RESEARCH ARTICLE

Evaluation of the Compound-microbes-preparation Based on Microflora Information as Inoculants in the Silage

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Abstract

Ensiling technique is becoming a common way to preserve fresh corn and grass. This study aimed at evaluating the silages' quality with a new type of inoculants designed based on the microflora information of the native silages. A total of 33 silage samples were analyzed for their microbial community structure by high throughput sequencing. A total of 73 dominant strains were isolated with a clear goal. The corn was ensiled by dominant strains (*Lactobacillus buchneri* II JC1-5: *Acetobacter pasteurianus* DHL-8 = 4:3; 10^9 CFU/g) as inoculants. In a 15-day trial period, the aerobic stability of this inoculated silage was significantly higher than the control group. There was a positive correlation between lactic acid content and pH/mold number (R^2 values of 0.64331 and 0.48584, respectively). Compared to the control group, ammonia nitrogen (NH_3-N), water soluble carbohydrates (WSC), neutral detergent fiber (NDF), and acid detergent fiber (ADF) decreased by 57.7%, 12.5%, 1.4%, and 7.5%, respectively; crude protein (CP) increased by 1.1%. Our results indicate that the new type of inoculants can improve the quality of the silage.

Keywords: silage, inoculants, microflora, high throughput sequencing.

1 Introduction

Silage is an important way for preserving the nutrient of forage, and an effective pretreatment technology, which reduces the risk of feedstock decay under anaerobic conditions [1–3]. Nowadays, corn silage has been widely used in Germany, Austria, and the Czech Republic [4, 5]. There is a growing realization that silage feeding can make animals healthier and also allows us to obtain safer animal-derived food. In China, the silage has shown a positive development trend in terms of policy support, market demand, technological advancement, and environmentally sustainable development. The market size of China's silage industry reached around 37.4 billion yuan in 2023, with a year-on-year increase of 7.47%. Not only that, but with the use of fermentation agents and the improvement of fermentation technology, the quality and yield of silage (especially corn silage) have been enhanced. The industry is committed to improving the nutritional value and economic benefits of feed by selecting suitable silage crop varieties and optimizing fermentation processes, thereby increasing the digestibility and palatability of feed and reducing nutritional losses. Meanwhile, the silage is an important source of nutrition for ruminants and has an profound effect on hosts' microbiota, which plays

Academic Editor:
Jie Ouyang

Submitted: 19 September 2024
Accepted: 21 October 2024
Published: 12 November 2024

Vol. 1, **No.** 1, 2024.
10.62762/ASFP.2024.516036

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Citation
Zhuang, X., Han, W., Xue, Z., & Miao, H. (2024). Evaluation of the Compound-microbes-preparation Based on Microflora Information as Inoculants in the Silage. *Agricultural Science and Food Processing*, 1(1), 29–37.

an strong role in animal growth performance and feed digestion. Such as, *Succinivibrionaceae* was negatively correlated with glycan biosynthesis and metabolism, metabolism of co-factors and vitamins, nucleotide metabolism, and translation while *Prevotellaceae* was positively correlated with amino acid metabolism, carbohydrate metabolism [6].

It has been widely accepted that the high quality silage depends on development of a favorable microflora under anaerobic conditions [7, 8]. The main nutritional components, including ammonia Nitrogen (NH_3-N), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and PH and so on, can be enhanced. Also, Good silo management and the use of silage inoculants can prevent the growth of fungi or yeast and pathogenic microorganisms that could cause aerobic spoilage [9, 10].For example, previous reports have analyzed the effect of a probiotic bacteriocin-producing strain of *Enterococcus faecium* on grass ensiling [11] and the antimicrobial activity of bovicin HC5 against pathogen microorganisms isolated from silages [12–14]. However, the majority of researches aim at the evaluation and detection for the silage with single microbial additive. Only few studies have focused on the design of the compound-microbes inoculants, and the reasons for their combination.

The objective of this study was to assess the bacterial community structure of the native silages, design the inoculants based on the dominant microflora, and evaluate effects for this silage including its quality.

