4.60^{Bb}$ | $28.9^{a}$ | $8.9^{b}$ | $0.3^{b}$ | ND | $49.5^{Bb}$ | $3.8^{cb}$ |
| **7** | **CON** | $4.50^{ca}$ | $45.8^{b}$ | $10.1^{b}$ | $0.3^{b}$ | ND | $41.2^{ca}$ | $6.2^{Ba}$ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **IN** | $3.90^{cb}$ | $65.1^{a}$ | $16.2^{a}$ | $0.4^{a}$ | ND | $30.8^{cb}$ | $4.9^{Bb}$ |
| **30** | **CON** | $4.15^{Da}$ | $62.5^{b}$ | $14.5^{a}$ | $0.3^{b}$ | ND | $31.5^{Da}$ | $7.9^{Aa}$ |
| | **IN** | $3.83^{Db}$ | $81.3^{a}$ | $22.8^{a}$ | $0.4^{a}$ | ND | $20.1^{Db}$ | $5.5^{Ab}$ |
| **60** | **CON** | $4.12^{Da}$ | $65.8^{b}$ | $15.1^{a}$ | $0.3^{b}$ | ND | $29.7^{Da}$ | $8.2^{Aa}$ |
| | **IN** | $3.81^{Db}$ | $85.2^{a}$ | $24.5^{a}$ | $0.4^{a}$ | ND | $18.3^{Db}$ | $5.8^{Ab}$ |

Table Footnotes:

[1]WSC: Water-Soluble Carbohydrates. [2]$NH_3$-N: Ammonia Nitrogen, expressed as a percentage of Total Nitrogen (TN). [3]ND: Not Detected. [4]CON: Control group (no additives). [5]IN: Inoculated group. Values are means of three replicates. $^{A}$, $^{B}$, $^{c}$, $^{D}$: Means in the same column with different uppercase superscripts differ significantly ($p < 0.05$) across different ensiling days for the same treatment. $^{a}$, $^{b}$: Means in the same row with different lowercase superscripts differ significantly ($p < 0.05$) between CON and IN treatments at the same ensiling day. $^{x}$: Indicates the initial value at day 0.

As shown in Table 1, a rapid decrease in pH was observed in both treatments within the first week. By day 7, the pH in the IN group had dropped to 3.90, significantly lower than the CON group's pH of 4.50. The pH continued to decrease and stabilize, reaching a final value of 3.81 in the IN group and 4.12 in the CON group after 60 days.

Lactic acid was the predominant organic acid produced during fermentation. Its concentration increased sharply in both groups, but the accumulation was faster and reached a higher final concentration in the IN group (85.2 g/kg DM) compared to the CON group (65.8 g/kg DM). Acetic acid concentration was notably higher in the IN group, likely due to the presence of the heterofermentative L. buchneri in the inoculant, reaching 24.5 g/kg DM by day 60, compared to 15.1 g/kg DM in the CON group. Propionic acid was detected in trace amounts, and butyric acid was below the detection limit in all samples, indicating the absence of undesirable clostridial fermentation.

The WSC content decreased progressively during ensiling as it was consumed by microorganisms. The consumption was more rapid and thorough in the IN group, with the final WSC content being 18.3 g/kg DM, compared to 29.7 g/kg DM in the CON group. The NH3-N concentration, an indicator of proteolysis, remained low in both treatments but was significantly lower in the IN group (5.8% of total nitrogen) than in the CON group (8.2% of total nitrogen) at day 60, suggesting better preservation of true protein in the inoculated silage.

3.2. Sequencing Data and Bacterial Diversity

After quality filtering and chimera removal, a total of 1,254,890 high-quality 16S rRNA gene sequences were obtained from the 30 silage samples, with an average of 41,830 sequences per sample. The rarefaction curves for all samples approached a plateau, indicating that the sequencing depth was sufficient to capture the majority of the bacterial diversity present [44].

