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# Effects of Different Additives on Fermentation Quality, Microbial Communities, and Rumen Degradation of Alfalfa Silage

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**Abstract:** This study examined the effects of different additives on the fermentation quality, nutrient composition, microbial communities, and rumen degradation of ensiled alfalfa. Six treatments were employed in which additives were applied to alfalfa on a fresh weight basis: CK (no additive), FA (0.6% formic acid), CaO (3% calcium oxide and 3% urea), LB ($1 \times 10^6$ cfu/g *Lentilactobacillus buchneri*), GLB (2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*), and FLB (2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*). After 60 days of ensiling, all treatments altered the bacterial communities, improved the fermentation quality, reduced dry matter (DM) and crude protein (CP) losses, and enhanced the rumen degradation of nutrients. The addition of LB increased the relative abundance of *Lactobacillus* spp. ($p < 0.05$), whereas GLB reduced ($p < 0.05$) the $NH_3$-N:TN ratio and elevated ($p < 0.05$) the concentrations of *Lactobacillus* and lactic acid content. The FA treatment reduced ($p < 0.05$) the pH, as well as the DM and CP degradations, while the CaO treatment increased the degradations of DM, acid detergent fiber, and neutral detergent fiber. We concluded that FA, LB, GLB, and FLB had beneficial effects on alfalfa fermentation, and that CaO increased alfalfa silage rumen degradation.

**Keywords:** alfalfa silage; additives; *Lentilactobacillus buchneri*; fermentation quality; rumen degradation; microbial diversity

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**Citation:** Ling, W.; Zhang, L.; Feng, Q.; Degen, A.A.; Li, J.; Qi, Y.; Li, Y.; Zhou, Y.; Liu, Y.; Yang, F.; et al. Effects of Different Additives on Fermentation Quality, Microbial Communities, and Rumen Degradation of Alfalfa Silage. *Fermentation* **2022**, *8*, 660. https://doi.org/10.3390/fermentation8110660

Academic Editors: Pascal Drouin and Érica Benjamim da Silva

Received: 14 October 2022
Accepted: 16 November 2022
Published: 21 November 2022

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## 1. Introduction

Alfalfa (*Medicago sativa*) is a quality forage for ruminants and is considered one of the foremost forages in the world. It has a high protein content, excellent nutritional qualities, and high yield, and it can be grown under a variety of conditions [1]. Ensiling can improve nutrient content and palatability via anaerobic fermentation by lactic acid bacteria (LAB), in which pH is reduced to suppress harmful microorganisms such as *Escherichia coli*, yeast, and molds [2]. However, producing alfalfa silage presents challenges. Alfalfa has low numbers of epiphytic LAB, low sugar substrate content, and high buffer capacity [3]. It is poorly digested in the rumen, resulting in poor utilization [4]. Moreover, the florescence of alfalfa usually occurs during the rainy season (May to July), associated with the East Asian monsoon climate in China, resulting in high water content of the alfalfa under natural weather conditions. Consequently, additives are considered among the feasible methods to deal with the high water content of the alfalfa to improve the quality of silage.

Fermentation inhibitors were the first of numerous additives to be applied to improve alfalfa silage. Inorganic acids, as additives, were corrosive and could endanger animal

health and pollute the environment; therefore, they were gradually replaced by organic acids. Formic acid lowers the pH during ensiling and lessens the nutrient loss [5]. It is particularly effective on forages with low water-soluble carbohydrate (WSC) content and high buffering capacity [6]; therefore, it is suitable in the production of alfalfa silage.

Microorganisms are employed to facilitate the fermentation process. In order to reduce pH during ensiling, LAB is added to increase lactic acid production [7]. *Lentilactobacillus buchneri*, a heterofermentative LAB, produces volatile fatty acids by fermentation. The dissociation levels of acetic and propionic acids are low under most ensiling conditions, thus allowing passive diffusion within the yeast or other microbial cytoplasm. In the cytoplasm, acetic and propionic acids are broken down into their corresponding salts [8]. Both acids are strong yeast inhibitors by affecting several metabolic pathways and by reducing yeast activity, which lessens silage aerobic spoilage [9]. Sugar additives provide WSC substrate for LAB to enhance fermentation [10]. Glucose is the most common sugar added to boost the quality of silage and can be utilized directly by LAB [11], while polysaccharides are hydrolyzed during fermentation to free sugars prior to being used by microorganisms. Being environmentally friendly, as well as functional (antibacterial, anti-inflammatory, and antioxidant), naturally active polysaccharides have been used widely [12,13]. Fucoidans inhibit harmful intestinal microorganisms and enhance host immunity, and they have been applied in a variety of animal production systems [14]. Few reports have been published on the impact of LB alone or in association with monosaccharides or polysaccharides on the production of silage.

Alkali treatment has been used commonly to improve the utilization of feed [15]. The low digestibilities of starch and neutral detergent fiber (NDF) were improved by feeding CaOH-treated maize silage to cows [16]. In a study by Allen et al. [17], fiber content decreased linearly with increasing supplementary alkali, indicating that this treatment could improve the low utilization of silage. Urea can be used as a nutrient additive for microbial protein synthesis [18], and a combination of urea and alkali improved the digestion of NDF and acidic detergent fiber (ADF) [19]. Most research on alkali treatment has focused on the improvement of utilization of silage by animals, with little research on the effect of alkali treatment on fermentation [20].

In the current study, alfalfa was treated with acid (formic acid), alkali (calcium oxide in combination with urea), and microorganisms (LB alone and LB in combination with glucose or fucoidan) to examine the effects on silage quality in terms of nutritional content, fermentation variables and rumen degradability.

## 2. Materials and Methods

### *2.1. Animal Care*

All procedures on the animals were approved by the College of Animal Sciences (College of Bee Science), Fujian Agriculture and Forestry University, and followed the recommendations of the European Commission (1997).