2 Materials and Methods

2.1 The Collection of Samples

33 silage samples were obtained from some rural households and ranches of North China (Table 1), which have not been inoculated by natural fermentation. The each sample was vacuum-packed immediately, and stored at 4°C.

2.2 Isolation and Genotypic Identification of Bacteria

The strains were isolated on MRS (1000 ml water, 20 g agar, 10 g glucose, 10 g peptone, 5 g yeast extract, 5 g sodium acetate, 2 g K_2HPO_4, 2 g diammonium citrate, 1 ml Tween 80, 0.58 g $MgSO_4{\cdot}7H_2O$, 0.25 g $MnSO_4{\cdot}H_2O$, pH 7.0) agar plates, which were identified by 16S rRNA gene sequencing [15, 16]. Additionally, reducing the use of MRS medium by 5–15% by weight and adding an equal proportion of solid residue from actinomycete fermentation as a culture medium has been used to reduce cultivation costs.

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2.4 Analysis of Microbial Diversity

Prior to bacterial DNA extraction, 10 g of the silage sample was suspended in 100 mL of sterilized saline solution and shaken for 20 minutes. The resulting supernatant was collected for subsequent experiments. Bacterial DNA was extracted from the silage samples using a DNA kit (Omega Bio-tek Inc., Norcross, GA, USA) following the manufacturer's instructions. Amplification of a 467 bp fragment from the V3-V4 region of the bacterial 16S ribosomal RNA gene was performed via PCR [17–19]. The PCR reaction mixture (20 μL) included 4 μL of 5μFastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, 10 ng of template DNA, and the necessary amount of UV-sterilized water. All PCR reagents were obtained from TransGen Biotech Co., Ltd. (Beijing, China). The PCR products were then sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for sequencing on the Illumina MiSeq platform.

The raw fastq files were processed by multiplexing and quality filtering using QIIME (version 1.17). Operational Taxonomic Units (OTUs) were clustered at a 97% similarity threshold with UPARSE (version 7.1, http://drive5.com/uparse/). Chimeric sequences were identified and eliminated using UCHIME. Taxonomic classification of each 16S rRNA gene sequence was conducted using the RDP Classifier (http://rdp.cme.msu.edu/) with a confidence threshold set at 97% [20–22].

2.5 Making Process and Storage of the Silage

According to the average result of the relative abundance of microflora, the inoculants were made

Table 1. The basic information of silage-samples.

No.	**Samples**[1]	**Sampling location (Latitude/ longitude)**[2]	**Basic properties of samples**	pH	**Number of viablebacteria**[3,4]
1	NM1	40.67/110.27	Blue-green	3.68	107
2	NM2	40.84/111.75	Blue-green	3.42	107
3	NM3	40.73/110.27	Yellowish green	4.17	107
4	NM4	42.25/118.88	Blue-green	3.63	108
5	NM5	39.90/111.68	Blue-green	3.79	107
6	NM6	42.25/118.88	Blue-green	3.87	107
7	NM7	40.27/111.19	Yellowish green	3.77	107
8	NM8	40.56/110.52	Blue-green	3.65	107
9	NM9	40.78/111.58	Blue-green	3.65	107
10	NM10	40.78/111.58	Blue-green	3.66	107
11	NM11	40.56/110.52	Blue-green	3.77	107
12	NM12	40.72/111.16	Blue-green	3.72	107
13	NM13	40.72/111.16	Blue-green	4.86	107
14	NM14	40.75/111.67	Blue-green	3.77	107
15	NM15	40.72/111.16	Blue-green	3.57	108
16	GS1	35.42/104.51	Yellowish brown	3.68	108
17	GS2	35.36/103.70	Yellowish green	3.78	107
18	GS3	35.57/104.60	Yellowish brown	5.68	105
19	GS4	38.62/103.09	Green	6.38	107
20	GS5	37.90/102.62	Blue-green	3.78	106
21	HLJ1	46.38/123.42	Tan	3.93	107
22	HLJ2	46.71/131.57	Yellowish brown	3.69	106
23	HLJ3	44.55/129.63	Yellowish brown	3.64	108
24	SD1	36.97/117.17	Blue-green	3.52	107
25	SD2	36.97/117.17	Blue-green	4.72	107
26	SD3	36.97/117.17	Blue-green	4.20	107
27	SD4	35.23/115.48	Yellowish brown	3.67	107
28	SD5	34.70/115.51	Blue-green	3.84	107
29	SD6	34.81/117.32	Blue-green	3.71	107
30	BJ1	40.42/116.72	Yellowish brown	3.83	105
31	TJ1	39.49/117.34	Blue-green	3.73	107
32	HN1	32.99/112.52	Blue-green	4.11	107
33	HB1	38.83/115.68	Blue-green	6.44	107