Alpha diversity, as measured by the Shannon and Chao1 indices, showed a clear trend over the ensiling period. The fresh corn stover (day 0) exhibited the highest bacterial diversity and richness. As fermentation progressed, both the Shannon and Chao1 indices decreased dramatically in both treatment groups, reaching their lowest points by day 7 and remaining low thereafter. This indicates that the anaerobic, acidic environment created during ensiling acted as a strong selective pressure, leading to a less diverse community dominated by a few well-adapted species. The IN group generally showed a slightly faster decline in diversity compared to the CON group.

Beta diversity analysis using PCoA based on weighted UniFrac distances revealed distinct clustering of samples according to the ensiling time. The day 0 samples formed a distinct cluster, separate from all ensiled samples. The communities from days 3 and 7 formed intermediate clusters, while the day 30 and day 60 samples clustered closely together, indicating that the microbial community had reached a relatively stable state by day 30. The PERMANOVA test confirmed that ensiling time had a highly significant effect on the

bacterial community structure ($p < 0.001$).

3.3. Bacterial Community Composition and Succession

The taxonomic composition of the bacterial communities shifted profoundly during fermentation. At the phylum level, the fresh corn stover (day 0) was dominated by Proteobacteria (55.6% relative abundance) and Firmicutes (28.3%), with smaller proportions of Bacteroidetes and Actinobacteria. As fermentation commenced, a rapid and dramatic shift occurred. By day 7, Firmicutes had become the overwhelmingly dominant phylum, accounting for over 95% of the community in both treatments, while the abundance of Proteobacteria plummeted to less than 2%.

This shift was even more pronounced at the genus level. The initial bacterial community on fresh stover was diverse, with the most abundant genera being Pantoea, Enterobacter, Pseudomonas, and Weissella. Following the onset of anaerobic conditions, the community composition changed drastically. By day 3, Weissella, a genus of heterofermentative LAB, became dominant. However, from day 7 onwards, the community was almost completely dominated by Lactiplantibacillus. In the mature silage (days 30 and 60), Lactiplantibacillus accounted for over 85% of the relative abundance in the CON group and over 90% in the IN group. The genus Lentilactobacillus was also detected at a higher abundance in the IN group (average 4.5% at day 60) compared to the CON group (<1%), consistent with the composition of the applied inoculant. The abundance of undesirable genera like Enterobacter and Clostridium was reduced to negligible levels after 7 days of ensiling.

3.4. Microbial Co-occurrence Networks and Keystone Taxa

To understand the interactions within the bacterial communities, co-occurrence networks were constructed for the initial (day 0) and late (day 60) fermentation stages. The network for the fresh stover was more complex, with 58 nodes (genera) and 215 edges (significant correlations), indicating a diverse and interconnected community. The network exhibited a modularity of 0.68, suggesting the presence of distinct ecological niches.

In stark contrast, the network for the mature silage (day 60) was much simpler and more fragmented. It contained only 22 nodes and 45 edges, with a lower modularity of 0.35. This structural simplification reflects the low-diversity community that resulted from the strong selective pressures of the silage environment.

In the day 60 network, Lactiplantibacillus emerged as the central hub and a clear keystone taxon. It had the highest number of connections (degree) and was involved in the majority of the significant positive correlations, primarily with other LAB genera like Lactococcus and Pediococcus. This suggests a synergistic or mutualistic relationship among the acid-tolerant bacteria that dominate the late stage of fermentation. Conversely, Lactiplantibacillus showed strong negative correlations with genera associated with spoilage or aerobic conditions, such as Pseudomonas and Acinetobacter, illustrating the principle of competitive exclusion.

