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Effect of *Bacillus* Additives on Fermentation Quality and Bacterial Community during the Ensiling Process of Whole-Plant Corn Silage

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Abstract: The aim of this study was to evaluate the effects of a complex *Bacillus subtilis* additive on the fermentation quality and bacterial community during the ensiling process of whole-plant corn silage (WPCS). The pH values of WPCS treated with the *B. subtilis* inoculant decreased faster than those of the control without inoculant, and significantly higher contents of lactic acid (LA) and acetic acid (AA) were observed. After 45 days of ensiling, the LA contents reached 7.95% (*w*/*w*). In the treatment group, the neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents decreased significantly compared to the control, and the degradation rates of the NDF and ADF were 26.52% and 27.34% after 45 days, respectively. The deoxynivalenol (DON) content in the treatment group decreased to 205.67 μg/kg, which was significantly lower than the content of 382.51 μg/kg in the control group. The results indicated the positive effect of the *B. subtilis* inoculant in improving WPCS fermentation, especially in terms of degrading linocellulose and removing DON. The analysis of the bacterial community indicated that the *B. subtilis* inoculant resulted in an increased abundance of *Lactobacillus*, which contributed to the enhancement of LA production. The increased abundance of *Bacillus* possibly played a role in the degradation of NDF and ADF and the reduction in DON. Therefore, the complex *B. subtilis* additive could be used for the production of high-quality WPCS.

Keywords: *Bacillus* additive; lignocellulose; deoxynivalenol; bacterial community; ensiling process; whole-plant corn silage

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1. Introduction

China's animal husbandry industry has been developing rapidly in recent years, and the scale of animal breeding is expanding [1]. However, the development of animal husbandry and feed production is very unbalanced [2]. The feed planting industry appears to be particularly weak, and natural grassland and cultivated forage can no longer meet the demands of the rapid development of the animal husbandry sector. Thus, whole-plant corn silage (WPCS), which possesses good palatability as well as a high protein and energy content, has been developed as an important source of coarse feed for ruminants [3].

The positive effects of using WPCS for actual ruminant production have been demonstrated previously. As reported earlier, WPCS could effectively improve the milk protein rate and milk yield of dairy cows and the production performance of beef cattle and mutton sheep [4,5]. Nevertheless, the digestibility of WPCS in the rumen of ruminants is usually less than 60% and even lower when the WPCS is harvested late, indicating that it has a low

utilisation rate [6]. The reason for this is that the corn stem and leaves, which account for about 55% of the total dry weight of WPCS, limits the digestion and ingestion of whole biomass. The main component of corn stover is lignocellulose, and its complex structure, which consists of cellulose, hemicellulose, and lignin, resists the WPCS degradation caused by microorganisms, thus affecting nutrient absorption.

On the other hand, during the ensiling process, the mold that is attached to the surface of chopped WPCS produces a variety of mycotoxins [7,8], potentially resulting in acute toxicity, subclinical disease, or immune suppression within livestock following ingestion [9]. While aflatoxin contamination can be effectively controlled by harvest management, deoxynivalenol (DON) is commonly detected in freshly harvested forage maize, incurring moderate to high levels of risk [10,11].

To enhance the ensiling quality of the WPCS, lactic acid bacteria (LAB) are commonly used as silage additives during fermentation to improve the ensiling process [12]. However, to improve the abovementioned problems in the WPCS forage application, LAB are not effective enough. More and more studies have attached great importance to other silage additives, such as *Bacillus subtilis* [13]. *B. subtilis* has the characteristics of strong resistance to adverse environments, and importantly, it can produce a broad spectrum of lignocellulolytic enzymes, thus leading to the degradation of lignocellulose and the enhancement of the nutritional quality of WPCS. *B. subtilis* has also been reported to be capable of degrading mycotoxins [14]. From these two perspectives of degrading lignocellulose and removing mycotoxins during fermentation, we isolated two *B. subtilis* strains from WPCS. *B. subtilis* CGMCC No. 21707 was able to promote the degradation of neutral detergent fibre (NDF) in corn stover, thus regulating the growth of cellulose-degrading bacteria in the rumen of cattle [15]. *B. subtilis* CGMCC No. 21708 was not only able to remove DON, but it was also able to produce fengycin and iturin A, which inhibited mold growth and thus reduced mycotoxin production [16].

In this study, *B. subtilis* CGMCC No. 21707 and *B. subtilis* CGMCC No. 21708 were mixed as a complex *B. subtilis* silage inoculant for WPCS fermentation to degrade the lignocellulose and reduce the mycotoxins content specifically. We hypothesised that the application of the complex *B. subtilis* inoculant could produce dual functions to enhance the silage fermentation quality. It was reported that profiling the microbial community would shed light on the silage ensiling process [17,18]. However, few studies have investigated the effects of *B. subtilis* inoculant on microbial communities during silage fermentation. Thus, to demonstrate the positive effects of the complex *B. subtilis* silage inoculant on WPCS fermentation quality, the lignocellulose degradation, and DON removal in WPCS were investigated during the ensiling process. The bacterial community was also analyzed to provide insight into the improvement of fermentation by the complex inoculant. The results could be used to direct the practical production of high-quality fodder.

