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The Effect of *Lactiplantibacillus plantarum* ZZU203, Cellulase-Producing *Bacillus methylotrophicus*, and Their Combinations on Alfalfa Silage Quality and Bacterial Community

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Abstract: This study assessed the effects of *Lactiplantibacillus plantarum* (ZZU203), cellulase-producing *Bacillus methylotrophicus* (CB), or their combination (ZZU203_CB) on the fermentation parameters of alfalfa after 10 and 60 days of ensiling. Additionally, the bacterial community compositions were analyzed using absolute quantification 16S-seq (AQS). The results showed that CB silage displayed a higher lactic acid (LA) concentration at 10 d, a higher abundance of *Lactobacillus*, and lower abundance of *Pediococcus*, *Enterococcus*, and *Weissella* than those in the control (CK) silage. Compared with CK silage, the ZZU203 silage increased LA concentration, fructose and rhamnose concentrations, and the abundance of *Lactobacillus*, and decreased pH value, ammoniacal nitrogen, acetic acid, neutral detergent fiber and acid detergent fiber concentrations, and the abundance of *Pediococcus*, *Enterococcus*, *Weissella*, *Hafnia*, and *Garciella* after 60 days of ensiling. In addition, ZZU203 and ZZU203_CB silage had a similar silage quality and bacterial community, while the inoculation of ZZU203_CB significantly promoted LA accumulation and the numbers of *Lactobacillus* at 10 d compared with ZZU203 silage. Therefore, ZZU203 or a combination of ZZU203 and CB can be used as potential silage additives to improve the silage quality of alfalfa.

Keywords: alfalfa silage; absolute quantification 16S-seq; fermentation quality; *Lactiplantibacillus plantarum*; cellulase-producing *Bacillus methylotrophicus*

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1. Introduction

Ensiling is an important technique for conserving crops in the livestock industry. With the rapid development of animal husbandry, silage production has been receiving increasing attention. The fermentation quality of silage, as a major source of roughage in ruminant feed worldwide, may directly affect the growth and production of ruminants and create economic benefits for farmers. Alfalfa is an excellent health feed with high protein content, a rich microbial and mineral content, and amino acid balance. To promote the revitalization of alfalfa production in China, alfalfa has become an important legume that is extensively cultivated. In the main alfalfa producing areas, due to the same period of rain and heat, alfalfa is vulnerable to the loss of rain and fallen leaves in the process of preparing alfalfa hay in the harvest season, so it is difficult to make high-quality hay. Therefore, ensiling alfalfa has become an ideal way to solve the above problems. Unlike grasses, alfalfa is hard to ensile due to its low water-soluble carbohydrate (WSC) content and high buffering capacity [1]. To accelerate the progress of lactic acid (LA) production and pH reduction during fermentation, additives are often added before alfalfa ensiling [2–4]. Cellulolytic

enzymes have been shown to be efficient fermentation activators by improving fiber degradation, increasing the WSC content, and achieving lactic-type fermentation [2,5,6]. However, the use of commercial cellulases increases the cost of ensiling, and the instability of enzymes also limits their application in silage [6].

Bacterial additives used in silages are known as inoculants and mainly include lactic acid bacteria (LAB), which are primarily responsible for silage preservation and are therefore the most widely studied [7,8]. Cao et al. [9] reported the effect of the inoculation of *Lactiplantibacillus plantarum* PS-8 on alfalfa silage fermentation, and found that PS-8 can improve silage quality by accelerating acidification; reducing the number of *Clostridium*, *Escherichia coli*, mold, and yeast; and increasing the content of protein and organic acid (except butyrate). Ce et al. [10] evaluated the effects of three inoculants of LAB (*Lactiplantibacillus casei*, *Lactiplantibacillus plantarum* and *Pelococcus pentosanus*) on the quality of silage, rumen in situ degradation, and in vitro fermentation of alfalfa silage, and found that the inoculants of LAB could improve the quality of silage and rumen in situ degradation. Recently, there have also been studies aimed at evaluating the potential of different kinds of non-LAB microorganisms as additives to improve the quality of silage [11,12]. Bai assessed the effects of antibacterial peptide-producing *Bacillus subtilis* (BS), *Lactiplantibacillus buchneri*, or their combination on alfalfa silage, and found that BS improved the fermentation and aerobic stability of alfalfa silage, and it also reduced proteolysis and dry matter (DM) loss [13]. Khota and Li believed that *Bacillus subtilis* could produce cellulase, increase the release of soluble sugar by hydrolyzing the plant cell wall, and improve the yield of LA, thus improving the nutritional quality of silage [14,15]. However, the dynamic changes and potential roles of these aerobic bacteria under anaerobic environmental conditions in the process of silage fermentation are rarely reported. In addition, our previous research, which aimed at understanding the characteristics of raw materials with a low WSC content of alfalfa, screened LAB ZZU203, which has excellent acid production performance, by using structural sugar and its metabolites and alfalfa powder, which can express cellulose-degradation-related enzyme genes in MRS and MRS-CMC media, and has a certain improvement effect on the quality of alfalfa silage [16]. Therefore, it is necessary to explore whether adding LAB and *Bacillus* with a fiber degradation function together can further improve the fermentation quality of alfalfa silage compared with adding LAB alone.

Silage is a complex activity involving microbial flora, such as LAB, spoilage bacteria, yeasts, molds, and *Bacillus*. The composition of a microbial community can directly affect the silage quality, and even further affect the rumen microbiota of ruminants [17]. There is an interaction effect between different microbial additives on the bacterial community structure of alfalfa silage, which is of great significance for the development of alfalfa silage additives. In the past decade, high-throughput sequencing has greatly expanded our understanding of bacterial communities in alfalfa. Eikmeyer and others first used NGS to study the bacterial population in silage, and compared the uninoculated silage with the silage inoculated with LAB, and observed the decrease in bacterial diversity in the silage process [18]. Guo used NGS technology to evaluate the effect of LAB isolated from rumen fluid and the feces of dairy cows on the microbial flora of alfalfa silage after 30 or 60 days of ensiling, and found that the abundance of *Lactobacillus* in silage treated with *Lactiplantibacillus plantarum* F1 increased [19]. Although this method is valuable for determining the relative abundance of different microbial groups in samples, it cannot accurately provide information on the abundance differences of target microorganisms between different samples. Recently, researchers began to use the absolute quantitative 16 S rRNA sequencing method to solve the technical challenges in this field. However, apart from the fact that Yang et al. [20] of our research group reported using absolute quantitative 16 S rRNA sequencing to evaluate the composition and dynamics of bacterial communities in alfalfa silage inoculated with and without *Lactiplantibacillus plantarum*, there are few studies on this technology to explore alfalfa silage bacterial communities.

Therefore, the main objective of this study was to evaluate effects of LAB, cellulase-producing *Bacillus*, and their combinations, on the fermentation parameters, sugar profile, lignocellulose degradation, and bacterial community of alfalfa silage.