1.In the naming of the samples, the letters represent the initials of the Chinese province names.
2.The latitude and longitude information from the Google map.
3.The bacteria were cultured on MRS ager plates.
4.The unit is CFU/g.

by two dominant microbes (*Lactobacillus buchneri* II JC1-5 and *Acetobacter pasteurianus* DHL-8), which were cultured anaerobically on MRS media at 37°C for 24 h after incubation (1%, v/v); and the number of viable cells reached up to 1.0×10^9 CFU and was reserved for the next experiment. The whole crop (fresh corn) was chopped into 2–3 cm pieces and placed in sterile fermentation bags, and assigned randomly to two experimental groups with 3 replicates: group A (Blank control) - the corn added with 5 ml sterile water per 50 g; group B - the corn added with 5 ml fermentation broth per 50 g. The finished product was obtained after 15 days of fermentation under the condition of constant temperature (25°C). Several indexes, including NH_3-N, WSC (water soluble carbohydrates), CP, NDF, ADF, L-lactic acid content, *Mold*, and pH, were detected and calculated for 0 d through 15 d [23].

2.6 Data Analysis

Significance tests were performed using SPSS 16.0 software. Data were compared by T test. Line fitting Analysis was performed using OriginPro software.

3 Results

3.1 Sampling and Sorting for Silage-samples

The basic information of silage samples is shown in Table 1. A total of 33 samples were obtained and labeled from different origins of 8 provinces/province-level municipalities of North China, including Beijing, Gansu, Hebe, Henan, Heilongjiang, Inner Mongolia, Shandong and Tianjin. The latitude distribution zone of samples(range from 32°N to 47°N), covers traditional agricultural and pastoral areas or the industrial feed production area; So these samples are representative. Moreover, the quality of silages was also very critical. In particular, the samples should be spontaneously and well fermented, such as good smell, acidic pH, and non-slight fungal contamination.

Table 2. Distribution of the stains in silage-samples.

Species[1]	Number of stains
Acetobacter ghanensis	5
Acetobacter pasteurianus	12
Klebsiella variicola	4
Lactobacillus acidophilus	1
Lactobacillus buchneri	6
Lactobacillus casei	2
Lactobacillus curvatus	1
Lactobacillus fermentum	3
Lactobacillus plantarum	20
Lactococcus lactis	8
Lysinibacillus sp.	2
Staphylococcus sp.	4
Weissella sp.	5

1.Forward primer:5′-GAGAGTTTGATCCTGGCTCAG-3′; reverse primer:5′-AAGGAGGTGATCCAGCCGCA-3′.[15]

3.2 Isolation, Identification, and Selection of Bacterial Strains

As shown in Table 2, a total of 73 potential strains were found from samples, which were identified through 16S rRNA gene sequencing. These strains included *Acetobacter ghanensis* (5 strains), *Acetobacter pasteurianus* (12 strains), *Klebsiella variicola* (4 strains), *Lactobacillus acidophilus* (1 strain), *Lactobacillus Buchneri* (6 strains), *Lactobacillus casei* (2 strains), *Lactobacillus curvatus* (1 strains), *Lactobacillus fermentum* (3 strains), *Lactobacillus plantarum* (20 strains), *Lactococcus lactis* (8 strains), *Lysinibacillus sp.* (2 strains), *Staphylococcus sp.* (4 strains) and *Weissella sp.* (5 strains). These strains were compared by the acid production detection, the growth rate detection and inhibition of Mold. L. buchneri strain II C1-5 and A. pasteurianus strain DHL-8 were selected as the inoculant stains. Both strains displayed rapid growth (generation time about 30 min), short lag phase (1.3h-1.7h) and a certain ability (broth of the strains) to inhibit *Penicillium* sp. (the data not shown).