### *2.2. Silage Preparation*

Alfalfa at the first flowering stage was harvested at the Agricultural Meteorological Experiment Station in Dingxi City, Gansu Province, China (35.47° N, 104.60° E, 2035 m above sea level) in May 2021. It was mowed manually at 2–3 cm above ground level, transported to the laboratory immediately, spread out evenly in a ventilated, cool building, and air-dried overnight. The alfalfa was cut manually into 2–3 cm lengths and treated with one of the following six additives: (1) distilled water (CK); (2) formic acid (FA, with 0.6% FA); (3) alkali treatment (CaO, 3% calcium oxide and 3% urea); (4) *L. buchneri* (LB); (5) glucose and *L. buchneri* (GLB, 2% glucose and *L. buchneri*); (6) fucoidan and *L. buchneri* (FLB, 2% fucoidan, where purity > 90% and *L. buchneri*). *L. buchneri* (BNCC187961, Beijing Beina Chuanglian Biotechnology Institute, Beijing, China) was added at $1 \times 10^6$ cfu/g in 10 mL of fermentation solution and sprayed on the alfalfa for the LB, GLB, and FLB treatments. For the CK, 10 mL of sterile water were sprayed on the alfalfa. Four hundred

grams of the treated alfalfa were placed in a polyethylene bag (248 mm × 344 mm), vacuum-sealed, and then fermented at room temperature (25 ± 3 °C) for 60 days. Each treatment had five replicates. In the week prior to ensiling, *L. buchneri* was inoculated in de Man Rogosa Sharpe medium (Fuzhou Mili Biotechnology Co., Ltd., Fuzhou, China) for bacterial counting under anaerobic incubation at 30 °C for 48 h to adjust the application rate to $1 \times 10^6$ cfu/g. A concentration of $1 \times 10^6$ cfu/g was chosen on the basis of several studies. In a meta-analysis by Arriola et al. [21], an application of *L. buchneri* at $10^5$ or $10^6$ cfu/g was optimal for improving silage quality and aerobic stability. Kung and Ranjit [22] reported that, for *L. buchneri* to be the dominant bacteria and improve silage quality, the added dose should be greater than $5 \times 10^5$ cfu/g, while Ranjit and Kung [23] reported that silage nutrient loss was reduced and aerobic stability was improved when *L. buchneri* was added at $1 \times 10^6$ cfu/g.

### *2.3. Fermentation Variables and Nutritional Content of Alfalfa Silage*

Ten grams of alfalfa ensiled for 60 days were taken from each bag and placed into a 100 mL wide-mouth conical flask with 90 mL of distilled water (1:9 material–liquid ratio), sealed, and stored at 4 °C. Samples were leached for 24 h, passed over four layers of saran wrap, and stored at −20 °C. The pH was measured using a pH meter (pHS-3D, Shandong China), and WSC content was determined by anthrone sulfuric acid colorimetry [24]. Total nitrogen (TN) was determined using a nitrogen analyzer (K9840 Kjeldahl, Hanon, Jinan, China), and crude protein (CP) was calculated as TN × 6.25 [25]. Phenol sodium hypochlorite colorimetry was used to determine ammonia nitrogen ($NH_3$-N) [26], and high-performance liquid chromatography identified volatile fatty acids [27]. The LAB was cultured in de Man Rogosa Sharpe medium (Fuzhou Mili Biotechnology Co., Ltd., Fuzhou, China). Alfalfa silages were oven-dried at 65 °C for 48 h to determine DM, and samples were finely ground. NDF and ADF concentrations were measured following Ke et al. [28].

### *2.4. DNA Extraction, PCR Amplification, and Sequencing*

After 60 days, ensiled alfalfa samples of all treatments, in triplicate, were placed into 50 mL sterile cryopreservation tubes and stored at −80 °C for DNA extraction. Total genomic DNA of bacteria was extracted following the instructions of the E.Z.N.A.® Kit (Omega Bio-tek, Norcross, GA, USA), and the quality of DNA extraction was determined by 1% agarose gel electrophoresis. Concentration and purity of DNA were determined using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR amplification and bioinformatics analysis of the samples were conducted by Shanghai Majorbio Bio-Pharm Technology Co (Shanghai, China). The 16S rRNA gene V3–V4 variable region was amplified [29].

Library construction was performed using a NEXTFLEX Rapid DNA-Seq kit in the following order: (1) splice ligation; (2) removal of spliced self-joined fragments by magnetic bead screening; (3) enrichment of library template by PCR amplification; (4) recovery of PCR products by magnetic beads to obtain the final library. Sequencing used Illumina's Miseq PE300 platform (Shanghai Maibo Biomedical Technology Co., Ltd., Shanghai, China). Sequences were clustered into operational taxonomic units (OTUs) using UPARSE software (http://drive5.com/uparse/, version 7.1, accessed on 13 October 2022), and chimeras were removed according to 97% similarity. Each sequence was annotated for species classification using the RDP classifier (http://rdp.cme.msu.edu/, version 2.2, accessed on 13 October 2022) and compared to the Silva 16S rRNA database (v138) with a set comparison threshold of 70%. Alpha diversity was adopted to analyze the species diversity of the samples according to five indices: Shannon diversity index, Simpson diversity index, Ace richness estimator, Chao1 richness estimator, and Good's coverage; QIIME (v1.9.1) was used to calculate all indices of the samples. Beta diversity analysis was applied to evaluate differences in species complexity among samples; principal coordinates analysis (PCoA), based on Bray–Curtis distance, demonstrated the distinct clusters among the six treatments, which were further analyzed by the Adonis test [30]. The Spearman correlation

analysis was test for relationships between variables, and the correlation between the main bacterial genera and the quality of silage was characterized using a heatmap. Microbial data analysis and mapping were performed on the Majorbio Bio-Pharm cloud platform (https://login.majorbio.com, accessed on 13 October 2022).

### *2.5. Ruminal Degradation*

Three Min Dong rumen-fistulated goats (body weight: 24.5 ± 2.0 kg) were used to measure ruminal degradabilities of nutrients in alfalfa ensiled for 60 days. The nylon bag method [31] was used to determine the degradabilities of DM, CP, NDF, and ADF. Prior to the study, the goats were dewormed and fed a 60:40 forage/concentrate diet at 9:00 a.m. and 6:30 p.m. daily, with water freely available.

The samples of each treatment were oven-dried at 65 °C and sieved through a 40 mesh screen. Four grams of each sample was placed in a nylon bag (8 cm × 15 cm) with a pore diameter of 48 µm, and sealed with a hand-operated impulse heat sealer (AIE—200, American International Electric, City of Industry, CA, USA). The bag, with a small string attached, was placed in the rumen 2 h before morning feeding. Duplicate bags were incubated in the rumen of each goat for 4, 8, 12, 24, 48, and 72 h. The material was removed from the bag, oven-dried at 65 °C to determine DM, and sieved through a 1 mm screen; the composition was determined as described previously. The nutrient degradation was calculated according to the rumen kinetic index model proposed by Ørskov et al. [32]. The effective degradation rate of the nutrient was calculated as follows:

$$y = a + b\ (1 - e^{-ct}), \quad (1)$$

$$ED = a + [bc/(c + k)], \quad (2)$$

where a is the fast degradation part (%), b is the slow degradation part (%), c is the slow fraction degradation rate (%), ED is the effective degradation rate (%), and k is the rate of efflux from the rumen (0.0235 $h^{-1}$) [33].