2. Materials and Methods

2.1. Silage Preparation

The first-cut alfalfa at the early bloom stage was manually harvested from Zhengzhou, Henan Province, China (temperate monsoon climate, 34.76° N, 113.65° E, altitude 110.4 m above sea level). The harvested forage was chopped into 1–2 cm pieces with a paper cutter (deli8015, Deli group Co., Ltd., Ningbo, China) in the laboratory within 40 min. Before ensiling, fresh forage was sampled before freezing at −80 °C for later analysis. The chopped alfalfa was treated with the following: (1) The group was treated with an equal volume of distilled water without inoculants (CK); (2) *Lactiplantibacillus plantarum* was applied in this group at a rate of 1×10^6 cfu g^{-1} of fresh weight (ZZU203); (3) *Bacillus methylotrophicus* was applied in this group at a rate of 1×10^6 cfu g^{-1} of fresh weight (CB); (4) ZZU203 combined with CB was applied in this group at a rate of 1×10^6 cfu g^{-1} of fresh weight (ZZU203_CB). Here, the CB strain could produce cellulase. To accurately trace the silage parameters such as degradation of the organic acids before and after fermentation, laboratory vacuum-packed mini silos have been frequently used for alfalfa silage [3,21,22]. Approximately 500 g of each of three replicates of chopped alfalfa were packed into polyethylene plastic bags (dimensions: 200 mm × 300 mm; Dongda, Zhengzhou, China), vacuumed, and sealed with a vacuum sealer (P-290, Shineye, Dongguan, China). The samples were ensiled for 10 and 60 d at room temperature (25 °C).

2.2. Analysis of Fermentation Parameters and Chemical Composition

Immediately after the bags were opened, the subsamples (10 g) were blended with 90 mL of sterilized water. The pH was measured with an electrode pH meter (Mettler Toledo Co., Ltd., Greifensee, Switzerland). The content of ammoniacal nitrogen (NH_3-N) in each silage sample was determined using the phenol-sodium hypochlorite colorimetry of Broderick, whose principle is to react NH_4^+ in the extraction solution with hypochlorite and phenol in a strong alkaline medium to produce a water-soluble indigo blue dye, whose color is in direct proportion to the NH_4^+ content in the solution [23]. Organic acid contents, which include LA, acetic acid (AA), propionic acid (PA), and butyric acid (BA), were quantified using high-performance liquid chromatography (HPLC, Waters Inc., Milford, MA, USA) with a UV detector (Waters Inc., Milford, MA, USA). The samples were filtered through the filter paper (Whatman No. 6) and the filtrate was refiltered using a 0.45 μm syringe filter before injection into HPLC. Organic acids were separated in a Carbomix H-NP 10:8% column (7.8 × 300 mm × 10 μm, Sepax Technologies, Inc., Santa Clara, CA, USA). The following conditions were used for HPLC: the mobile phase was 0.0254% H_2SO_4 under isocratic elution, with a flow rate of 0.6 mL min^{-1} and column temperature of 55 °C, and detected at 214 nm. [16]. The DM weights of the fresh alfalfa and silage materials were determined following oven drying at 65 °C for 48 h [24]. The oven-dried samples were then milled through a 1.0 mm sieve prior to further chemical analyses. The WSC was determined by anthrone colorimetry [25]. The neutral detergent fiber (NDF, using heat-stable α-amylase) and acid detergent fiber (ADF) concentrations were determined according to the method of Van Soest et al., using an Ankom A2000 I fiber analyzer (Ankom Technology, Fairport, NY, USA); The specific method is that the silage is boiled with neutral detergent, the insoluble residue is NDF, the silage is treated with acid detergent, and the remaining residue is ADF [26]. Nonstructural carbohydrate contents, which include glucose, fructose, galactose, and rhamnose, were measured using high-performance ion chromatography with a Dionex ICS3000 system (HPIC, Thermo Fisher Scientific, Wilmington, DE, USA) with an amperometric detector and conductivity detector; nonstructural carbohydrates were separated in CarboPac PA10 pellicular anion-exchange resin column (250 × 4 mm), CarboPac PA10 guard column (50 × 4 mm), AS11

analytical column (250 mm× 4 mm) and AG11 guard column (50 × 4 mm). The following conditions were used for HPIC: the mobile phase was 25 mM NaOH, with a flow rate of 1.0 mL min^{-1} [27].

2.3. Bacterial Community Analyses

Subsamples (10 g) of each fresh or silage sample were shaken well with 90 mL of sterile phosphate-buffered saline at 180 rpm for 1 h. The solution was filtered through four layers of medical gauze and the filtrates were centrifuged at 8000× *g* for 15 min at 4 °C to collect the microbial pellet for DNA extraction [28]. Total DNA was extracted using a Bacterial DNA Kit D3350-02 (Omega Biotek, Norcross, GA, USA). After DNA extraction, DNA concentration and quality was evaluated using 1% agarose gel electrophoresis and a Nano Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The PCR amplifications of the V3–V4 regions of the bacterial 16 SrDNA gene were performed using Primer F (Illumina adapter sequence 1+ CCTACGGGNGGCWGCAG) and Primer R (Illumina adapter sequence 2+ GACTACHVGGGTATCTAATCC) [29]. The PCR products were extracted from a 2% agarose gel and purified using AgencourtAMpure XP nucleic acid purification magnetic beads to obtain an original library of samples. The general procedure of absolute quantification 16S-seq (AQS) was outlined by Smets et al. [30] and Tkacz et al. [31]. Briefly, synthetic chimeric DNA spikes were designed with variable regions lacking identity to nucleotide sequences deposited in public databases. This allows the robust tracing of spike-in reads in 16S-seq data from any microbial samples. With known amounts of synthetic chimeric DNA spikes added to the samples, these spikes could be used as internal standards for absolute quantification. Nine different synthetic chimeric DNA spikes with four different concentrations (10^3, 10^4, 10^5, and 10^6 of copies of internal standards) were added to the sample DNA pools. The amplicon sequencing of 16 Sr DNA was conducted using the Miseq platform (Genesky Bio-Tech Co., Ltd., Shanghai, China) after the purification and quantification of the PCR products. All the raw reads were checked using FLASH2 (version 2.2.00), and low-quality sequences (quality scores below 20) were discarded according to the QIIME quality control process (version 1.7.0). Operational taxonomic units were clustered using Uparse (version 7.0.1001) at 97% similarity. For AQS, the synthetic chimeric DNA spikes were filtered out and reads were counted. The copy numbers were then rectified based on the ribosomal RNA operons (rrn) DataBase using the procedure described by Stoddard et al. [32] and Wu et al. [33]. The specific step is to use the classification information annotated by OTU to search the rrnDB to obtain the 16 S rRNA copy number of each OTU, and then use the abundance information to carry out the weighted average of the abundance information and copy number to obtain the weighted average of the final sample copy number. The taxonomy assignment of representative sequences was performed with the Ribosome Database Project [34].

2.4. Statistical Analyses

The data of fermentation parameters were statistically analyzed using the GLM procedure of IBM SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Statistical differences in parameters between the days were determined in accordance with Duncan's multiple comparison test, and effects were considered significant when p was < 0.05. The alpha diversities of samples, the Shannon index, and the Chao richness estimator were determined using Mothur (version 1.30.1). β Diversity analysis of samples, including principal component analysis (PCA), was performed using R software (version 2.15.3). Taxonomic classification at different levels was performed using the Ribosome Database Project (version 2.2), algorithm to classify the representative sequences of each OTU.