2. Materials and Methods

2.1. Inoculant Preparation

The silage inoculant was produced in the Hebei Feed Microorganism Technology Innovation Center, Baoding, China. It was composed of *B. subtilis* CGMCC No. 21707 and *B. subtilis* CGMCC No. 21708 in the ratio of 1:1. Production procedures were performed according to the Technical regulation for the production of agricultural microbial inoculants (NY/T 883-2004) and Feed additives, *Bacillus subtilis* (NY/T 2131-2012). Briefly, 5.0 L of seed culture from each strain was inoculated into 1000 L of medium containing 5.0 kg of peptone, 7.0 kg of bean cake powder, 7.0 kg of corn flour, 7.0 kg of glucose, 0.7 kg of $MnSO_4$, 0.7 kg of K_2HPO_4, and 0.7 kg of $CaCl_2$. The pH of the medium was adjusted to 7.2 via the addition of NaOH prior to use. After culture at 37 °C for 24 h, it was centrifugated and dried into a powder.

2.2. Silage Production

Whole-plant corn (*Zea mays* L. Zhengdan 958) was harvested at half milk-line in the No. 3 experimental farm of Hebei Agricultural University. The dry matter (DM) and deoxynivalenol (DON) contents of the fresh whole-plant corn was 31.90% and 27.17 μg/kg of fresh weight (FW). The neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP), and starch contents in the whole-plant corn were 41.17%, 29.64%, 8.52%, and 35.12% of DM, respectively. The harvested whole-plant corn was collected and transported to the feed processing site of the Hebei province Feed Microorganism Technology Innovation Center and then chopped to be 1–2 cm in size with a forage cutter (Baoding Golden Land Ecological Engineering Co., Ltd., Baoding, China).

The silage inoculant was added at a dosage of 9 $\log_{10}$ CFU/kg (fresh weight) to chopped whole-plant corn, which was used as the treatment group (T). The same amount of distilled water was added to the whole-plant corn and designated as the control group (C). Whole-plant corn was put into 30 cm × 40 cm polyethylene plastic bag, and the bags were pressed and vacuum-sealed. There were 100 silos for each treatment, and 3 silos were opened and sampled after 1, 3, 5, 10, 30, and 45 days of ensiling. The WPCS taken from each silo was divided into two portions. One portion was used to evaluate the quality of the silage by measuring the fermentation parameters. The other portion was stored in a freezer at −80 °C immediately and used for microbial diversity analysis.

2.3. pH and Organic Acid Analyses

For the pH and organic acid analyses, a total of 20 g samples were homogenised in 180 mL of distilled water and stirred for 30 min and then filtered through 4 layers of medical gauze. The pH value of the filtrate was measured using a pH meter (E-201-D, Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China). The lactic acid (LA), acetic acid (AA), and butyric acid (BA) in the filtrate were analysed using a High-Performance Liquid Chromatography machine (Agilent 1260, Agilent Technologies Inc., Palo Alto, CA, USA). Experimental conditions: SB-AQ C_{18} column (4.6 mm × 250 mm); mobile phase A (Methanol): mobile phase B (0.01 mol/L $(NH_4)_2HPO_4$, pH = 2.70) =3:97; flow rate was 1.0 mL/min; injection volume was 20 μL; detection wavelength was 210 nm; and column temperature was 25 °C.

2.4. Nutrient Analyses

The DM contents of the WPCS were calculated based on the fresh weight and dry weight after oven-drying at 65 °C for 72 h. After drying, the samples were ground through a 1 mm screen using a waring blender for nutrient analyses. The nitrogen content was determined using a Kjeldahl apparatus (K9860, Hanon Instruments Co., Ltd., Jinan, China), and the CP was calculated by multiplying the N content by 6.25. The NDF and ADF were determined according to the methods described by Van Soest [19] using a fully automatic fibre meter (A2000i, Ankom Technologies Inc., Macedon, NY, USA). The starch was determined by means of polarimetry (Polax 2 L, Atago®, Tokyo, Japan).

2.5. DON Analyses

The DON content in the WPCS was determined by means of ToxinFast® DON ELISA kit (Meizheng Bio-Tech Company, Beijing, China). A total of 5.0 g comminuted samples (particle size < 1 mm) was homogenised in 25 mL of 60% methanol solution, blendedfor 5 min, and then centrifuged for 5 min at 4000 r/min. A quantity of 1 mL supernatant extract was added into 4 mL deionized water and vortexed for 5 s to mix well. Then, a 50 μL sample was taken for the DON quantitative analysis according to the manufacturer's instructions.

2.6. Bacteria Community Analyses

Silage samples amounting to 10.0 g were put into sterilised eluent (0.9% NaCl + 0.1% Tween-80) and blended at 100 r/min for 2 h, and then 4 layers of gauze were used to filter

the supernatant. The precipitate was obtained by centrifugating at 5000 r/min for 15 min. The total genome DNA of the microbes was extracted from the precipitate by using a DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). The quality and concentration of the DNA were detected using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The bacterial 16S rRNA genes were amplified via PCR using the primers 340F (5′-CCTACGGGNBGCASCAG-3′) and 805R (5′-GACTACNVGGGTAT CTAATCC-3′). The bacterial 16S amplicon was sequenced with the Hiseq2500 (Illumina, San Diego, CA, USA) according to the description of Guo et al. [15]. The quality-filter and cluster analyses were performed. The 16S rRNA sequencing data were analysed using the online Majorbio Cloud Platform (www.majorbio.com (accessed on 6 March 2020)).