### *2.6. Statistics Analysis*

One-way analysis of variance (ANOVA) and Duncan's multiple comparison were used to analyze the data (SPSS 25.0, Chicago, IL, USA), with $p < 0.05$ accepted as the level of significance. Values of the deterioration constants a, b, and c in the numerical exponential model were determined using a non-linear dynamic model (SAS, Cary, NC, USA).

## 3. Results and Discussion

### *3.1. Characteristics of Fresh Alfalfa*

The alfalfa had a pH of 6.28, a DM content of 19.3%, a WSC content of 6.04% DM, and an epiphytic LAB of 4.15 Lg cfu/g (Table 1). These conditions were not optimal for the LAB to become the dominant bacteria during fermentation [34]. With a moisture content greater than 700 g/kg fresh matter, the concentrations of WSC and LAB were reduced, the decline in pH was constrained, and the silage was affected negatively [35,36]. It is difficult for the pH to be lowered with a low DM content of the alfalfa. A high DM content before silage production is advantageous for improving silage quality. Consequently, wilting of alfalfa is often practiced prior to the production of silage. However, the continuous rain and high air humidity during the main rainy season in late spring to summer in East China makes it difficult to wilt alfalfa to optimal dry matter level [37]. Drying crops using mechanical methods is effective in reducing crop water content; however, this process generally requires substantial energy and is very expensive [38,39]. Therefore, results from the present study are very relevant to large parts of China.

**Table 1.** Nutrient and microbial composition of fresh alfalfa.

| Variable | Measurement |
|---|---|
| pH | 6.28 |
| Dry matter (% FW) | 19.3 |
| Water-soluble carbohydrate (% DM) | 6.04 |
| Crude protein (% DM) | 28.9 |
| Neutral detergent fiber (% DM) | 39.6 |
| Acid detergent fiber (% DM) | 28.8 |
| Lactic acid bacteria (Lg cfu/g FW) | 4.15 |
| Aerobic bacteria (Lg cfu/g FW) | 4.38 |
| Yeast and Molds (Lg cfu/g FW) | 5.81 |

FW, fresh weight; DM, dry matter.

### *3.2. Fermentation Variables and Nutritional Content of Alfalfa after 60 Days of Ensiling*

The pH of alfalfa silage was lowest with the addition of 0.6% formic acid ($p < 0.05$, Table 2), which was due to the acidification by formic acid and the high lactic acid content ($p < 0.05$, Table 2). Formic acid directly acidified the silage, accelerated the ensiling process, enabled the rapid start of the LAB fermentation stage, and reduced the competition of undesirable microorganisms for the substrate WSC, thus increasing the lactic acid content and reducing the pH [40]. The alkali treatment, in contrast, increased pH and inhibited LAB activity, thus reducing lactic acid production. With the microbial treatments, the pH of GLB was lower ($p < 0.05$), that of LB was not altered ($p > 0.05$), and that of FLB was higher ($p < 0.05$) compared to CK. These differences could be explained by the high lactic acid content in the GLB treatment (3.89% DM, Table 2) and low lactic acid content in the LB and FLB treatments (2.02% and 2.01% DM, respectively). WSC, as a substrate for LAB fermentation, can multiply LAB to produce lactic acid and reduce pH, as well as improve silage fermentation quality [41]. The WSC content of LB, GLB, and FLB treatments differed significantly ($p < 0.05$, Table 2). The WSC content in the GLB treatment increased due to the addition of 2% glucose, which enhanced the activity of LAB and produced more lactic acid, while the LB treatment had lower WSC content ($p < 0.05$, Table 2), as was reported in the study by Bai et al [3]. Fucoidans require a certain reaction time to dissolve into monosaccharides [42]. Consequently, in the FLB treatment, small amounts of WSC were provided to LAB during ensiling, and, because of the lag in time, large amounts (4.15% DM) of WSC were left as unused residue. Furthermore, the dissolution process of fucoidan consumed acid and, thus, increased the pH.

FA had the lowest ($p < 0.001$) acetic acid content (0.12% DM) among treatments. Due to its broadly antibacterial properties, FA inhibited the activity of microbes producing acetic acid during ensiling. The acetic acid content was correlated with the relative abundances of *Enterococcus* and *Lactococcus*, which is consistent with the results of Zong et al. [43]. The reduced acetic acid content could prompt a shift in rumen fermentation to propionic acid and reduce methane emission [44]. A recent study reported that propionic acid prevented the use of nutrients by undesirable microorganisms and promoted fermentation [45]. The acetic acid contents in the LB, GLB, and FLB treatments were lower than in CK ($p < 0.05$), while the propionic acid content was greater. This was likely due to the capability of LB to degrade lactic acid into acetic acid and 1,2-propanediol and, subsequently, generate propionic acid [46]. In addition, a negative feedback relationship exists between propionic acid and acetic acid, where propionic acid suppresses the activity of acetic acid-producing microorganisms, thereby reducing the acetic acid content [47]. Adding 0.6% formic acid reduced the butyric acid content and the $NH_3$-N:TN ratio ($p < 0.05$) when compared with CK, which was also reported by Zhao et al. [40]. Formic acid, due to its widespread antimicrobial properties, inhibits undesirable microorganisms such as yeasts and *Clostridium perfringens*, which could grow under anaerobic conditions and break down sugars, organic acids, and proteins to produce butyric acid and $NH_3$-N [48]. The LB, GLB, and FLB treatments reduced the $NH_3$-N:TN ratio ($p < 0.05$), which could be linked to the increase

in propionic acid content that inhibited the growth of undesirable microorganisms. The FLB treatment did not reduce the butyric acid content, which was likely due to the lower propionic acid content (0.53% DM) than in the other treatments. Adding 3% calcium oxide and 3% urea inhibited the fermentation of harmful bacteria and reduced ($p < 0.05$) butyric acid and the $NH_3$-N:TN ratio, which is consistent with the findings of Cook et al. [16].