3.3 Diversity Profiling of the Pacterial Microbiota

Further characteristics of the silage samples' bacterial microbiota, including Similarity and Relative content analysis [24–29], are shown in Figure 1. By using the Illumina MiSeq technology to complete 16S rRNA gene sequencing, the large and detailed bacterial profiles were obtained at *phylum* and *genus* (even to *species*) level for each sample. Together, from all 33 samples, 4 *phyla* and 46 *genera* were identified. At the *phylum* level, the most bacteria were *Firmicutes* and *Proteobacteria* (87.9%), followed by *Bacteroidetes* and *Actinobacteria* as well as other minor *phyla*.

As shown in Figure 1, the major bacterial *genera* (relative abundance of >1%) in silages mainly belonged to *Lactobacillus* sp. (41.4%) and *Acetobacter* sp. (32.4%), *Comamonas* sp. (2.2%), *Pseudomonas* sp. (2.0%), *Bacillus* sp. (1.9%), *Acinetobacter* sp. (1.7%), *Stenotrophomonas* sp. (1.5%), *Kurthia* sp. (1.4%), *Enterococcus* sp. (1.3%), *Lactococcus* sp. (1.2%). Minor *genera* that had a relative abundance of <1% included *Sphingobacterium* sp. (0.9%), *Pediococcus* sp. (0.6%), *Corynebacterium* sp. (0.5%), *Lysinibacillus* sp. (0.5%), *Enterobacter* sp. (0.4%) and others.

The silages from different regions showed a clear consistency in their bacterial community with two predominant *genera* of *Lactobacillus* sp. and *Acetobacter* sp. (73.8%; 4:3 at average), contrasting with other *genera*. Based on these results, it can be assumed that: if the inoculants are designed based on the proportion of the two kinds of dominant bacteria (*Lactobacillus* sp. and *Acetobacter* sp.) for silage fermentation, the quality of the industrialized silage may show no significant difference or even better than the native silage, and the quality control may become easier.

3.4 Evaluation of the Complex-stains Inoculants

The compound strains (*L. buchneri* II JC1-5: *A. pasteurianus* DHL-8 ≈ 4:3) were sprayed onto the test corn for a 15-day fermentation period. The results indicated that (as shown in Table 3 and 4):