All sequences were homologously clustered into operational taxonomic units (OTUs) based on 97% sequence similarity by using the QIIME UCluster method. To determine the species classification information corresponding to each OTU, the RDP classifier Bayesian algorithm was used for the taxonomic analysis of representative OTU sequences with 97% similarity, and they were then compared to the 16S rRNA database. The community species composition of each sample was measured at the domain, kingdom, phylum, class, order, family, and genus levels. The microbial diversity in the silage was analysed according to the α diversity index, including the Shannon index, the Chao1 index, and the community Coverage index. Principal component analysis (PCA) was performed on each group of samples to study the similarities and differences in the community composition. The linear discriminant analysis effect size (LEfSe) was used to compare the two groups at the genus level at days 10, 30, and 45.

2.7. Statistical Analyses

The experimental protocol had a 2 × 6 factorial design with 2 groups and 6 ensiling times. The data associated with fermentation quality was analysed using the general linear model procedure of SPSS 20.0. Tukey's test was also used for pair-wise mean comparisons. Student's T-test was used to analyse the relative abundance of *Bacillus*. Significance was considered at $p < 0.05$.

3. Results

3.1. Dynamic Changes in pH and Organic Acids during the Ensiling Process

The pH values of WPCS in both the control and treatment groups decreased rapidly during the first 5 days of ensiling and then remained stable during the following ensiling time (Table 1). After 5 days, the pH of the treatment group decreased to 3.7, which was significantly lower than the pH of the control group. It took 3 days for the pH to decrease below to 4.0, and that was 2 days faster than the control group. Interestingly, there were no notable differences in the pH values between the treatment and control groups from 10 days to 45 days.

Table 1. Dynamic changes in the pH and organic acids in the two groups during the ensiling process.

Groups	Days	pH Values	LA (%)	AA (%)	BA (%)
C	1	5.64 ^{a}	1.95 ^{a}	0.32 ^{a}	-
	3	4.25 ^{c}	2.62 ^{b}	0.35 ^{a}	-
	5	3.95 ^{de}	2.79 ^{b}	0.32 ^{a}	-
	10	3.83 ^{fg}	3.11 ^{c}	0.38 ^{a}	-
	30	3.88 ^{def}	3.89 ^{d}	0.43 ^{a}	-
	45	3.85 ^{ef}	3.82 ^{d}	0.44 ^{a}	-
T	1	4.55 ^{b}	2.66 ^{b}	0.75 ^{b}	-
	3	3.97 ^{d}	5.02 ^{e}	1.21 ^{c}	-
	5	3.74 ^{g}	5.78 ^{f}	1.40 ^{d}	-
	10	3.79 ^{fg}	6.32 ^{g}	1.45 ^{d}	-
	30	3.81 ^{fg}	7.88 ^{h}	1.92 ^{e}	-
	45	3.80 ^{fg}	7.95 ^{h}	1.98 ^{e}	-
SEM		0.072	0.268	0.078	-
p-Value					
G		<0.01	<0.01	<0.01	
D		<0.01	<0.01	<0.01	
G × D		<0.01	<0.01	<0.01	

C, control group, WPCS without inoculant; T, treatment group, WPCS treated with *B subtilis* inoculant; LA: lactic acid; AA: acetic acid; BA: butyric acid. SEM, standard error of the means. "-", undetected; G, inoculant group; D, ensiling time; G × D, the interaction between inoculant group and ensiling time. Values with different lowercase ($^{a–h}$) letters indicate significant differences ($p < 0.05$).

The LA and AA contents of both the control and treatment groups substantially increased after ensiling for 3 days, which probably resulted in the rapidly decrease of pH values (Table 1). After 3 days, the LA and AA contents increased steadily. During the entire ensiling process, the LA and AA contents in the treatment group were significantly higher than that in the control group. The LA content reached the maximum of 7.95% (*w*/*w*), and the AA content reached 1.98% (*w*/*w*) after 45 days, respectively. There was no accumulation of BA during the entire ensiling process in the two groups.

3.2. Dynamic Changes in Chemical Compositions during the Ensiling Process

The NDF and ADF contents in both the control and treatment groups decreased throughout the entire ensiling process (Table 2), and the decrease was greater in the treatment group, reaching significant levels between 3 days and 45 days. After 45 days of ensiling, the NDF and ADF degradation rates in the treatment group were 26.52% and 27.34%, respectively. The degradation rates in the control group were only 15.18% and 9.31%, respectively. The results suggest that the *B. subtilis* inoculant could effectively degrade the lignocellulose in WPCS. There were no distinct differences in the CP and starch contents between the two groups, indicating that the *B. subtilis* inoculant did not lead to the loss of nutrient components during the ensiling process.

Table 2. Dynamic changes in the neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP), and starch contents in the two groups during the ensiling process.

Groups	Days	NDF (%)	ADF (%)	CP (%)	Starch (%)
C	1	40.51 ^{a}	29.21 ^{a}	8.88	35.62
	3	38.83 ^{b}	28.53 ^{ab}	8.72	34.49
	5	37.23 ^{cd}	28.44 ^{b}	8.57	35.12
	10	36.28 ^{de}	27.92 ^{b}	8.35	35.54
	30	35.21 ^{ef}	27.22 ^{c}	8.35	35.26
	45	34.92 ^{f}	26.88 ^{c}	8.05	35.89
T	1	39.52 ^{ab}	28.24 ^{b}	8.88	35.15
	3	37.55 ^{c}	26.52 ^{c}	8.95	36.25
	5	35.62 ^{ef}	24.98 ^{d}	8.91	35.25
	10	32.54 ^{g}	22.24 ^{e}	8.98	35.99
	30	31.56 ^{h}	21.93 ^{f}	9.01	35.62
	45	30.25 ^{i}	21.55 ^{f}	9.00	36.20
SEM		0.403	0.330	0.115	0.518
p-Value					
G		<0.01	<0.01	0.204	0.208
D		<0.01	<0.01	0.085	0.105
G × D		<0.01	<0.01	0.125	0.226

C, control group, WPCS without inoculum; T, treatment group, WPCS treated with *B. subtilis* inoculant; SEM, standard error of the mean; G, inoculant group; D, ensiling time; G × D, the interaction between the inoculant group and the ensiling time. Values with different lowercase ($^{a-i}$) letters indicate significant differences ($p < 0.05$).