**Table 2.** Fermentation variables and nutrient contents of alfalfa silages after 60 days.

| Variables | Treatment | | | | | | *p*-Value |
|---|---|---|---|---|---|---|---|
| | CK | FA | CaO | LB | GLB | FLB | |
| Fermentation variables | | | | | | | |
| pH | 5.83 ± 0.02 $^{c}$ | 4.47 ± 0.01 $^{e}$ | 8.62 ± 0.14 $^{a}$ | 5.72 ± 0.09 $^{cd}$ | 5.64 ± 0.01 $^{d}$ | 6.14 ± 0.02 $^{b}$ | <0.001 |
| Lactic acid (%DM) | 2.13 ± 0.07 $^{c}$ | 3.55 ± 0.01 $^{b}$ | 0.13 ± 0.02 $^{e}$ | 2.02 ± 0.01 $^{d}$ | 3.89 ± 0.07 $^{a}$ | 2.01 ± 0.01 $^{d}$ | <0.001 |
| Acetic acid (%DM) | 0.43 ± 0.01 $^{a}$ | 0.12 ± 0.00 $^{e}$ | ND | 0.33 ± 0.01 $^{b}$ | 0.22 ± 0.02 $^{d}$ | 0.28 ± 0.02 $^{c}$ | <0.001 |
| Propionic acid (%DM) | 0.34 ± 0.01 $^{e}$ | 0.90 ± 0.00 $^{b}$ | 0.37 ± 0.02 $^{e}$ | 0.71 ± 0.02 $^{c}$ | 1.08 ± 0.05 $^{a}$ | 0.53 ± 0.04 $^{d}$ | <0.001 |
| Butyric acid (%DM) | 0.05 ± 0.00 $^{ab}$ | 0.04 ± 0.00 $^{abc}$ | 0.01 ± 0.00 $^{d}$ | 0.04 ± 0.00 $^{c}$ | 0.04 ± 0.01 $^{bc}$ | 0.05 ± 0.01 $^{a}$ | <0.001 |
| $NH_3$-N:TN ratio | 13.6 ± 0.08 $^{a}$ | 0.59 ± 0.06 $^{f}$ | 1.84 ± 0.05 $^{e}$ | 10.5 ± 0.23 $^{c}$ | 6.27 ± 0.05 $^{d}$ | 11.2 ± 0.47 $^{b}$ | <0.001 |
| LAB (Lg/cfu/gFW) | 6.52 ± 0.06 $^{c}$ | 6.15 ± 0.02 $^{d}$ | 4.02 ± 0.01 $^{e}$ | 6.96 ± 0.03 $^{b}$ | 7.00 ± 001 $^{b}$ | 7.16 ± 0.02 $^{a}$ | <0.001 |
| Chemical composition | | | | | | | |
| Dry matter (%FW) | 18.9 ± 0.68 $^{d}$ | 22.9 ± 0.93 $^{b}$ | 28.6 ± 0.50 $^{a}$ | 19.2 ± 0.84 $^{d}$ | 22.4 ± 0.82 $^{bc}$ | 21.1 ± 0.65 $^{c}$ | <0.001 |
| Crude protein (%DM) | 17.3 ± 0.51 $^{e}$ | 29.1 ± 0.38 $^{b}$ | 35.2 ± 0.25 $^{a}$ | 20.6 ± 0.46 $^{d}$ | 24.3 ± 0.16 $^{c}$ | 19.7 ± 1.66 $^{d}$ | <0.001 |
| WSC (%DM) | 2.57 ± 0.22 $^{d}$ | 3.39 ± 0.20 $^{c}$ | 4.82 ± 0.36 $^{a}$ | 1.73 ± 0.12 $^{e}$ | 2.68 ± 0.18 $^{d}$ | 4.15 ± 0.11 $^{b}$ | <0.001 |
| NDF (%DM) | 39.4 ± 1.06 $^{a}$ | 36.9 ± 0.77 $^{ab}$ | 26.4 ± 2.91 $^{c}$ | 39.7 ± 2.10 $^{a}$ | 34.5 ± 1.70 $^{b}$ | 40.0 ± 0.44 $^{a}$ | <0.001 |
| ADF (%DM) | 31.7 ± 0.07 $^{a}$ | 24.4 ± 0.21 $^{e}$ | 19.3 ± 0.56 $^{f}$ | 28.8 ± 0.46 $^{c}$ | 25.7 ± 0.15 $^{d}$ | 30.0 ± 0.77 $^{b}$ | <0.001 |

Values are means ± SE. Means with different letters within columns differ from each other ($p < 0.05$); FW, fresh weight; DM, dry matter; $NH_3$-N:TN, ammonia nitrogen/total nitrogen ratio; LAB, lactic acid bacteria; WSC, water-soluble carbohydrate; NDF, neutral detergent fiber; ADF, acid detergent fiber; CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*; ND, not detected.

When compared to CK, the FA treatment reduced the NDF and ADF and increased the DM and CP contents ($p < 0.05$, Table 2), as reported in a previous study [49]. The contents of DM and CP were also higher in the LB, GLB, and FLB treatments than in CK, which could be due to the reduction in harmful microorganisms and, consequently, reduction in the degradations of DM and CP during ensiling. LB, GLB, and FLB treatments decreased the ADF content ($p < 0.05$), but only GLB also decreased the NDF content ($p < 0.05$), when compared to CK. The CaO treatment had the highest DM and CP and lowest NDF and ADF contents ($p < 0.05$) of all treatments, which is consistent with the results of Allen et al. [17]. The higher CP content ($p < 0.05$) could be linked to the urea added with the CaO [50]. The reduction ($p < 0.05$) in ADF and NDF contents could be traced to the ability of CaO to disrupt the plant cell-wall structure and break down the ester bonds between lignin and cellulose. Alkali treatment has been used to improve fiber digestibility and improve the utilization of roughage [16,51]. In addition, the WSC content of the CaO treatment was the highest among all treatments, which was likely due to the high degradations of NDF and ADF.

The FA treatment improved the fermentation quality, but the quantity of LAB was lower (6.15 vs. 6.52 Lg/cfu/g FW) than in CK. The GLB treatment improved the quality of alfalfa silage to a greater extent than the LB and FLB treatments. The FLB treatment also improved the quality of alfalfa silage, but the fucoidan could not be used directly as a fermentation substrate by LAB, in contrast to glucose. Despite the promising application of alkali treatment in reducing the fiber content and energy utilization, it was less effective from the fermentation point of view, as the high pH inhibited the activities of LAB.

### *3.3. Effect of Different Additives on the Bacterial Communities of Alfalfa Silage*

A total of 1194 OTUs were obtained by clustering at the 3% dissimilarity level (Figure 1A). The numbers of OTUs in CK, FA, GLB, FLB, LB, and CaO were 146, 335, 104, 157, 147, and 260, and the unique numbers were 4, 105, 3, 10, 4, and 53, respectively,

among which there were 56 core OTUs. Rarefaction curves plateaued, indicating that the sequencings were saturated and that all microorganisms were identified (Figure 1B).