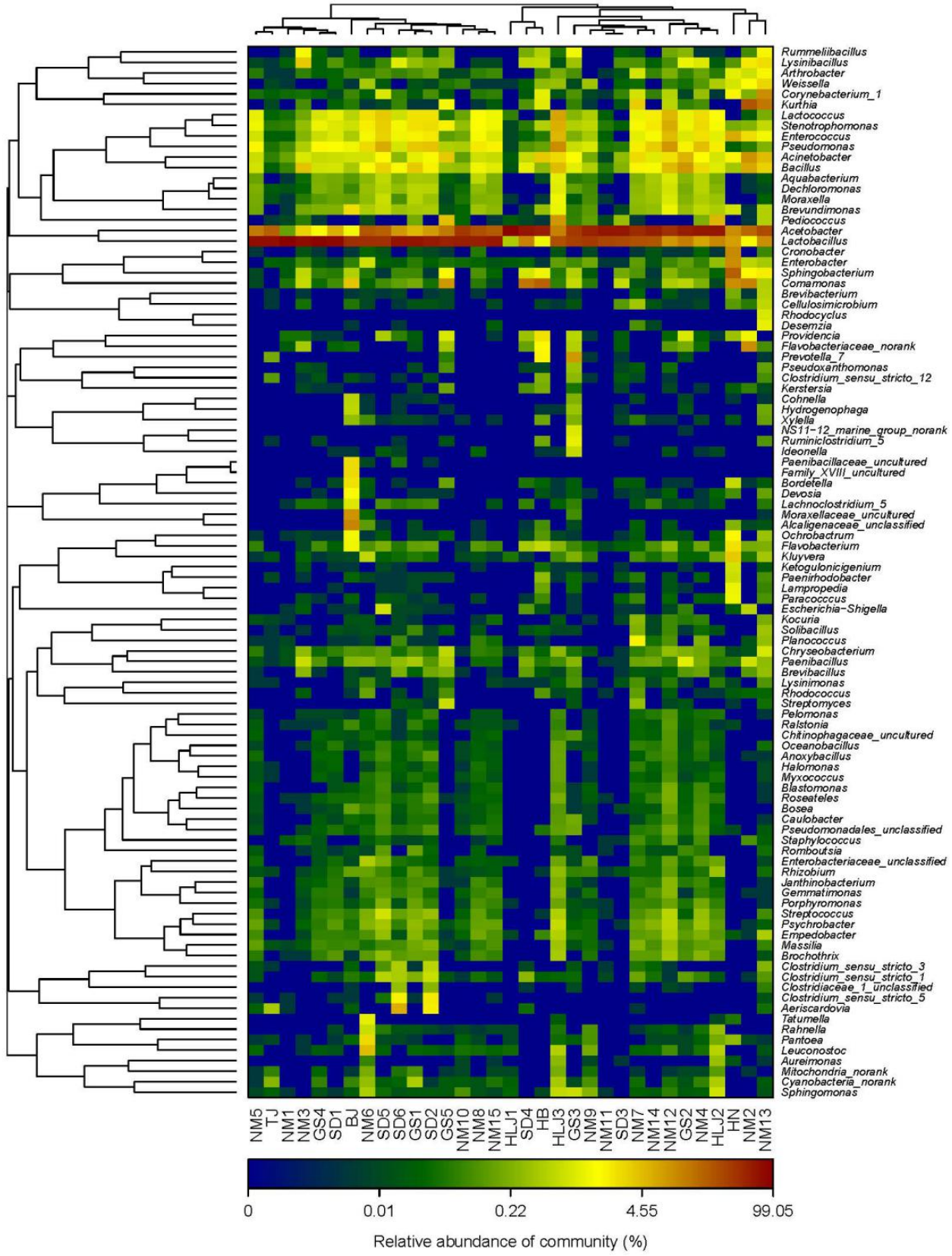

Figure 1. Heat-map of samples using genus-level distributions based on 16S rRNA gene sequencing
In 33 samples, the relative abundance of *Lactobacillus* sp. and *Acetobacter* sp. is, $n \leq 50\%$: 4 samples; $50\% \leq n \leq 70\%$: 3 samples; $70\% < n \leq 80\%$: 10 samples; $80\% < n \leq 90\%$: 3 samples; $90\% \leq n$: 11 samples.