3.3. Dynamic Changes in DON Content during the Ensiling Process

The DON content in both the control and treatment groups increased rapidly between days 1 and 10, and then began to decrease until plateauing after 30 days (Table 3). Significantly lower DON contents were observed in the treatment group compared to the control after 1 day of ensiling. The highest DON content was 499.42 μg/kg in the control group after 10 days, which was 200 μg/kg higher than it was in the treatment group ($p < 0.01$). After 45 days, the DON content in the treatment group was only 205.67 μg/kg, indicating DON was efficiently removed by the *B. subtilis* inoculant during the ensiling process.

Table 3. Dynamic changes in the deoxynivalenol (DON) content in the two groups during the ensiling process.

Groups	Days	DON (μg/kg)
C	1	32.67 ^{a}
	3	150.11 ^{c}
	5	401.32 ^{fg}
	10	499.42 ^{h}
	30	415.32 ^{g}
	45	382.51 ^{f}
T	1	42.52 ^{a}
	3	102.42 ^{b}
	5	289.56 ^{e}
	10	298.93 ^{e}
	30	191.31 ^{d}
	45	205.67 ^{d}
SEM		19.94
p-Value		
G		<0.001
D		<0.001
G × D		<0.001

C, control group, WPCS without inoculum; T, treatment group, WPCS treated with *B. subtilis* inoculant; SEM, standard error of the mean; G, inoculant group; D, ensiling time; G × D, the interaction between inoculant group and ensiling time. Values with different lowercase ($^{a-h}$) letters indicate significant differences ($p < 0.05$).

3.4. Dynamic Changes in Bacterial Communities during the Ensiling Process

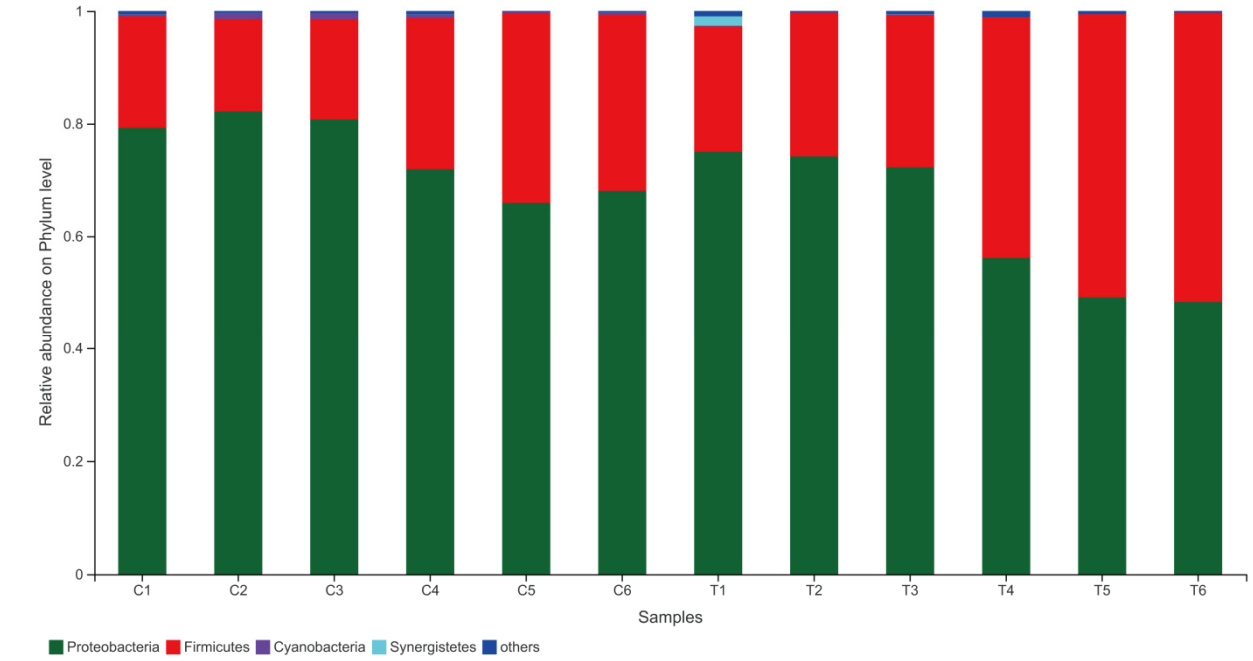
To gain insight into the WPCS ensiling process, the dynamic changes in the bacterial communities in the WPCS were analysed. The alpha diversity of the bacteria during the ensiling process is shown in Table 4. The *B. subtilis* inoculant significantly affected the abundance and richness of the bacterial community in the WPCS. According to the Chao1 index, the abundance of the bacterial community in the treatment group was lower than that of the control group during the entire ensiling process, apart from at 5 days. According to the Shannon index, the diversity of the bacterial community was higher in the treatment group compared to the control group after 1 day of ensiling, while it was higher in the control group after 10, 30, and 45 days.