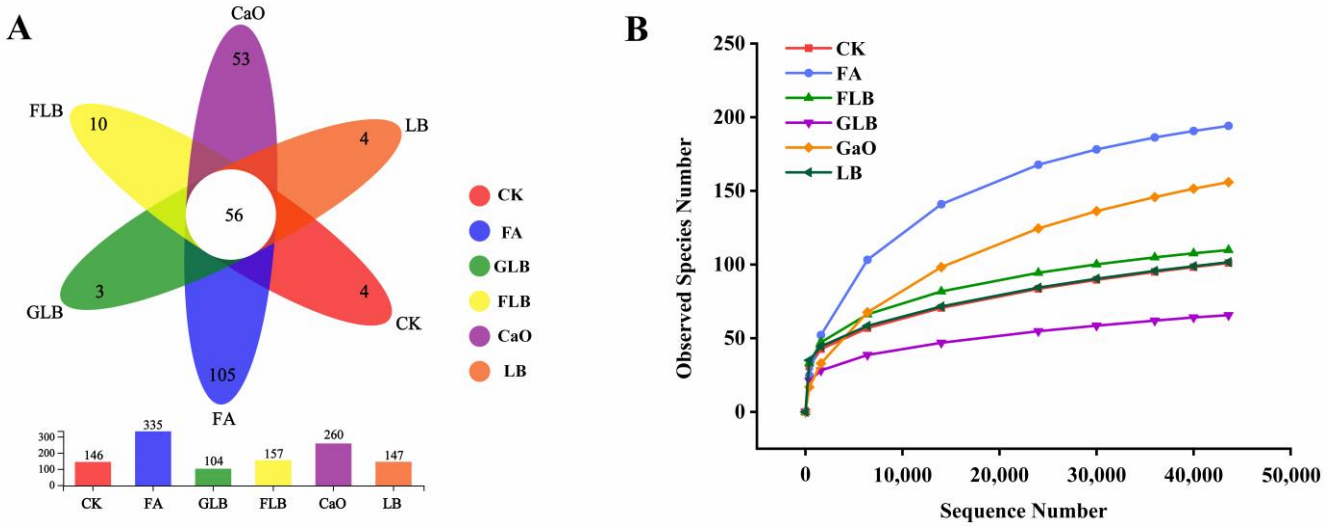



**Figure 1.** Petal plots illustrating the extent of overlapping bacteriological operational taxonomic units (OTUs) in the six treatments. Each petal denotes a treatment, the number in the middle represents the OTUs common to all treatments, and the number on the petal represents the OTUs specific to that treatment. The bar plots present the total bacteriological OTUs in six treatments (**A**). Observed rarefaction curves for species indices. The horizontal coordinate is the number of sequencing strips selected randomly from a sample, and the vertical coordinate is the number of OTUs that could be constructed on the basis of the number of sequencing strips to reflect the sequencing depth. Different treatments are represented by different colors (**B**). CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

#### 3.3.1. Microbial Diversity of Alfalfa Silage

The alpha diversity of bacterial communities in each treatment is presented in Table 3. Good's coverage for each treatment was 1, which indicates that sequencing assays were adequate to cover most of the bacteria. The Simpson, Ace, and Chao indices in the FA and CaO treatments increased, while the Shannon index decreased, indicating that the FA and CaO treatments were able to increase bacterial richness while decreasing diversity. The decrease in Shannon's index in the FA treatment could be a result of the low pH (Table 2), which was reported to be the primary contributor in restricting bacterial diversity [28]. Similarly, Jiang et al. [52] reported a decrease in Shannon's index and an increase in Simpson's index with the addition of formic acid to whole maize silage. The results of CaO treatment might be due to the high pH, which suppressed the growth of various microorganisms [51]. The Ace and Chao 1 indices were lower for the GLB treatment than CK, indicating that the bacterial richness was reduced. This could be explained by the higher contents of lactic and propionic acids in the GLB treatment ($p < 0.05$, Table 2), which inhibited the activity of harmful bacteria during fermentation and, ultimately, decreased bacterial diversity [53,54]. As in the current study, Drouin et al. [55] reported that the microbial diversity decreased, and the quantity of LAB increased in corn silage after adding *L. buchneri*.

PCoA analysis illustrated that the bacterial communities in the different treatments were significantly separated and different, implying the presence of different bacterial communities [56] (Figure 2).

**Table 3.** Bacterial alpha diversity indices in alfalfa silage.

| Treament | Items | | | | |
|---|---|---|---|---|---|
| | Shannon | Simpson | Ace | Chao 1 | Coverage |
| CK | 2.68 ± 0.13 $^{a}$ | 0.11 ± 0.02 $^{b}$ | 179.6 ± 76.0 $^{ab}$ | 152.1 ± 47.5 $^{b}$ | 1 |
| LB | 2.81 ± 0.04 $^{a}$ | 0.10 ± 0.01 $^{b}$ | 175.4 ± 45.6 $^{ab}$ | 136.9 ± 12.1 $^{b}$ | 1 |
| GLB | 2.36 ± 0.09 $^{a}$ | 0.14 ± 0.02 $^{b}$ | 130.1 ± 16.4 $^{b}$ | 98.3 ± 10.0 $^{b}$ | 1 |
| FLB | 2.63 ± 0.08 $^{a}$ | 0.12 ± 0.01 $^{b}$ | 139.3 ± 27.3 $^{b}$ | 137.6 ± 29.0 $^{b}$ | 1 |
| FA | 1.56 ± 0.46 $^{b}$ | 0.39 ± 0.22 $^{ab}$ | 228.4 ± 4.2 $^{a}$ | 219.7 ± 8.8 $^{a}$ | 1 |
| CaO | 1.17 ± 0.90 $^{b}$ | 0.57 ± 0.35 $^{a}$ | 221.4 ± 28.1 $^{a}$ | 213.2 ± 24.4 $^{a}$ | 1 |

Values are means ± SE. Means with different letters within a column differ from each other ($p < 0.05$); CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