3. Results and Discussion

3.1. Chemical Characteristics of Alfalfa before Ensiling

The chemical characteristics of alfalfa before ensiling are shown in Table 1. Wilting for about 24 h increased the DM contents of alfalfa to 39.20%, which was in the range of the

ideal level (30–40%) for silage preparation. The DM content of raw material has an essential influence on silage quality [35]. The silage with a high DM content has low moisture, which could inhibit the growth of undesirable microorganisms. The contents of NDF (389.79 g/kg DM) and ADF (274.98 g/kg DM) evaluated in this study conform to the normal range of the NDF and ADF content of alfalfa [19,36]. In the process of alfalfa silage, the important material base of LAB fermentation is WSC. However, when the WSC content is insufficient, the LA content produced by LAB fermentation is very small and cannot be accumulated rapidly, which makes the harmful microorganisms in the silage multiply. Therefore, the content of WSC in raw materials is an important factor affecting the fermentation quality of silage. To assure acceptable fermentation quality, the WSC content is usually around 60–80 g/kg DM [37]. Generally, many studies show that the WSC of alfalfa is lower than the minimum fermentation requirements, and natural ensiling is difficult. However, our study showed that the WSC contents (76.11 g/kg DM) met the recommended requirements. This was related to the harvest generation and harvest time of the alfalfa. The alfalfa in this study was harvested for the first time in its early flowering period this year. Generally, the nutrition in this period is very rich, so it is sufficient for full fermentation during ensiling. The initial contents of glucose, fructose, galactose, and rhamnose were 9144.42 mg/kg DM, 14,019.40 mg/kg DM, 1152.59 mg/kg DM, and 69.70 mg/kg DM, respectively. It can be seen that glucose and fructose account for a large proportion of the WSC.

Table 1. Chemical composition of fresh alfalfa.

Item	Alfalfa
pH	6.52
Dry matter (%)	39.20
Neutral detergent fiber (g/kg DM)	389.79
Acid detergent fiber (g/kg DM)	274.98
Water soluble carbohydrate (g/kg DM)	76.11
Rhamnse (mg/kg DM)	69.70
Galactose (mg/kg DM)	1152.59
Glucose (mg/kg DM)	9144.42
Fructose (mg/kg DM)	14,019.40

3.2. The Fermentation Parameters of Alfalfa Silage after Ensiling for 60 Days

The fermentation parameters after ensiling for 60 days are listed in Figure 1. The interaction of treatment of inoculants (T) × days of ensiling (D) had a significant ($p < 0.05$) effect on all fermentation parameters (Figure 1). The first stage of silage fermentation is aerobic respiration period. The longer the period lasts, the greater nutrition loss will be. Therefore, when making silage, the aerobic respiration period must be shortened as much as possible. In addition, rapidly increasing the content of LA and rapidly reducing the pH value (<pH 5.5) can inhibit the activity of plant respiratory enzymes and inhibit the activity of mold and spoilage bacteria, thus reducing the fermentation temperature and reducing the loss of protein, dry matter, and energy. As the oxygen is exhausted and the silage enters into anaerobic fermentation, anaerobic microorganisms gradually become the dominant group of bacteria, mainly LAB and butyrate bacteria. Butyrate bacteria are harmful bacteria in the silage process, which are not acid tolerant. If the LAB grows rapidly, the growth of butyrate bacteria can be inhibited by rapidly reducing the pH value to below 4.7. The fast accumulation of LA and decline in pH are important indicators for assessing silage fermentation quality [19]. In this study, we observed that the ZZU203-inoculated silages (ZZU203 silage and ZZU203_CB silage) had lower pH values compared with the CK and CB silage ($p < 0.05$); the pH values of CK and CB silage were, respectively, 6.13 and 5.91 at 10 d and 5.31 and 5.19 at 60 d, while the pH value of ZZU203 silage and ZZU203_CB silage all decreased rapidly to about 4.7 at 10 d, indicating that the silage fermentation process was accelerated by inoculating ZZU203, and a similar phenomenon was shown in Yang's report [20]. Although the WSC concentration in fresh materials was

considered sufficient for full fermentation in this study, the pH value of ZZU203 silage (4.66) and ZZU203_CB silage (4.69) at 60 d was still higher than the optimal level (4.2). This was due to the relatively high buffer capacity of alfalfa compared with grain feed [36,38]. Kung et al. [39] reported that the LA concentration of high-quality silage was >50 g/kg DM and accounted for more than 60% of the total organic acid. Compared with the CK silage, all treatments of inoculants significantly ($p < 0.05$) increased the concentrations of LA at 10 d. The highest LA concentrations were observed in silage inoculated with ZZU203_CB at 10 d (63.11 g/kg DM). The reasons for this phenomenon were that on the one hand, inoculating ZZU203 can improve the abundance of LAB, while on the other hand, inoculating cellulase-producing *Bacillus methylatrophy* accelerates the degradation of structural sugar, providing more WSC for the growth of LAB, so this treatment group can produce more LA during the fermentation process. Similar results were also found in other studies [13]. With the extension of silage time, the LA concentrations of ZZU203 silage and ZZU203_CB silage at 60 d were lower than those at 10 d, which was related to the fact that the WSC was at a low level at 60 d. At this time, some LA-degrading bacteria begin to metabolize LA, resulting in the consumption of LA. Our results showed that the AA concentration in silage was at desirable nutritional level (<30 g/kg DM) [39] and the LA concentration was higher than the AA concentration during ensiling, indicating that the fermentation process tended toward homo-fermentation. PA and BA contents in alfalfa silage were not detected in this study, which is desirable because their presence is considered a waste of metabolic energy [40] and indicates the presence and activity of spoilage microorganisms [41]. NH_3-N is generally considered to be the result of amino acid deamination and decarboxylation, which reduces the nutritional value of silage in the ensiling process. Kung et al. [39] reported that the effects of plant and microorganism proteolytic enzymes were typical causes of NH_3-N accumulation. The NH_3-N concentration of ZZU203 silage and ZZU203_CB silage was significantly ($p < 0.05$) lower compared with CK silage and CB silage. One of the reasons was that most plant protein hydrolases in alfalfa silage showed high activities at pH 5.0–6.0 [42], and the pH value of CK silage and CB silage was in this range. In addition, another possible reason was that the inhibition of ZZU203 or combined ZZU203 and CB on proteolytic microorganisms was strong in these two silages.