Table 4. Alpha diversity of bacterial diversity in two groups.

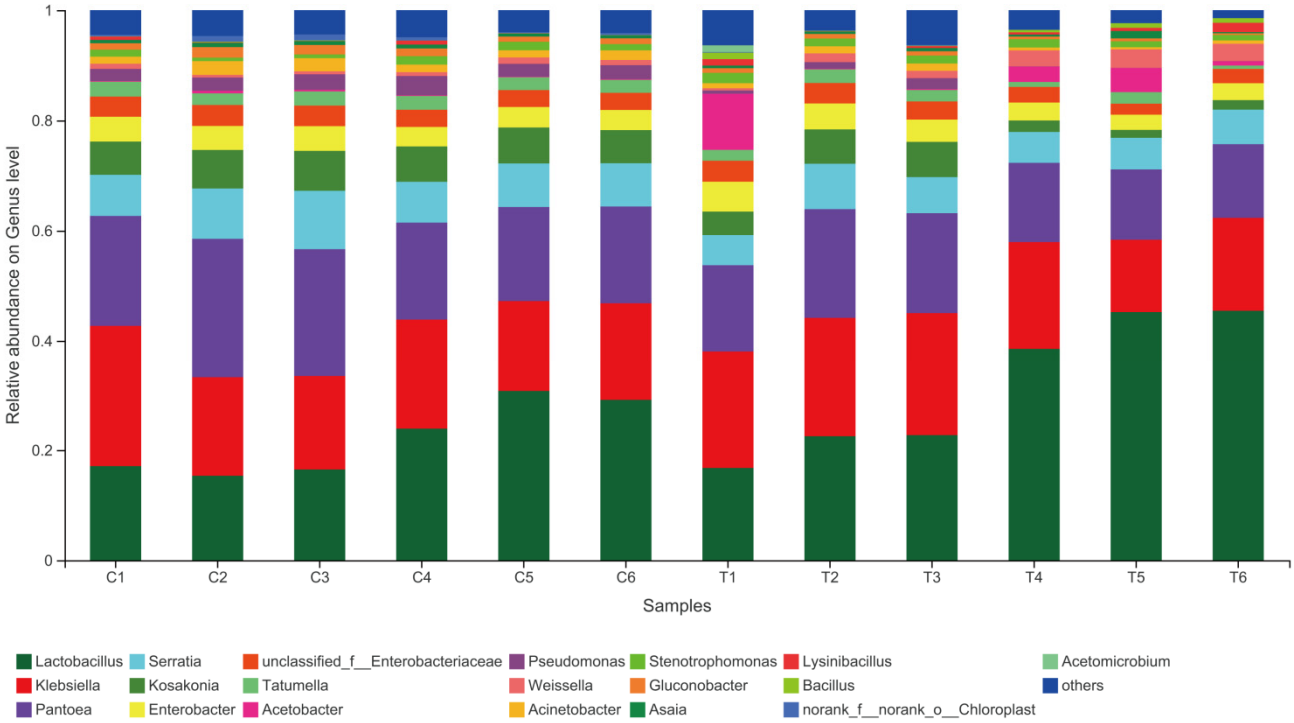
Groups	Days	Reads	OTU	Shannon	Chao1	Coverage (%)
C	1	62,067.33	898	3.04	758.67	99.65
	3	61,457.67	705	3.09	632.37	99.72
	5	63,284.33	678	3.08	613.16	99.73
	10	61,296.67	903	3.11	707.33	99.68
	30	64,128.33	526	2.93	467.68	99.82
	45	56,734.67	702	2.99	570.96	99.74
T	1	59,743.00	848	3.25	640.05	99.71
	3	63,531.67	624	2.94	518.76	99.79
	5	63,846.33	834	3.08	617.08	99.77
	10	69,875.00	648	2.73	533.82	99.82
	30	72,961.67	559	2.63	459.89	99.86
	45	68,830.33	479	2.60	453.64	99.84

C, control group, WPCS without inoculum; T, treatment group, WPCS treated with *B. subtilis* inoculant.

The relative abundance of bacteria during the WPCS ensiling process was observed (Figure 1). At the phylum level, the dominant phyla in the control and treatment groups were Firmicutes and Proteobacteria, which accounted for more than 97% of all the microorganisms (Figure 1A). Among them, Proteobacteria was the most predominant phylum, the percentage of which decreased in both groups as the ensiling time continued, and the proportion of Firmicutes increased. In the control group, the abundance of Proteobacteria decreased from 79.23% to 68.06% after 45 days, and in the treatment group, it decreased from 74.97% to 48.31%. Correspondingly, the abundance of Firmicutes in the treatment group was 51.41% after 45 days, which was significantly higher than that in the control group. Additionally, Cyanobacteria and Synergistetes were also annotated, but their abundance was less than 1%, and no significant differences were observed between the control and treatment groups.



(**A**)



(**B**)

Figure 1. Relative bacteria abundance in two groups at phylum (**A**) and genus (**B**) levels: C, control group, WPCS without inoculant; T, treatment group, WPCS treated with *B. subtilis* inoculant. The numbers 1, 2, 3, 4, 5, and 6 represent days 1, 3, 5, 10, 30, and 45 of the ensiling process.