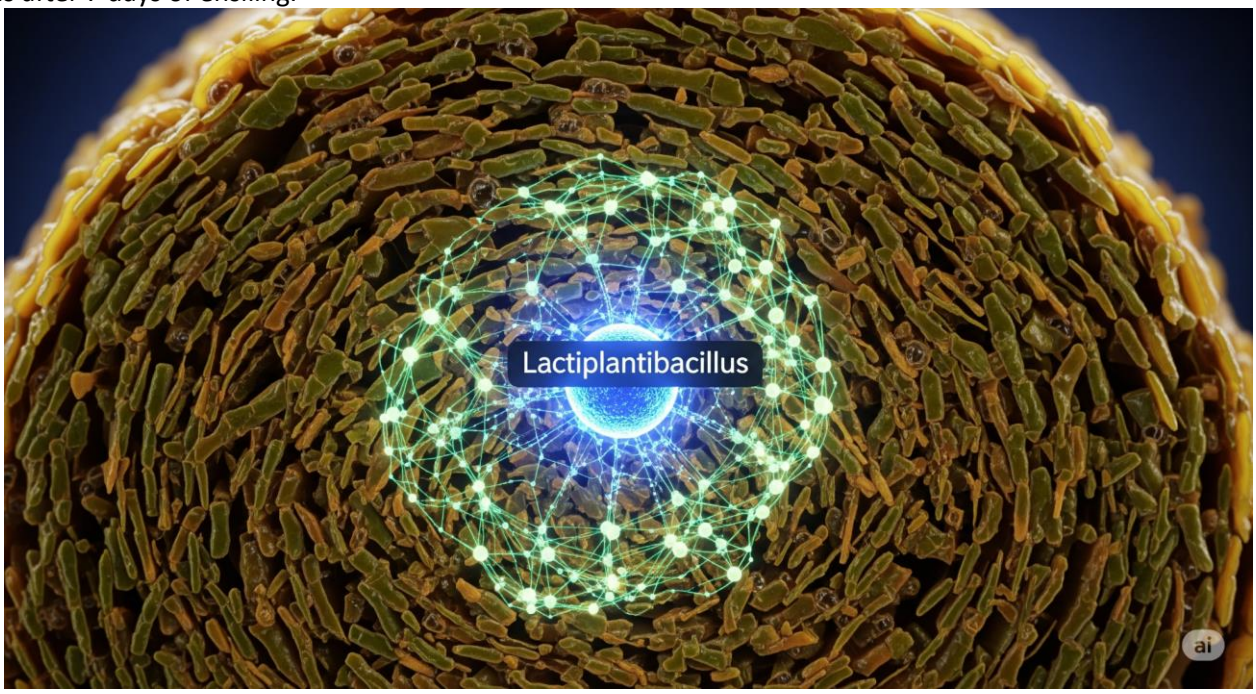


**Figure 1: A conceptual visualization of the mature silage microbiome, illustrating the central role of**

***Lactiplantibacillus* as the keystone taxon within the microbial co-occurrence network. The blue central node represents *Lactiplantibacillus*, while the interconnected green and yellow nodes represent other symbiotic bacteria within the fibrous corn stover matrix.**

3.5. Functional Potential of the Bacterial Community

The functional capabilities of the silage microbiome were predicted using PICRUSt2. A clear shift in the metabolic potential of the community was observed as fermentation progressed. At KEGG Level 2, the most significant changes were observed in "Carbohydrate Metabolism," "Amino Acid Metabolism," and "Metabolism of Cofactors and Vitamins."

Compared to the fresh stover community, the mature silage community showed a significant enrichment in pathways related to carbohydrate metabolism. Specifically, at Level 3, pathways such as "Glycolysis / Gluconeogenesis," "Pentose phosphate pathway," and "Starch and sucrose metabolism" were significantly upregulated. This aligns perfectly with the primary function of silage bacteria, which is to ferment available sugars into organic acids [48, 55]. Conversely, pathways related to "Amino Acid Metabolism," particularly those involved in amino acid degradation and putrefaction, were significantly downregulated in the mature silage. This predicted functional shift corresponds with the lower NH3-N concentrations observed in the well-fermented silage, indicating better protein preservation. The functional profile of the IN group showed a slightly stronger enrichment of carbohydrate metabolism pathways compared to the CON group, reflecting its enhanced fermentation efficiency.

3.6. Assessment of Potential Pathogenic Bacteria

The high-throughput sequencing data were screened for the presence of genera known to contain opportunistic pathogens relevant to livestock. On the fresh corn stover (day 0), several such genera were detected at notable abundances, including Pseudomonas (8.2%), Enterobacter (12.5%), Clostridium (1.8%), and Listeria (0.5%).