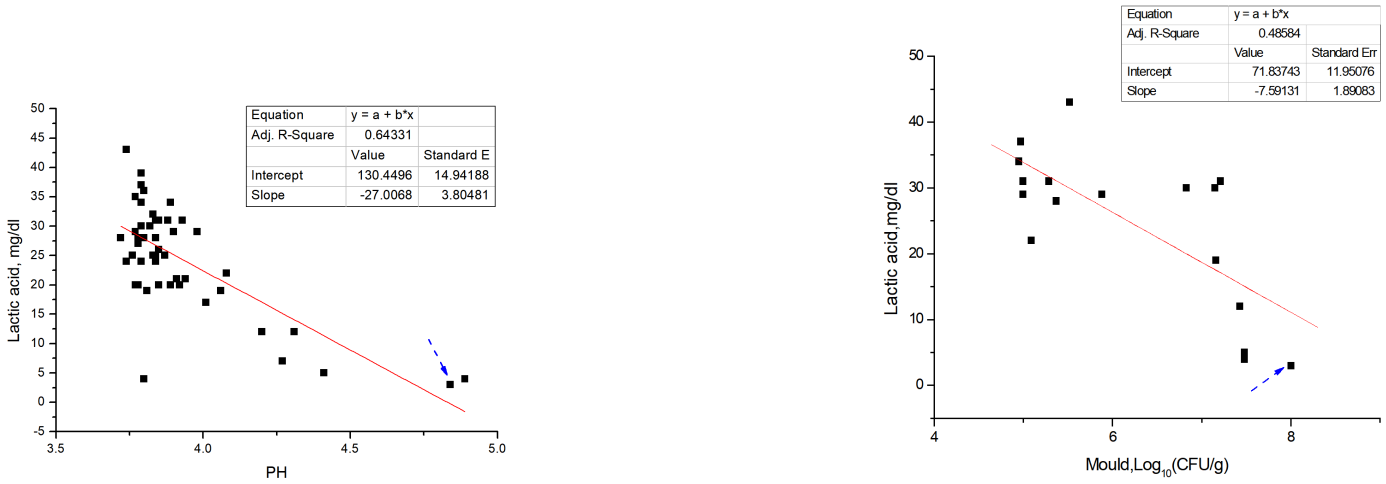

Figure 2. Correlation between the lactic acid content and change of pH (a) or and Mold number (b) Black spots are the data of test corn in 0 d-15 d; Red line is the fitting curve; Blue arrow points the control data point.

Compared to group A, the acidity of group B decreased more rapidly (pH < 4.0), and the lactic acid yield increased significantly (32 g/dl). The silage of group B had significantly less mold than that of group A. Furthermore, there was a certain correlation between pH and lactic acid yield (R^2 = 0.64331, Figure 2(a)) or between lactic acid yield and *Mold* (R^2 = 0.48584, Figure 2(b)). It's worth noting that this was the coexistence of fungal contamination and bacterial contamination. Although *L. buchneri* II JC1-5 and *A. pasteurianus* DHL-8 were two most dominant bacteria (up to 10^8 CFU/g) in test samples, the undesired microbes also increased with storage time, mainly as *Bacillus pumilus* and *Bacillus atrophaeus*.

After a 15-day trial period, the nutrient content of this silage, including NH_3-N, WSC, CP, NDF, and ADF, changed in various degrees. Compared to the control group, NH_3-N, WSC, and ADF decreased by 57.1%, 12.5%, and 7.5%, respectively; CP increased by 1.1%, NDF showed no significant change in inoculated silage (pH=3.64±0.02). Moreover, the standard deviation of these indices was significantly reduced. The results suggested that the quality of inoculated silage is improved obviously and more stable.

4 Discussion

Silage is a traditional method for preserving cattle feed over extended periods, especially when fresh forage is scarce or unavailable, due to the combined effects of low pH and the antimicrobial properties of short-chain organic acids [10]. The fermentation process of silage is primarily driven by microbial activity. Since the early 1990s, the use of microbial starters, particularly lactic acid bacteria (LAB), has been extensively studied as an effective alternative to chemical ensiling [30].

Therefore, investigating the microbial species in silages has become an area of interest quite early on people's field of interest. Many microbes were isolated from silages, such as *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides*, *Pediococcus acidilactici* and *Enterococcus faecalis* [31–33]. In recent years, with the cost reduction of high throughput sequencing and genomics, the use of these technologies has become more common and easier. The time can be largely shortened for detecting and analyzing the overall microbial profile of a sample, and the taxonomic resolution of species is enhanced markedly. By applying the Illumina MiSeq sequencing technology, our study analyzed the bacterial flora of 33 silage samples from North China, and obtained the important microbial community information, that could not be acquired by culture-dependent method, especially for low-abundance (<1%) of microorganisms. In terms of the design of the inoculants, we hypothesized that the quality of the silage mainly depends on the number and proportion of dominant species. Therefore, *Lactobacillus* sp. and *Acetobacter* sp. were of particular concern. Additionally, the application of mixed microbial strains to silage feed has a beneficial impact on its quality and is also a trend. Different types and quantities of microorganisms produce a variety of metabolic products, thereby exhibiting different functions, ultimately leading to the formation of mature silage products. For example, in a wet brewers grains-treated silage of southeast China, the counts of

Table 3. The nutrient content of 2 corn silages.