The compositions of the bacterial communities in the WPCS at the genus level are shown in Figure 1B. The results indicated that the main bacteria in the communities were *Lactobacillus*, *Klebsiella, and Pantoea*. During the first 5 days of ensiling in the control group, the abundance of *Klebsiella* decreased, and *Pantoea* increased to 23.19%. After 5 days,

Lactobacillus increased, and its abundance was 29.11% after 45 days. However, in the treatment group, the dominant genus changed from *Klebsiella*, which had an abundance of 21.33%, to *Lactobacillus* as the ensiling time prolonged. The abundance of *Lactobacillus* reached 45.46% after 45 days.

The relative abundance of *Bacillus* was very low in all the silage samples; therefore, we conducted a statistical analysis on the relative *Bacillus* abundance (Table 5). A higher relative abundance of *Bacillus* was observed in the treatment group than in the control group.

Table 5. Relative abundance of *Bacillus* during the ensiling process.

Days	Relative Abundance (%)		SEM	*p*-Value
	C	T		
1	0.049	1.088	0.254	<0.001
3	0.052	1.111	0.202	<0.001
5	0.043	1.003	0.215	<0.001
10	0.052	1.251	0.203	<0.001
30	0.053	1.439	0.185	<0.001
45	0.054	1.433	0.198	<0.001

C, control group, WPCS without inoculant; T, treatment group, WPCS treated with *B. subtilis* inoculant.

The beta diversity was analysed using principal component analysis (PCA), and significant differences in the bacterial compositions in the WPCS were observed at different ensiling times (Figure 2). The microbial samples from the two groups were mainly divided into two clusters along the direction of PC1. The samples taken at 10, 30, and 45 days in the treatment group gathered in a cluster, and the samples taken at 1, 3, and 5 days in the treatment group, and all the control samples gathered in another cluster. The separation of the bacterial composition after 10 days in the treatment group indicated that there was a large dynamic migration in the bacterial communities that was likely the result of the effects of the *B. subtilis* inoculant. This conclusion was determined because the changes in the control group were not obviously observable.

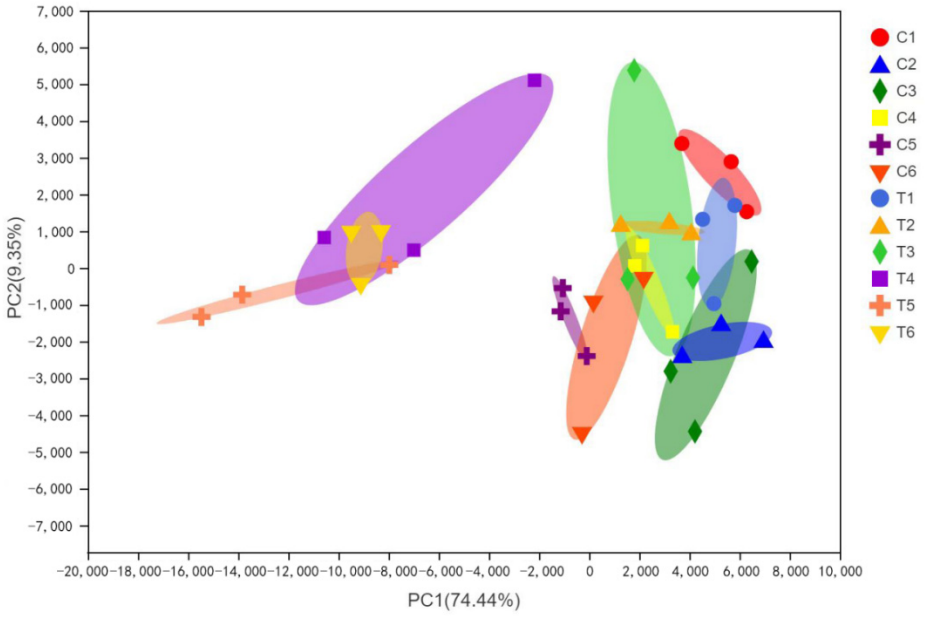
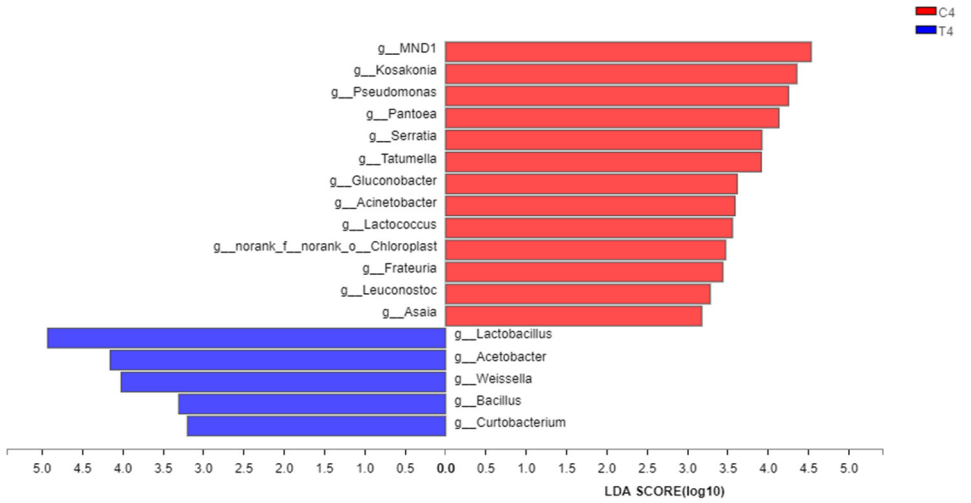
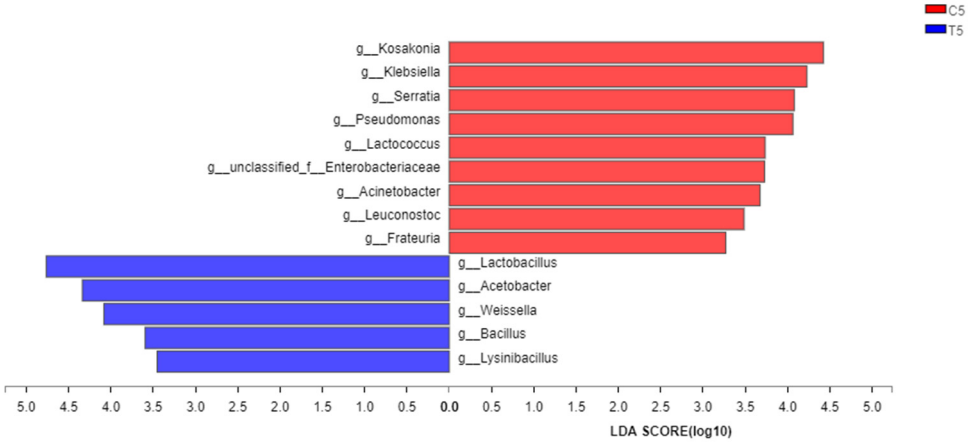