3.3. Nonstructural Carbohydrate Components of Alfalfa Silage

The content of nonstructural carbohydrate compositions in alfalfa silage with different additives after ensiling is given in Figure 2. The treatment of inoculants (T), days of ensiling (D), and their interaction significantly ($p < 0.05$) affected the contents of glucose, fructose, galactose, and rhamnose in alfalfa silage. Compared with the initial nonstructural carbohydrate contents (Table 1), the content of WSC, glucose, galactose, and fructose in each group decreased sharply, and the content of rhamnose also decreased ($p < 0.05$) after 10 days of ensiling. Compared with silage for 10 days, the contents of WSC and fructose of all silage groups decreased significantly ($p < 0.05$) at 60 d. At 10 d, compared with the CK silage, the content of fructose and rhamnose in CB silage increased significantly ($p < 0.05$), while the content of WSC and glucose decreased significantly ($p < 0.05$); in ZZU203 and ZZU203_CB silages, the contents of WSC and glucose were all significantly lower than those in CK silage ($p < 0.05$) at 10 d. This was related to the large number of microorganisms in the additive silage groups, resulting in a large consumption of nonstructural carbohydrate. Shao et al. [43] showed that glucose was the substrate that LAB preferentially used. After the beginning of silage, LAB proliferated and used glucose to produce LA. The content of LA increased gradually, while the content of glucose decreased gradually. After 60 days of ensiling, the contents of glucose, fructose, and rhamnose recorded in both ZZU203 and ZZU203_CB silage were higher than those in CK or CB silage ($p < 0.05$). This was due to the fact that the ZZU203 screened in previous studies can release WSC and sugar through the enzymatic degradation of structural carbohydrate components or acid hydrolysis of structural carbohydrates, or it can save the consumption of WSC by inhibiting the growth

of silage microorganisms. Interestingly, CK and CB silage had higher galactose contents than those in ZZU203 and ZZU203_CB silage ($p < 0.05$). This indicated that the ability of the epiphytic LAB on the alfalfa material and CB to ferment arabinose and galactose was weaker than that of ZZU203 and combined ZZU203 and CB.

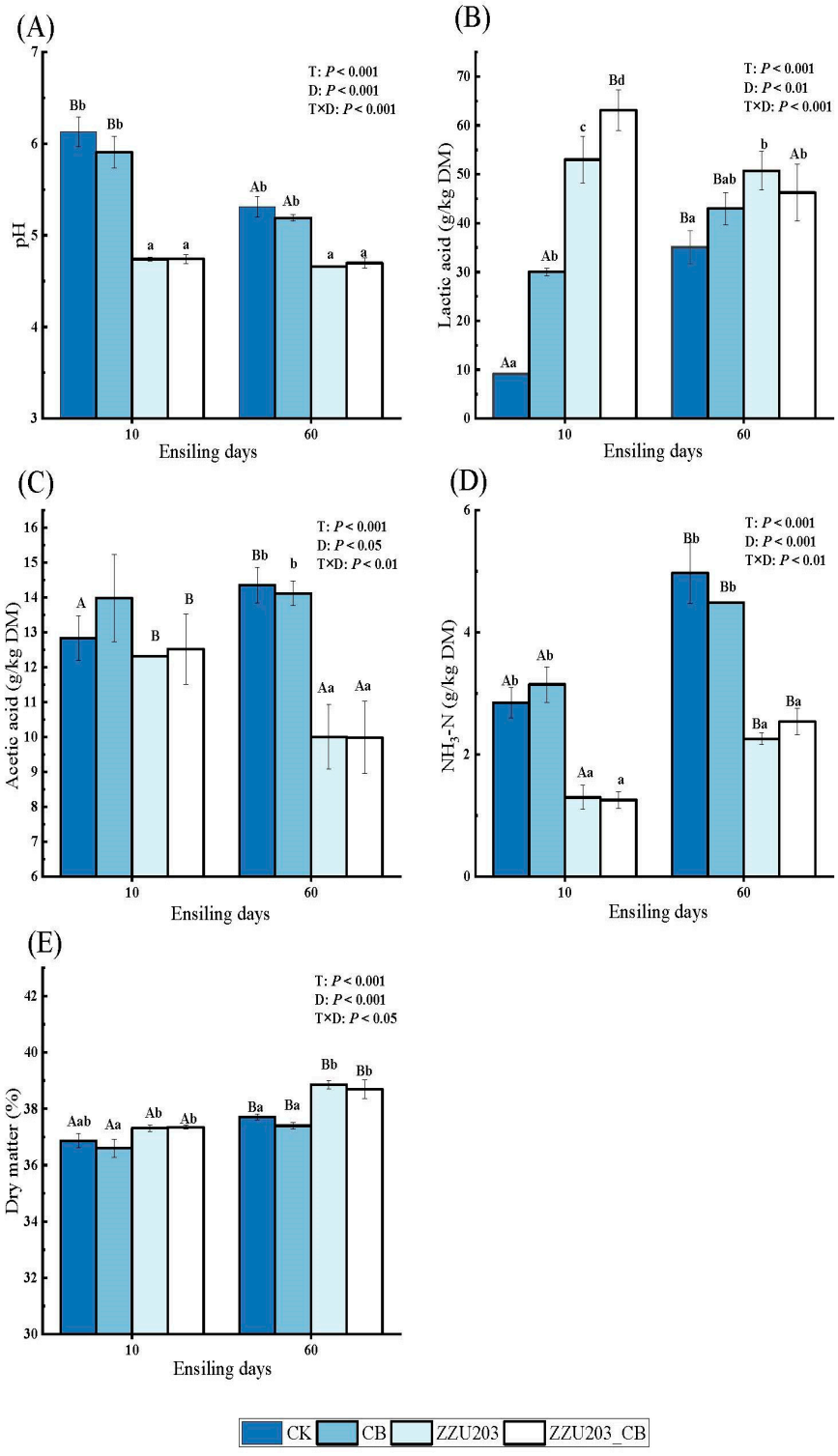

Figure 1. Changes in pH (**A**), Lactic acid (LA) (**B**), Acetic acid (AA) (**C**), NH_3-N (**D**) and Dry matter (DM) (**E**) in alfalfa silages during ensiling for 60 d. Values with different superscript lowercase letters show significant differences between treatments on the same ensiling day, while values with different superscript capital letters show significant differences between ensiling days with the same treatment ($p < 0.05$). NH_3-N, ammoniacal nitrogen.