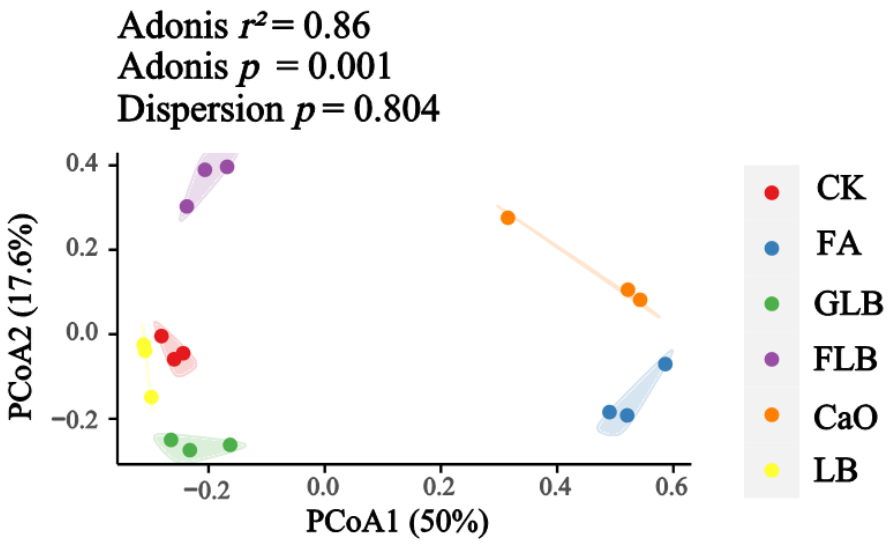



**Figure 2.** Principal coordinates analysis (PCoA) plot based on the Bray–Curtis dissimilarity matrix of the bacterial communities in alfalfa silages. Adonis: permutational Multivariate analysis of variance (MANOVA). CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

#### 3.3.2. Composition of Bacterial Communities in Alfalfa Silage

The relative abundances of the bacteria at the phylum level are presented in Figure 3, and those at the genus level are presented in Figure 4. The differences in microbial communities among treatments, determined by the linear discriminant analysis (LDA) effect size (LEfSe) method (LDA score > 4.5), are presented in Figure 5. Firmicutes and Proteobacteria were the dominant phyla in each treatment, except for the CaO treatment. In the CK treatment, Peptostreptococcales–Tissierellales was the most abundant order, and *Sedimentibacter* was the most abundant genus (Figure 5).

The FA treatment had the highest relative abundance of Proteobacteria (51.7%) of all treatments (Figure 3), while *Delftia* (49.6%) and *Bacillus* (25.5%) were the dominant genera in the treatment (Figure 4). *Delftia* is a plant growth-promoting bacterium, which not only increases the absorption of nutrients, but also resists pathogens [57]. Several *Bacillus* spp. produce ferulic acid esterase, which disrupts the ester bonds between cell walls and releases side-chains to form a polysaccharide skeleton, thus improving the rumen degradation of feed [58]. This could explain the decrease in NDF (36.9% DM) and ADF (24.4% DM) contents in the FA treatment after 60 days of ensiling (Table 2). In the CaO treatment, Cyanobacteria (69.2%) and Proteobacteria (25.1%) accounted for 94.3% of the total number of bacteria, while the relative abundance of Firmicutes was only 5.34% (Figure 3). The relative abundances of *Lactobacillus* and *Pediococcus* increased ($p < 0.05$) in GLB and these

two bacterial genera were the highest among treatments (Figure 4), which was consistent with the LAB count results (Table 2). *L. buchneri* promoted lactic acid fermentation by increasing the LAB content; thus, the GLB treatment also had the highest lactic acid content (Table 2). *Clostridium_sensu_stricto_18* was higher in CK, LB, and FLB than in the other treatments with 1.85%, 1.02%, and 2.25%, respectively (Figure 4). These results could be explained by the butyric acid content of FLB (0.04% DM) and the higher $NH_3$-N:TN ratio in the CK, LB, and FLB treatments (13.6, 10.5, and 11.2, respectively) (Table 2). *Clostridia* are divided into two physiological groups: glycolytic *Clostridia*, which produce butyric acid, and proteolytic *Clostridia*, which degrade proteins to $NH_3$-N [59,60]. In general, most of the bacteria during fermentation were among *Lactobacillus*, *Pedicoccus*, *Lactococcus*, *Weisseria* and *Leuconostoc* [53,61].

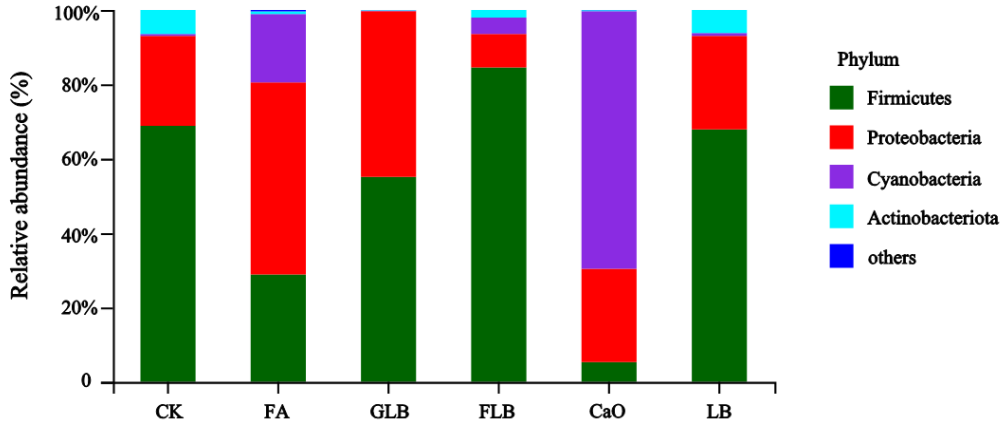



**Figure 3.** Bacterial communities and relative abundances at the phylum level in alfalfa silage after 60 days of fermentation. CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