Figure 2. Principal component analysis (PCA) of bacterial communities in two groups during ensiling (n = 3): C, WPCS without inoculant; T, WPCS treated with *B. Subtilis* inoculant. The numbers 1, 2, 3, 4, 5, and 6 represent the day 1, 3, 5, 10, 30, and 45 of ensilage time.

Due to the clear separation of the bacterial composition between the control and treatment groups, we investigated the differences in the bacterial communities between the two groups after 10, 30, and 45 days of ensiling via linear discriminant analysis effect size (LEfSe) at the genus level. After 10 days, *Kosakonia*, *Pseudomonas* and *Farnish* were enriched in the control group, while *Lactobacillus*, *Acetobacter*, *Weissella*, *Bacillus,* and *Curtobacterium* were more abundant in the treatment group (Figure 3A). After 30 days, *Kosakonia*, *Klebsiella*, *Serratia*, *Pseudomonas*, and *lactococcus* were enriched in the control group, and *Lactobacillus*, *Acetobacter*, *Weissella*, *Bacillus,* and *Lysinibacillus* were more enriched in the treatment group (Figure 3B). After 45 days, *Mycobacterium*, *Kosakonia,* and *Pseudomonas* were more abundant in the control group, while *Lactobacillus*, *Weissella*, *Lysinibacillus*, *Bacillus,* and *Acetobacter* were more abundant in the treatment group (Figure 3C).

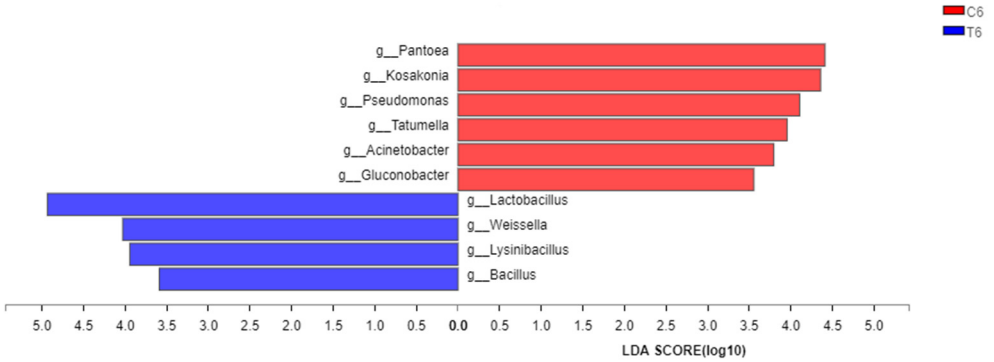


(**A**)



(**B**)

Figure 3. *Cont.*



(**C**)

Figure 3. Comparison of genus differences in the bacterial communities between the two groups on Days 10 (**A**), 30 (**B**), and 45 (**C**) of ensiling: C, whole-plant corn silage without inoculum; T, whole-plant corn silage treated with *Bacillus subtilis*. The numbers 4, 5, and 6 represent 10, 30, and 45 days of ensilage time.

4. Discussion

Silage additives are usually used to ensure that *Lactobacillus* is the dominant community as quickly as possible during the ensiling process or to prolong the secondary fermentation time and improve silage quality [12]. However, silage quality is still limited by lignocellulose and mycotoxin. In this study, *B. subtilis* inoculant was applied as the additive for the WPCS ensiling to reduce the contents of lignocellulose, which can improve the utilization of the WPCS, thus reducing the corn stover burning as waste. Meanwhile, the reduction of DON by application of the inoculant can protect the health of livestock, thus enhancing their production performance. These are beneficial for the ecological environment and husbandry economy.

The LA content increased significantly in the treatment group compared to the control group during the ensiling period. As reported earlier, the *B. subtilis* inoculant can consume the remaining oxygen during the early stages of ensiling, thus creating an anaerobic environment, which is conducive to the fermentation of the LA-producing bacteria that are attached to the plant surface [20]. The AA content also increased, which was probably due to the degradation of the lignocellulose in the WPCS into pentoses, leading to an increase in the AA content of the silage [21].