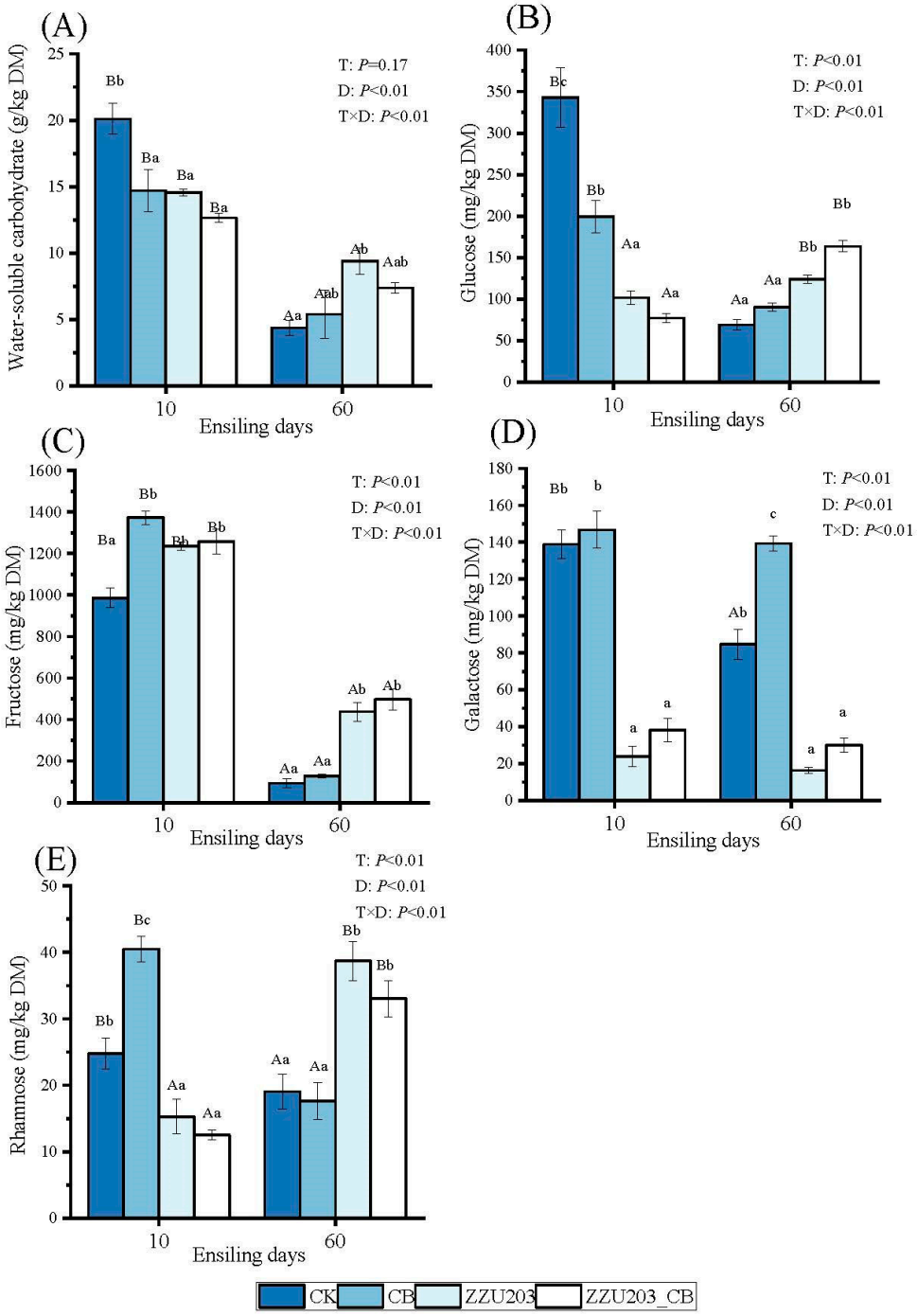

Figure 2. Changes in the concentrations of water-soluble carbohydrate (WSC) (**A**), Glucose (**B**), Fructose (**C**), Galactose (**D**) and Rhamnose (**E**) in alfalfa silages during ensiling for 60 d. Values with different superscript lowercase letters show significant differences between treatments on the same ensiling day, values with different superscript capital letters show significant differences between ensiling days with the same treatment ($p < 0.05$).

3.4. Structural Carbohydrate Components of Alfalfa Silage

The structural carbohydrate compositions of alfalfa silages after treatment with different additives are shown in Figure 3. The content of NDF and ADF in the CK silage and CB silage showed an increasing tendency during the 60 days of ensiling, which may be due to the relatively increased content of structural carbohydrate components with the degradation and consumption of organic substances during the silage process. Similar

results were found in the report of Liu et al. [44], who found that the contents of structural carbohydrates in the LAB-treated and control silages increased gradually during 60 days of ensiling. At 10 d, the content of NDF in the ZZU203_CB silage was the lowest, which was significantly different from that in the CK silage and CB silage ($p < 0.05$), but not significantly different from that in the ZZU203 silage ($p > 0.05$); there was no significant difference in the content of ADF in each group. After 60 days of ensiling, there was no significant difference in the NDF and ADF content between CK silage and CB silage. The content of NDF of LAB-treated silages was significantly ($p < 0.05$) lower than that of CK silage and CB silage. The content of ADF of ZZU203 was the lowest, which was significantly different from that in CK silage and CB silage ($p < 0.05$). During ensiling, the mechanism of structural carbohydrate degradation was mainly related to acid solubilization of the structural carbohydrates and the direct hydrolysis of the lignocellulosic content by fibrolytic enzymes. A low pH was observed in the ZZU203 silage and ZZU203_CB silage and the high reduction in the NDF contents recorded in the same silages could be attributed to lignocellulosic degradation by the organic acids produced during ensilage, which was similar to our previous research results, which was related to the ability of ZZU203 to produce acid using structural sugar [16], indicating that the addition of ZZU203 silage is conducive to the degradation of NDF.