	Non-inoculated silage (Group A)	Inoculated silage (Group B)
Ammonia nitrogen[1]	0.07 ± 0.01^a	0.03 ± 0.00^b
Water soluble carbohydrates	2.80 ± 0.11^a	2.45 ± 0.07^b
Crude protein	8.12 ± 0.15^a	8.21 ± 0.07^b
Neutral detergent fiber	51.32 ± 0.51	50.60 ± 0.21
Acid detergent fiber	32.56 ± 1.20^a	30.12 ± 0.45^b

1. The unit is %.
2. Within a given row, differing small letter superscripts indicate a significant difference ($p<0.05$) between the values. Identical small letter superscripts indicate no significant difference ($p>0.05$), the same as in Table 4.

Table 4. Statistical changes of pH, lactic acid content and Mold[1].

Group	5d			10d			15d		
	pH	Lactic acid, mg/dl	Mold, $Log_{10(cfu/g)}$	pH	Lactic acid, mg/dl	Mold, $Log_{10(cfu/g)}$	pH	Lactic acid, mg/dl	Mold, $Log_{10(cfu/g)}$
A	4.84	1	5.00 ± 0.00^a	4.24	8	7.75 ± 0.04^a	4.10	10	8.00 ± 0.00^a
B	4.20	12	$<4.00\pm0.02^b$	3.91	28	$<4.00\pm0.05^b$	3.78	32	$<4.00\pm0.01^a$

1. Treatments are as follows:
group A: the corn added with 5ml sterile water per 50g;
group B: the corn added with 5ml fermentation broth (Microbes number=1.0×109CFU) per 50g.

epiphytic lactic acid bacteria and aerobic bacteria and yeasts in raw materials were all below 1×10^6 cfu/g, and they all show varying degrees of increase after the ensiling process [4].

During fermentation, microbes anaerobically produce acids, primarily lactic and acetic, as the main end products of central carbohydrate metabolism, leading to a reduction in the surrounding pH. These anaerobic and acidic conditions inhibit the growth of harmful microorganisms such as *Listeria*, *Clostridia*, yeasts, and fungi [34]. Additionally, there is a significant correlation between silage quality and factors such as NH_3-N, WSC, CP, NDF, ADF, and pH. For instance, lower ADF levels enhance digestion and metabolism in ruminants [35], while maintaining a pH below 4.2 is critical for preventing the proliferation of contaminating microorganisms that could threaten animal health or enter the human food chain [36]. Compared to the control group, the inoculated silage demonstrated improved properties post-fermentation. Specifically, NH_3-N, WSC, and ADF were reduced by 57.1%, 12.5%, and 7.5%, respectively, while CP content increased by 1.1%, resulting in a final pH of 3.64±0.02. Moreover, no significant mold growth was observed in the inoculated silage within 15 days.

In summary, the present findings indicated that the inoculants designed based on dominant strains (*Lactobacillus buchneri* II JC1-5 and *Acetobacter pasteurianus* DHL-8, in a ratio of approximately 4:3; 1.0×10^9 CFU/g) can effectively improve the quality of the silage.

5 Conclusion

This study employed Illumina MiSeq high-throughput sequencing to analyze the microbial diversity and composition of 33 silage samples from North China, with a particular focus on low-abundance microorganisms. The findings indicate a strong correlation between silage quality and the presence and relative abundance of key microbial species. The targeted inoculant design, utilizing *Lactobacillus buchneri* II C1-5 and *Acetobacter pasteurianus* DHL-8 in a ratio of approximately 4:3, significantly enhanced the nutritional profile and fermentation characteristics of the silage. Specifically, the inoculated silage exhibited reductions in NH_3-N, WSC, and ADF by 57.1%, 12.5%, and 7.5%, respectively, while CP increased by 1.1%, resulting in a pH of 3.64±0.02, all of which were

superior to the control group. Furthermore, there was no significant mold growth observed in the inoculated silage within 15 days post-fermentation. These results underscore the importance of optimizing inoculant design and application in improving silage feed quality, providing valuable insights for future silage production practices.

Conflicts of Interest

The authors declare no conflicts of interest.

Funding

This work was supported without any funding.

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