The pH values in both the control and treatment groups decreased with the prolonged ensiling time due to LA and AA accumulation. This was in agreement with the study of Bai et al. [20]. The pH values decreased faster than those of the control, and this was in agreement with the change contents in the acid content between these two groups. However, after 45 days of ensiling, the pH values in these two groups were similar, which is consistent with the results of Oliveira et al.'s meta-analysis [22]. It has been reported that microbial inoculum either increases or does not affect silage pH [23,24]. However, the results of the present study suggest that the application of the *B. subtilis* inoculant could rapidly decrease the pH during the initial stages of ensiling at the very least, which can inhibit the growth of undesirable microbes, thus reducing nutrient loss [25,26].

The NDF and ADF content in both the control and treatment groups decreased with the prolonged ensiling time, which was probably the result of the hydrolysis of the digestible cell wall fraction by the organic acids produced during ensiling [27]. The fungi- and LA-producing bacteria could also have contributed to the degradation of NDF and ADF to a certain extent [12,28]. Significantly lower NDF and ADF contents were observed in the treatment group, and this was consistent with our previous study, which indicated that *B. subtilis* inoculation decreased the NDF and ADF contents in fermented corn stover [15]. Bai et al. [20,29] also reported that *B. subtilis* inoculation decreased the NDF and ADF contents in both WPCS and alfalfa silage. The increased accumulation of organic acids

possibly contributed to this. However, on the other hand, *B. subtilis* had the ability to degrade lignocellulose, something that has been widely reported. As described earlier, *B. subtilis* can produce cellulase, hemicellulase, or other enzymes such as feruloyl esterase to hydrolyse the structural carbohydrates in plants during silage fermentation [30]. In this study, *B. subtilis* CGMCC No. 21707 was screened from the feces of *Protaeti brevitarsis*. It was acknowledged that *P. brevitarsis* especially likes to eat fresh straw. Many cellulose degradation enzymes were observed in the hindgut of *P. brevitarsis*, which was determined based on the analysis of bacterial communities [31].

The DON content increased gradually in the early stages of silage with the extension of the silage time and reached the maximum at 10 days and then gradually decreased. It has been reported that the mycotoxin content changed unsteadily during the ensiling and may increase, decrease, or remain the same [8]. However, the overall DON levels in the treatment group were lower than they were in the control group. The low pH during the early stages of the ensiling process could inhibit the growth of mold, thus reducing mycotoxin formation. Several studies have shown that mycotoxin production is reduced due to the inhibition of the growth of molds, which was probably caused by a low pH, nutrient depletion, and microbial competition [32,33].

In addition to the effect of pH, *B. subtilis* CGMCC No. 21708 could not only degrade DON, it could also inhibit the growth of *Fusarium graminearum*, which may play a double role in reducing DON. In recent years, there has been more and more reports on mycotoxin degradation by *Bacillus* [34,35]. However, studies on the effectiveness of these microorganisms in actual environments are limited, especially in the ensiling process, and some strains may even have potentially negative effects on the ensiling process or on pathogenicity [36,37]. In this study, the *B. subtilis* inoculant had a positive effect on the reduction in the DON content as opposed to a negative effect, suggesting its potential application in practical silage production.

The diversity and richness of bacteria during the ensiling process decreased in both groups, but the degree of the decrease was higher in the treatment group. As reported by Eikmeyer et al. [38], a decrease in bacterial diversity was observed in the LAB-inoculated silage during the ensiling process by high throughput sequencing. Similarly, Bai et al. [20,29] revealed that silage inoculated with *B. subtilis* decreased the bacterial diversity in alfalfa silage and WPCS. This was probably due to the low pH during the early ensiling period inhibiting the growth of undesirable microorganisms and consequently decreasing the bacterial diversity. It was also possibly due to the inhibition caused by antibacterial substances produced by *B. subtilis* CGMCC No. 21708.

Gradually, Firmicutes became the most predominant phylum as the silage process progressed, which is consistent with the studies of Bai et al. [20] and Yang et al. [39]. The relative abundance of Firmicutes in the treatment group was greater than that in the control group at every point, indicating the dominant growth of Firmicutes, as seen in the growth of the genera *Lactobacillus*, *Weissella*, and *Bacillus*. Similar results were previously reported by Keshri et al. [40].

Lactobacillus and *Weissella* commonly play an important role in LA accumulation and pH decline, improving the ensiling process [41,42]. Guan et al. [43] reported that *Lactobacillus* dominated in the early stages of the corn ensiling process without silage inoculants. In our study, the relative abundances of *Lactobacillus* increased gradually and became the predominant genus in both groups, especially from 10 to 45 days of ensiling. This is probably the reason for the increase in the LA content and decrease in the pH values. Meanwhile, a higher relative abundance of *Bacillus* was observed in the treatment group than in the control group, indicating *Bacillus* growth during the ensiling process. Considering the effect of *Bacillus* on lignocellulose degradation and DON removal, the increased abundance of *Bacillus* probably played a role in improving the silage quality from these two perspectives.

5. Conclusions

The application of a complex *B. subtilis* inoculant during WPCS ensiling increased the lactic acid and acetic acid contents and further decreased the pH values. Importantly, the *B. subtilis* inoculant played a role in lignocellulose degradation and DON removal in WPCS since the NDF and ADF contents significantly decreased, and the DON content was reduced. The analysis of the bacterial community indicated that the complex *B. subtilis* inoculant resulted in the increased abundance of *Lactobacillus* and *Bacillus* during the ensiling process, which contributed to enhanced acid production, NDF and ADF degradation, and DON removal. The results suggest that the complex *B. subtilis* inoculant could improve the ensiling quality of WPCS and could potentially be applied for the production of high-nutrient feed.

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