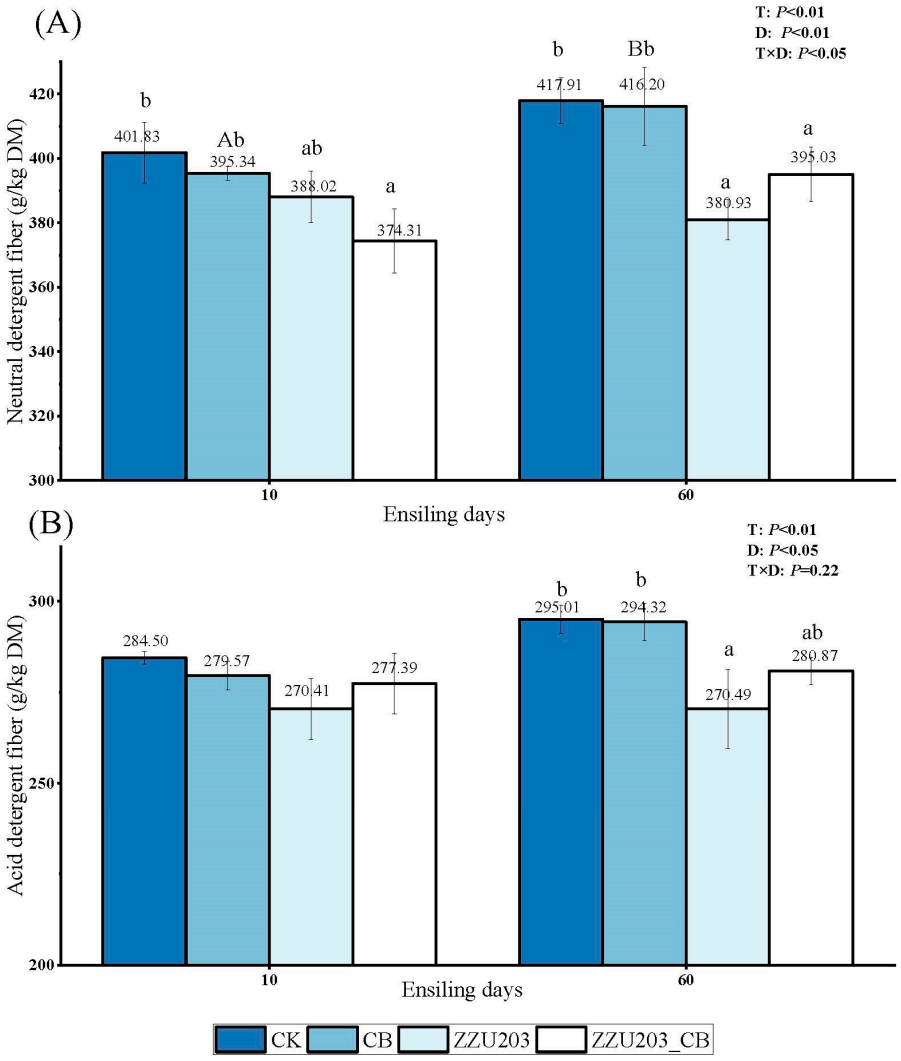

Figure 3. Changes in neutral detergent fiber (NDF) (**A**) and acid detergent fiber (ADF) (**B**) in alfalfa silages during ensiling for 60 d. Values with different superscript lowercase letters show significant differences between treatments on the same ensiling day, while values with different superscript capital letters show significant differences between ensiling days with the same treatment ($p < 0.05$).

3.5. Bacterial Community after 60 d of Ensiling

Relative quantification 16S-seq (RQS) has been widely used to detect microbial communities in silages [45–48]. However, the sole application of RQS was misleading when exploring bacterial community dynamics through the ensiling process or across multiple treatments, since fluctuations in the absolute abundance of a certain microbial group may not cause a significant change in the relative abundance of the taxon when the total abundance of the bacterial community is not fixed [49]. Yang et al. reported that AQS accurately illustrated the dynamics in the absolute abundance of the bacterial community in alfalfa silage using synthetic chimeric DNA spikes, and uncovered more sequencing information than RQS by analyzing alpha and beta diversities of bacterial communities [20]. In this study, high-throughput analyses determined the bacterial community compositions in alfalfa silage. After low-quality, short, ambiguous and singleton reads were excluded, the valid sequences were clustered into 1033 operational taxonomic units based on a 97% sequence identity. Gene copies in per ng DNA of the samples were calculated via a standard curve formula based on a 99% coefficient of determination (R2), which indicated that sequencing depth was sufficient for revealing the complete bacterial diversity. Alpha diversity of the bacterial community is shown in Figure 4. Lower ($p < 0.05$) values of Chao 1 and Shannon indexes were observed in silages than in pre-ensiled materials, which indicated that substantial selection occurred during the ensiling process and this was due to the large increase in some bacteria with good adaptability to the conditions of ensiling [50]. Similar trends were also reported by Zheng et al. [38]. The effects of inoculation of CB alone on community diversity and richness in alfalfa silage are similar to those of CK silage during silage fermentation. This is consistent with the results of similar fermentation parameters in two silage groups. The diversity of bacteria decreased in ZZU203 and ZZU203_CB silages, compared to those in the CK and CB silages. Similarly, Ogunade et al. [45] revealed that silage inoculated with L. plantarum had decreased bacterial diversity due to the increased relative abundance of the predominant genus (*Lactobacillus*). Polley et al. [51] reported that the microbial community was less diverse when the dominant bacteria were abundant. Mendez-Garcia et al. [52] reported that a low pH was the main factor underlying the limited microbial diversity. This might explain the decreased bacterial diversity in silage with a ZZU203 inoculant, as the inoculation significantly reduced the pH level in alfalfa silage ($p < 0.05$), which accelerated the growth of the desirable bacteria in this study.

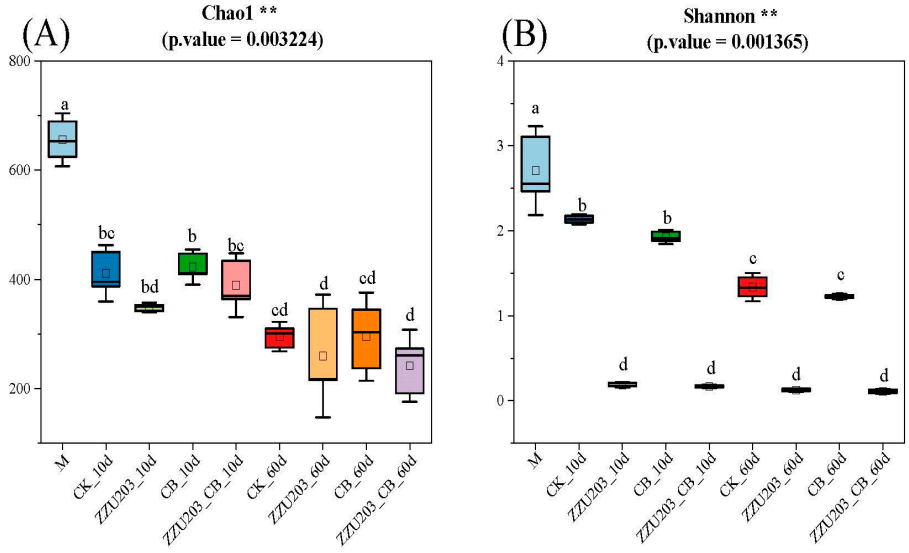

Figure 4. Box-plots of Chao 1 (**A**) and Shannon indices (**B**) of bacterial communities in alfalfa silage. Values with different small letters indicate significance over groups illustrated via absolute quantification 16S-seq (AQS; ** $p < 0.01$).

To analyze the distribution and structure of bacterial communities in different silage treatments at different storage times, PCA analysis based on OTU level was used in this study (Figure 5). Component 1 and component 2 could explain 67.22% and 27.29% of the total variance, respectively. Significant separation and differences in bacterial communities were observed between fresh and ensiled materials, indicating that there were significant differences between the microbial communities in fresh materials and silage. All silages were divided into four clusters, which suggested that exogenous additives and ensiling time had significant impacts on the bacterial community of alfalfa silage. Ni, Minh et al. considered that the variation in the microbial community could explain the difference in silage quality [53].

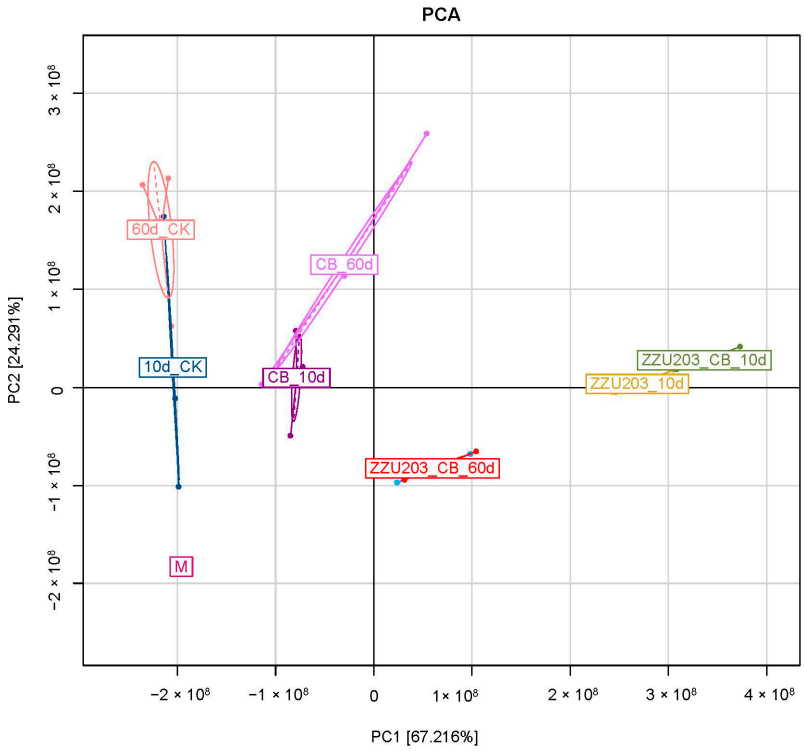

Figure 5. Principal coordinate analysis of bacterial communities in alfalfa silage based on the results illustrated by AQS.