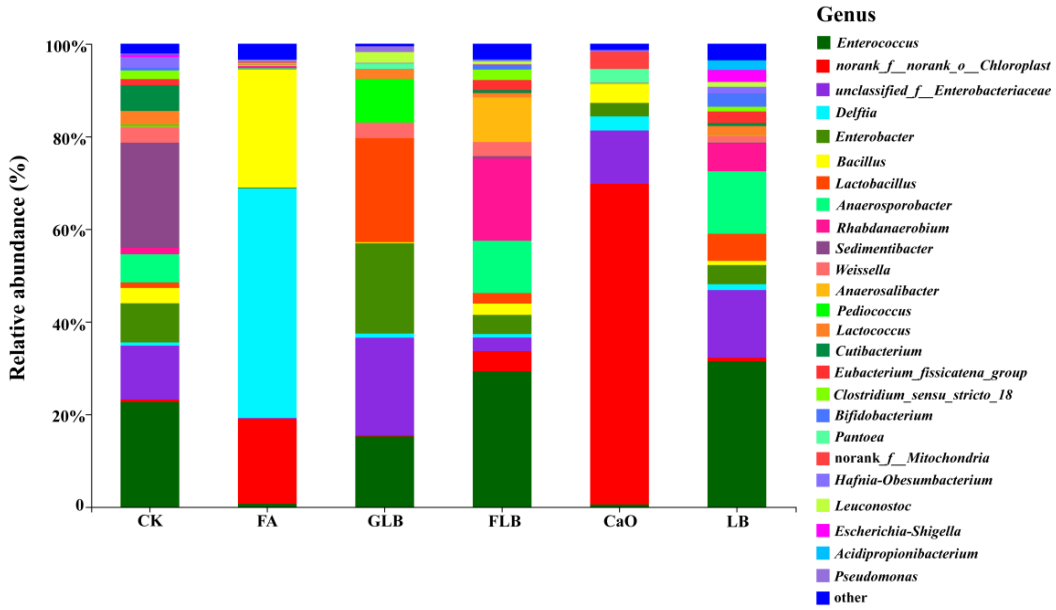



**Figure 4.** Bacterial communities and relative abundances at the genus level in alfalfa silage after 60 days of fermentation. CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

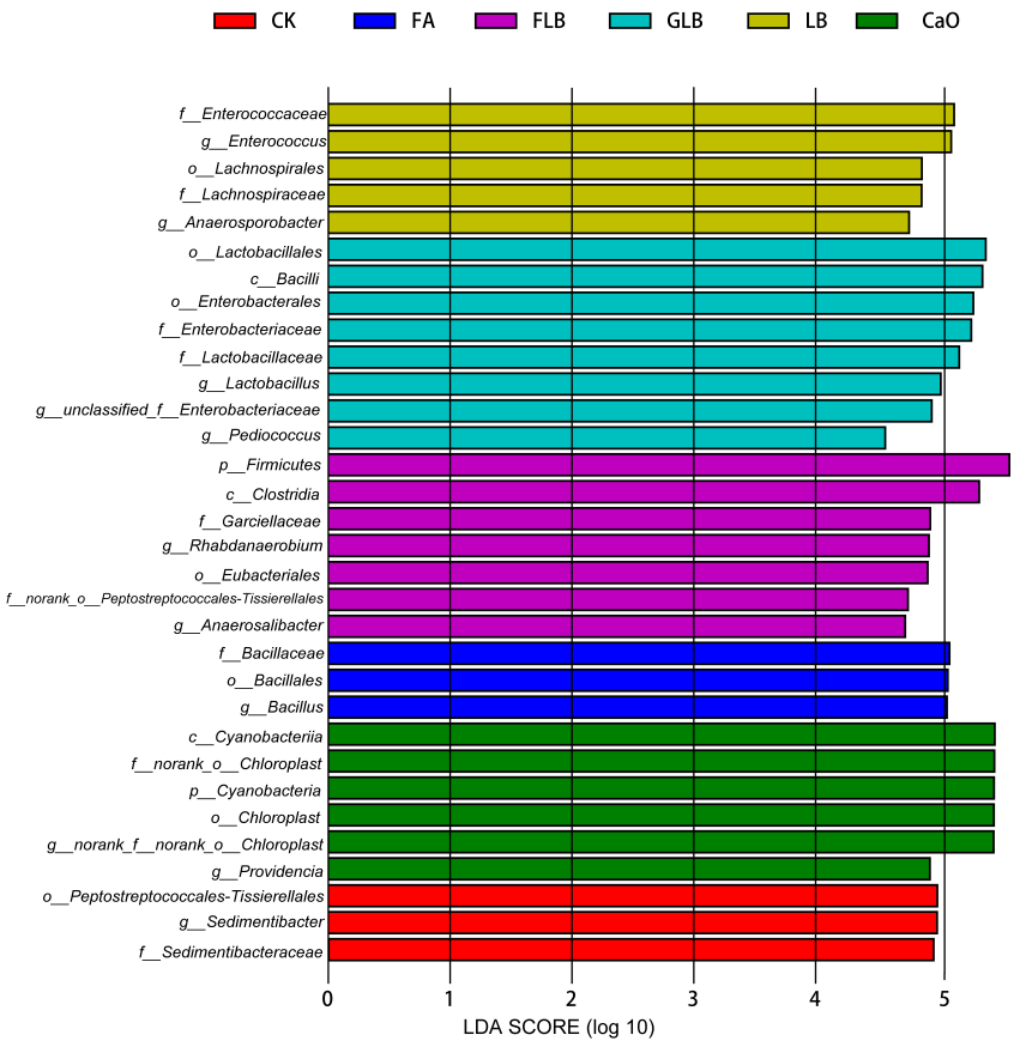



**Figure 5.** The differentially abundant bacterial taxa identified by linear discriminant analysis effect size (LEfSe) among treatments (CK, FA, CaO, LB, GLB, and FLB) and their cladograms. LDA, linear discriminant analysis. CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

### 3.3.3. Correlations between Relative Abundance of Bacteria and Nutrient Content or Fermentation Variables of Alfalfa Silage

The relationships between the relative abundance of bacteria at the genus level and silage variables were tested by Spearman's correlation coefficient, using 20 genera of bacteria (Figure 6). *Enterococcus* was correlated positively with the contents of NDF, ADF, and acidic acid, as well as with the ratio of $NH_3$-N:TN, and negatively with the contents of WSC, DM, and CP. In addition, *Bifidobacterium* ($r = -0.79$ and $-0.77$), *Cutibacterium* ($r = -0.80$ and $-0.79$), *Sedimentibacter* ($r = -0.75$ and $-0.80$), *Clostridium_sensu_stricto_18* ($r = -0.76$ and $-0.75$), *Eubacterium_fissicat*ena_group ($r = -0.70$ and $-0.71$), *Anaerosporobacter* ($r = -0.67$ and $-0.60$), *Rhabdanaerobium* ($r = -0.60$ and $-0.62$), *Weissella* ($r = -0.53$ and $-0.80$), and *Lactococcus* ($r = -0.77$ and $-0.68$) were correlated negatively with DM and CP contents. *Pantoea*, *norank_f__ norank_ o__ Chloroplast* and *norank_ f__ Mitochondria* were correlated positively with DM ($r = 0.53$, 0.61, and 0.61) and CP ($r = 0.63$, 0.59, and 0.55) contents (Figure 6). The relative abundances of *Pantoea* and *norank_f__ Mitochondria* in all treatments were low (Figure 4). However, the relative abundance of *norank_ f__ norank_ o__ Chloroplast* was the highest in the CaO (69.3%) and FA (18.4%) treatments, which could explain the higher DM and CP contents in these treatments than in CK (Table 2). Further-