During the ensiling process, the relative abundance of these potential pathogens decreased dramatically. By day 7, the acidic environment had suppressed their growth effectively. In the mature silage at day 60, the combined relative abundance of these four genera was less than 0.1% in both treatment groups. Pseudomonas, a versatile and often pathogenic bacterium [53], was virtually eliminated. Clostridium, responsible for butyric acid fermentation and potential toxin production, was also reduced to below detection limits. This demonstrates that successful lactic acid fermentation is a powerful tool for enhancing the hygienic quality of the feed and mitigating the risk of transmitting pathogens to livestock.

## 4. DISCUSSION

4.1. Microbial Succession Drives Effective Silage Fermentation

This study provides a comprehensive view of the microbiological and chemical transformations that occur during the ensiling of corn stover. The results clearly demonstrate that a successful fermentation is predicated on a rapid and predictable microbial succession. The initial epiphytic community on fresh stover was diverse and dominated by Proteobacteria, including genera like Enterobacter and Pantoea. These facultative anaerobes are common on plant surfaces and can initiate fermentation, but they are relatively inefficient, often producing a mix of acids, alcohols, and CO$_2$, and can contribute to proteolysis [40, 46]. Their dominance is transient, as they are quickly outcompeted once anaerobic conditions are established and the pH begins to drop.

The first phase of active fermentation was characterized by the proliferation of Weissella, a genus of heterofermentative LAB. This is a common observation in the early stages of silage fermentation for various crops, as Weissella species are known for their ability to grow rapidly under a wide range of temperatures and pH values [20]. However, the crucial shift occurred between days 3 and 7, where the community transitioned to an overwhelming dominance by Lactiplantibacillus. This genus, particularly L. plantarum, is renowned for its acid tolerance and homofermentative metabolism, which efficiently converts hexoses into two molecules of lactic acid [42, 48]. This metabolic efficiency is the primary driver behind the rapid pH drop observed in our results, which is the hallmark of high-quality silage production [41, 45]. The inoculation (IN group) accelerated this process, leading to a faster pH decline and higher final lactic acid concentration, confirming the efficacy of using elite LAB strains to steer the fermentation process [22, 43]. The lower final pH and NH3-N content in the IN group highlight a more efficient preservation of nutrients and inhibition of proteolytic activity, consistent with previous studies on inoculated silages [49, 50].

4.2. Ecological Interactions Revealed by Co-occurrence Networks

The co-occurrence network analysis provided valuable insights into the changing ecological relationships

within the silage microbiome. The complex and highly connected network of the fresh material reflects a diverse ecosystem with numerous potential interactions. However, the harsh, selective environment of the silo—characterized by anaerobiosis and low pH—dismantles this complex structure, leading to a much simpler and more specialized community. This ecological principle, where environmental stress reduces diversity and complexity, is well-documented in other extreme environments [28, 30].

The emergence of Lactiplantibacillus as the undisputed keystone taxon in the mature silage network underscores its central role in not just dominating the community by numbers, but also in structuring it. Its positive correlations with other beneficial LAB suggest a cooperative microenvironment where these acid-producing bacteria thrive. More importantly, its strong negative correlations with genera like Pseudomonas provide a clear illustration of competitive exclusion. This is likely mediated through multiple mechanisms: the production of lactic acid creates an environment too acidic for neutrophilic bacteria to survive; competition for limited substrates (WSC) starves out less efficient fermenters; and the potential production of bacteriocins by some L. plantarum strains can directly inhibit competitors [42]. This network-level perspective moves beyond simple abundance data to reveal the functional architecture of the microbial community, confirming that the dominance of LAB actively suppresses spoilage and undesirable microorganisms [14, 51, 57].

### 4.3. Functional Metagenomics Corroborates Fermentation Biochemistry

The functional predictions generated by PICRUSt2 strongly support the observed chemical and microbial data, providing a mechanistic link between community composition and ecosystem function. The significant upregulation of pathways related to carbohydrate metabolism in the mature silage microbiome is the genetic blueprint for the observed chemical changes. The enrichment of glycolysis and sucrose metabolism pathways directly reflects the intense conversion of plant sugars into lactic acid, which is the biochemical engine of the ensiling process [55]. This finding aligns with other studies that have used PICRUSt2 to explore the functional potential of silage communities, confirming its utility in this context [56, 58].