It is well known that the majority of bacteria involved in the LA fermentation of silage belong to the genera *Lactobacillus*, *Pedicoccus*, *Weissella*, and *Enterococcus* [48,54]. In this study, fresh alfalfa had a low abundance of epiphytic LAB. Therefore, adding LAB to the silage makes *Lactobacillus* become the dominant bacteria to accelerate the LA fermentation, reduces the environmental pH value, and inhibits the growth of harmful microorganisms, thus reducing the loss of protein and dry matter in the silage, which is an effective way to improve the quality of alfalfa silage. At 10 d, the CK silage exhibited a complex bacterial community composition, including *Hafnia* (1.56×10^8 copies/ng DNA), *Pediococcus* (1.48×10^8 copies/ng DNA), unassigned (1.07×10^8 copies/ng DNA), *Lactobacillus* (1.0×10^8 copies/ng DNA), *Enterococcus* (9.50×10^7 copies/ng DNA), and *Weissella* (6.12×10^7 copies/ng DNA); *Pediococcus* (3.24×10^8 copies/ng DNA) was the predominant genus in 60 d silage without inoculation, followed by *Lactobacillus* (1.14×10^8 copies/ng DNA), *Weissella* (4.83×10^7 copies/ng DNA), *Enterococcus* (4.72×10^7 copies/ng DNA), and *Garciella* (8.63×10^6 copies/ng DNA). The bacterial community composition of CB silage was similar to that of CK silage, while the difference was that the number of *Lactobacillus* in CB silage (2.01×10^8 copies/ng DNA at 10 d, 2.71×10^8 copies/ng DNA at 60 d) was higher. The increase in the number of *Lactobacillus* in silage inoculated with CB was due to the degradation of lignocellulose and the release of more WSC for the growth and

utilization of *Lactobacillus* in the early stage of silage. *Lactobacillus* can make better use of nutrients in raw materials and reduce the loss of nutrients compared with other typic LAB. The higher the number of *Lactobacillus*, the faster the acid production rate, the higher the LA content, and the lower the pH value, reducing the decomposition of protein and reducing the content of NH_3-N. In the current study, ZZU203 and ZZU203_CB silages showed increased abundance of *Lactobacillus* compared with those in the CK silage at 10 and 60 d (Figure 6). Yang et al. made a similar report in which *Lactobacillus* outnumbered all other genera after inoculation with *Lactiplantibacillus plantarum* [29]. Similar results were reported by Yan et al. [4], and they also found that the abundance of *Lactobacillus* was higher in the LAB-treated silage compared with that in the CK silage. *Lactobacillus* was the predominant genus of the bacterial community in the ZZU203 and ZZU203_CB silages in both periods (96.88%, 97.18% at 10 d and 98.27%, 98.31% at 60 d, respectively.) (Figure 6A), and there was no significant difference in the relative abundance of *Lactobacillus* between the two silages. However, after AQS was used to show the dynamics and effects of inoculation on the total amount of bacterial DNA, the highest abundance of *Lactobacillus* was found in the silage with the combined addition of ZZU203 and CB at 10 d (Figure 6B), which indicates that the combined additive has a synergistic effect on the growth of *Lactobacillus*. However, the abundance of *Lactobacillus* was no different in the ZZU203 silage compared with ZZU203_CB silage at 60 d. This was due to the ideal silage conditions such as adequate WSC, suitable storage temperature, and strict anaerobic control in this study; ZZU203_CB silage failed to play to the advantage of combined additives at 60 d. We speculate that when the conditions are not conducive to ensiling, the combined addition of ZZU203 and CB may have a better potential to make *Lactobacillus* become the dominant bacteria to accelerate the LA fermentation, inhibit the growth of harmful microorganisms, and reduce the loss of protein and dry matter to improve silage quality. The abundance of *Lactobacillus* was reduced in the inoculated group at 60 d compared with 10 d silage. This is consistent with the reduction in LAB count with the prolonged ensiling time reported by Yang et al. in alfalfa silage [20,55]. One reason for this reduction might be the lack of fermentation substrates [56]. *Pedicoccus*, *Enterococcus*, and *Weissella* were usually found in naturally fermented silages, which can initiate LA fermentation in the early stage of ensiling [42,50]. They were cocci-shaped LAB and generally not acid tolerant. Yang et al. reported an increase in *Pedicoccus*, *Enterococcus*, and *Weissella* at the early stage of alfalfa ensiling, and *Pedicoccus*, *Enterococcus*, and *Weissella* were later outcompeted by *Lactobacillus* under low pH conditions [57]. Cai et al. [42] showed that some cocci-shaped LAB could not grow below pH 4.5. In this study, the pH condition in the 60 d_CK silage (5.31) was still high, which explained the dominance of *Pedicoccus*, *Enterococcus*, and *Weissella* at 60 d in the CK silage. Enterobacteriaceae is a common genus during natural silage fermentation [47,58]. During the ensiling process, the presence of Enterobacteriaceae is undesirable, because they may cause nutrition loss [47]. *Hafnia* belongs to Enterobacteriaceae. Yang et al. [20] reported that *Hafnia* consumed nitrogen sources in silage and transformed them into alkaline products such as biogenic amines and other NH_4^+ compounds, which increased the pH level in silage. In this study, the results (Table 2) showed that a significantly higher abundance of *Hafnia* was observed in CK silage at 10 d on genus level ($p < 0.05$), which partly explain the higher pH value and NH_3-N content of the CK silage after 10 days of ensiling. The abundance of *Hafnia* was decreased after inoculation with ZZU203 and ZZU203_CB, indicating that adding ZZU203 silage can effectively inhibit the growth and reproduction of *Hafnia*, which could also explain the relatively low NH_3-N in ZZU203 and ZZU203_CB silages.

Garciella is an anaerobic and thermophilic bacterial genus of the class *Clostridia*, and is undesirable in silage because it can lead to excessive protein degradation, DM loss, and BA production [59]. In this study, *Garciella* became the dominant genus at 60 d only in the CK silage, indicating that the addition of ZZU203 silage could effectively inhibit the growth and reproduction of *Garciella*, thereby significantly reducing the content of NH_3-N and the loss of DM.