Furthermore, the predicted downregulation of amino acid degradation pathways is equally significant. Excessive proteolysis during ensiling is undesirable as it reduces the protein value of the feed and produces ammonia and biogenic amines, which can decrease palatability and be detrimental to animal health. The suppression of these pathways, mirrored by the low NH3-N concentrations, indicates that the LAB-dominated community effectively preserved the protein fraction of the corn stover. This demonstrates that the shift in microbial community structure has direct and favorable consequences for the nutritional quality of the final product. The functional profile provides a powerful confirmation that the observed microbial succession is not just a change in names and numbers, but a fundamental shift in the collective metabolic capability of the ecosystem towards efficient preservation.

### 4.4. Mitigating Pathogenic Risk through Lactic Acid Fermentation

Feed safety is a critical concern in livestock production. Fresh forages can be contaminated with a variety of microorganisms from soil, manure, and the environment, including opportunistic pathogens such as Listeria, Clostridium, and certain species of Pseudomonas and Enterobacter [52, 53]. Our results confirmed the presence of several of these potentially pathogenic genera on the fresh corn stover. If allowed to proliferate, these microorganisms could pose a significant risk to animal health, leading to diseases, reduced productivity, and in severe cases, mortality.

This study powerfully demonstrates that a well-managed lactic acid fermentation is an effective critical control point for reducing this microbial risk. The rapid acidification of the silage mass creates a hostile environment that most pathogens cannot tolerate. The final pH of <4.2 achieved in both treatments is below the growth threshold for most clostridia and pathogenic enterobacteria [49]. Our sequencing data confirmed this, showing a dramatic reduction in the relative abundance of all major potential pathogens to negligible levels in the mature silage. This process of competitive exclusion and environmental modification is a classic example of biological control. By ensuring the dominance of beneficial LAB, the ensiling process not only preserves nutrients but also acts as a robust sanitation step, transforming a microbially unpredictable raw material into a hygienically safe and stable feed product. This aspect is crucial for the sustainable intensification of livestock farming, ensuring that the utilization of alternative feed resources like corn stover does not compromise animal welfare or food safety [57, 58].

### 4.5. Limitations and Future Directions

While this study provides a comprehensive analysis, certain limitations should be acknowledged. Firstly, the study was conducted at a laboratory scale, and fermentation dynamics can sometimes differ in large

farm-scale silos. Future research should validate these findings under practical farm conditions. Secondly, 16S rRNA gene sequencing provides information on community composition and predicted function but does not measure the actual metabolic activity or gene expression. Future studies employing metatranscriptomics and metaproteomics could provide a more direct link between microbial genes and their real-time functional roles. Finally, PICRUSt2 is a predictive tool based on reference genomes, and its accuracy can be limited by the availability of relevant genomes in the database. Despite these limitations, the congruence between our chemical, microbiological, and bioinformatic data lends strong support to our conclusions.

Future research should focus on designing and testing synergistic microbial consortia as inoculants, potentially combining highly efficient acid producers with strains that produce antifungal compounds to improve aerobic stability upon feed-out. Exploring the impact of different corn stover hybrids and harvest times on the epiphytic microbiome and subsequent ensilability would also be a valuable avenue of investigation.

## 5. CONCLUSIONS

This study successfully integrated chemical analyses with advanced microbiological and bioinformatic techniques to provide a holistic understanding of the fermentation process in corn stover silage. Our findings revealed a clear and desirable microbial succession, transitioning from a diverse epiphytic community to one overwhelmingly dominated by the keystone taxon Lactiplantibacillus. This shift was directly responsible for the rapid acidification and efficient preservation of the silage. The co-occurrence network analysis elucidated the competitive and synergistic relationships that structure the mature silage microbiome, while functional predictions confirmed that the community's metabolic potential becomes highly specialized for carbohydrate fermentation. Crucially, the study demonstrated that this LAB-driven fermentation is a highly effective method for mitigating the risks associated with potential pathogenic bacteria present on the fresh crop. By managing the microbial ecology of silage, it is possible to transform a low-value agricultural residue into a high-quality, safe, and valuable feed resource for livestock.

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