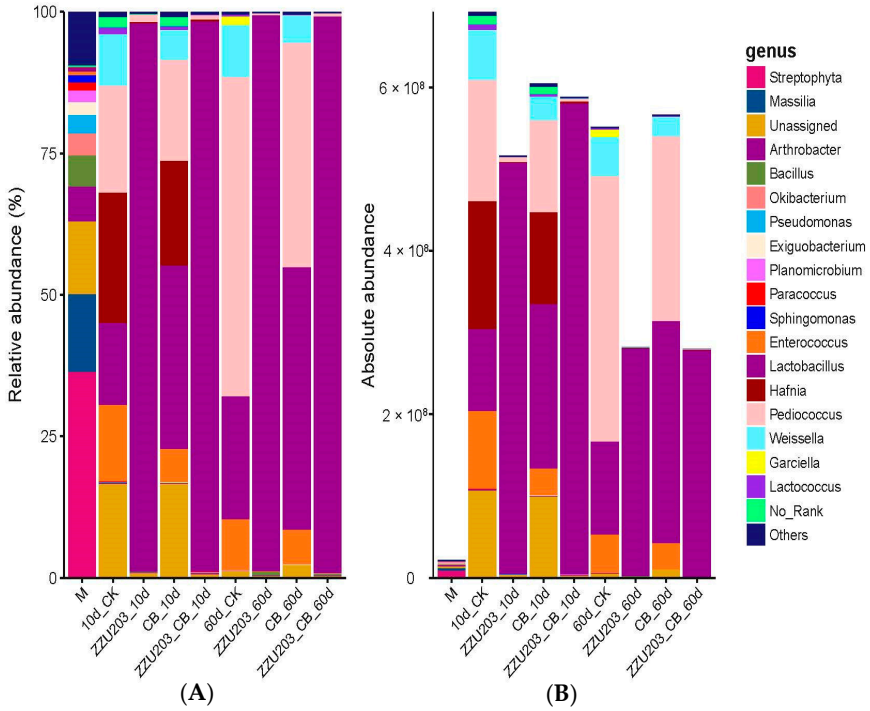

Figure 6. Bar plots of bacterial communities in alfalfa silage illustrated using RQS (**A**) and AQS (**B**).

Table 2. Bacterial communities in alfalfa silage illustrated using RQS and AQS of genera at 10 and 60 days.

Genus	Treatments	RQS (%)		AQS (Copies/ng DNA)	
		Ensiling Days (d)			
		10	60	10	60
Lactobacillus	CK	14.51 ^{aA}	21.76 ^{aB}	1.0×10^{8} ^{a}	1.14×10^{8} ^{a}
	CB	32.42 ^{bA}	46.37 ^{bB}	2.01×10^{8} ^{bA}	2.71×10^{8} ^{Bb}
	ZZU203	96.88 ^{c}	98.27 ^{c}	5.04×10^{8} ^{cB}	2.77×10^{8} ^{bA}
	ZZU203_CB	97.18 ^{c}	98.31 ^{c}	5.76×10^{8} ^{dB}	2.78×10^{8} ^{bA}
Pediococcus	CK	18.85 ^{bA}	56.38 ^{cB}	1.48×10^{8} ^{cA}	3.24×10^{8} ^{cB}
	CB	17.85 ^{bA}	39.63 ^{bB}	1.12×10^{8} ^{bA}	2.26×10^{8} ^{bB}
	ZZU203	1.26 ^{a}	<1.00 ^{a}	3.35×10^{6} ^{aB}	5.93×10^{5} ^{aA}
	ZZU203_CB	<1.00 ^{a}	<1.00 ^{a}	3.34×10^{6} ^{a}	1.05×10^{6} ^{a}
Hafnia	CK	23.08 ^{bB}	<1.00 ^{A}	1.56×10^{8} ^{cB}	2.18×10^{5} ^{bA}
	CB	18.44 ^{bB}	<1.00 ^{A}	1.12×10^{8} ^{bB}	3.25×10^{5} ^{bA}
	ZZU203	<1.00 ^{a}	<1.00	7.53×10^{5} ^{a}	8.96×10^{4} ^{a}
	ZZU203_CB	<1.00 ^{a}	<1.00	1.90×10^{6} ^{a}	7.47×10^{4} ^{a}
Enterococcus	CK	13.48 ^{cB}	8.95 ^{bA}	9.50×10^{7} ^{cB}	4.72×10^{7} ^{bA}
	CB	5.79 ^{b}	6.12 ^{b}	3.21×10^{7} ^{b}	3.29×10^{7} ^{b}
	ZZU203	<1.00 ^{a}	<1.00 ^{a}	1.16×10^{5} ^{a}	6.79×10^{4} ^{a}
	ZZU203_CB	<1.00 ^{a}	<1.00 ^{a}	1.17×10^{5} ^{a}	5.32×10^{4} a
Weissella	CK	9.08 ^{c}	9.13 ^{c}	6.12×10^{7} ^{c}	4.83×10^{7} ^{c}
	CB	5.28 ^{b}	4.85 ^{b}	2.92×10^{7} ^{b}	2.43×10^{7} ^{b}
	ZZU203	<1.00 ^{a}	<1.00 ^{a}	1.31×10^{5} ^{a}	7.15×10^{4} ^{a}
	ZZU203_CB	<1.00 ^{a}	<1.00 ^{a}	1.64×10^{5} ^{a}	5.65×10^{4} ^{a}
Lactococcus	CK	1.2 ^{bB}	<1.00 ^{A}	7.43×10^{6} ^{cB}	1.41×10^{6} ^{bA}
	CB	<1.00 ^{a}	<1.00	3.31×10^{6} ^{b}	4.26×10^{5} ^{b}
	ZZU203	<1.00 ^{a}	<1.00	1.28×10^{4} ^{a}	1.59×10^{3} ^{a}
	ZZU203_CB	<1.00 ^{a}	<1.00	5.36×10^{4} ^{a}	9.90×10^{2} ^{a}
Garciella	CK	<1.00 ^{A}	1.55 ^{bB}	5.36×10^{4} ^{cA}	8.63×10^{6} ^{bB}
	CB	<1.00	<1.00 ^{a}	1.63×10^{4} ^{b}	3.16×10^{4} ^{a}
	ZZU203	<1.00	<1.00 ^{a}	1.91×10^{4} ^{b}	7.56×10^{3} ^{a}
	ZZU203_CB	<1.00	<1.00 ^{a}	7.63×10^{3} ^{a}	7.34×10^{3} ^{a}

Values with different superscript lowercase letters show significant differences between treatments on the same ensiling day, while values with different superscript capital letters show significant differences between ensiling days in the same treatment ($p < 0.05$).

4. Conclusions

In conclusion, the inoculation of *Lactiplantibacillus plantarum* ZZU203 accelerated LA fermentation, inhibited NH_3-N accumulation, and decreased pH value in alfalfa silage, contributing to an increase in the abundance of *Lactobacillus*. Moreover, the combination of ZZU203 and CB promoted LA accumulation and the numbers of *Lactobacillus* at 10 d compared with ZZU203 inoculation alone. Therefore, ZZU203 or the combination of ZZU203 and CB could be used as the potential silage additives to improve the silage quality of alfalfa.

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