more, *norank_f__ norank_ o__ Chloroplast* was correlated positively with WSC ($r$ = 0.71) and negatively with ADF ($r$ = −0.56), which could be linked to the degradation of ADF and the generation of WSC. *Lactobacillus* and *Delftia* were correlated positively with propionic acid content (r = 0.49 and 0.50, Figure 6). The highest relative abundances of *Lactobacillus* and *Delftia* were in LB and FA (Figure 4), which also had a higher content of propionic acid than the other treatments ($p < 0.05$, Table 2). As a heteromorphic fermented LAB, *Weissella*, which produces lactic acid and acetic acid [62], was correlated positively with the content of lactic acid (Figure 6). *Weissella*, *Lactococcus*, *Bifidobacterium*, *Cutibacterium*, and *Sedimentibacter* were correlated positively with acetic acid content ($r$ = 0.76, 0.78, 0.81, and 0.76, respectively) and the $NH_3$-N:TN ratio ($r$ = 0.67, 0.67, 0.74, and 0.80, respectively). Both plant enzymes and microbial activities could produce $NH_3$-N, and rapid acidification of *Lactococcus* could reduce $NH_3$-N [63].

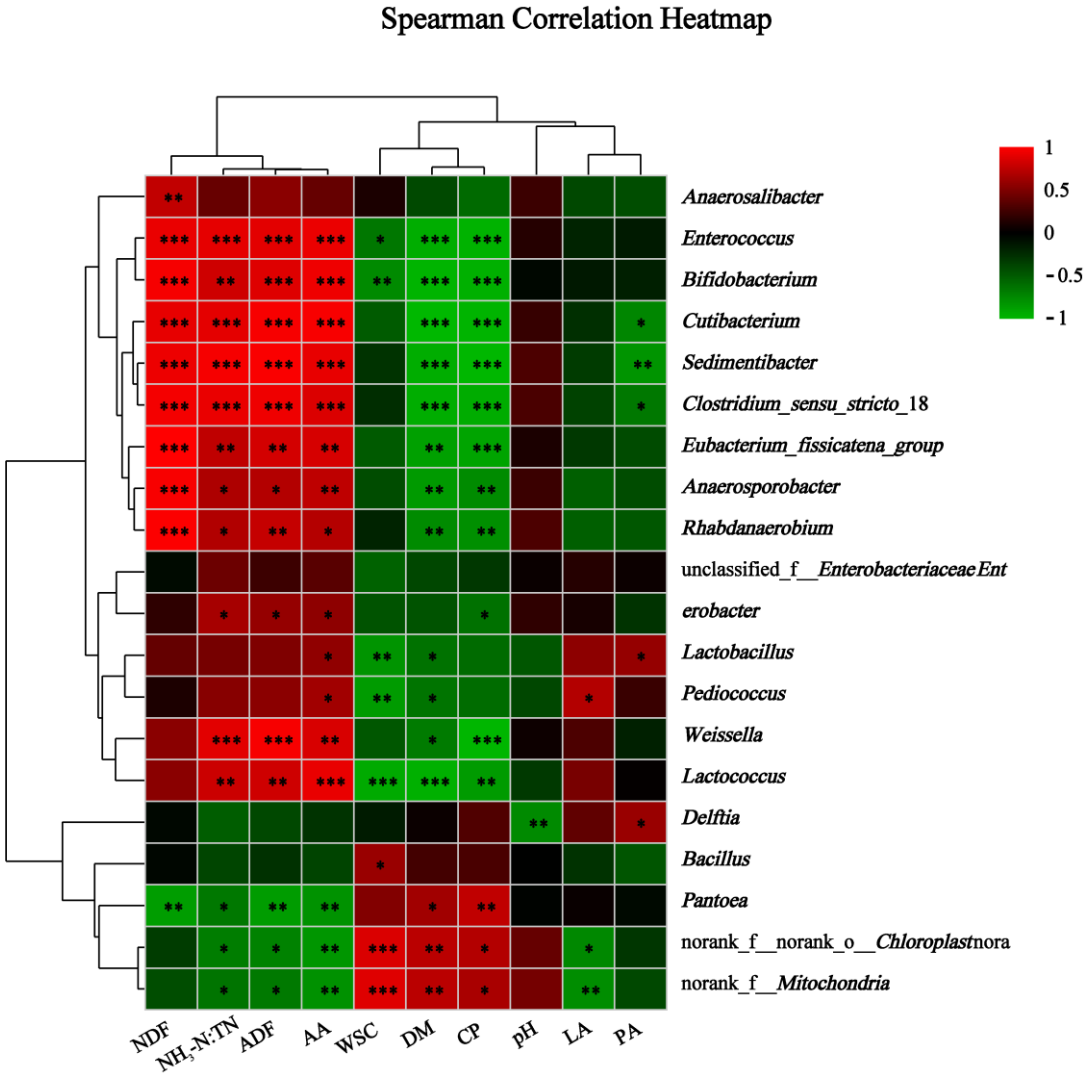



**Figure 6.** Heatmap displaying the correlations between the relative abundances of bacteria at the genus level and the fermentation variables of alfalfa silage. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DM, dry matter; $NH_3$-N:TN, ammonia nitrogen:total nitrogen ratio; CP, crude protein; WSC, water-soluble carbohydrate; NDF, neutral detergent fiber; ADF, acid detergent fiber; LA, lactic acid; AA, acidic acid; PA, propionic acid.

### *3.4. Rumen Degradation of Alfalfa Silage*

The degradations of DM, CP, ADF, and NDF in all treatments increased gradually with an increase in time, but the rate of increase differed in each time period (Figure 7).

The FA treatment had a higher DM degradation at 72 h than the other treatments ($p < 0.05$, Figure 7), and a higher ($p < 0.05$) fast degradation fraction (a%) and effective degradation rate (ED%) than CK (Table 4). Zhang et al. also reported that the addition of formic acid increased the digestibility of DM [64]. In addition, the DM degradation and the DM instant fraction and effective degradation at 72 h were higher ($p < 0.05$) in the GLB treatment than in CK, as was also found by Wang et al. [58]. In contrast, DM degradation in the CaO treatment was lower ($p < 0.05$) than in CK, which could be explained by the high pH, which was above the optimal range for rumen microorganisms. However, CP degradation at 72 h was highest in the CaO treatment, since this treatment contained more CP, due to non-protein N, than the other treatments [48]. Furthermore, the CaO treatment had the highest WSC content after 60 days of ensiling, which provided energy for rumen microbial activity and promoted rumen microbial digestion and absorption. The addition of urea has also been reported to contribute to feed degradation [65].

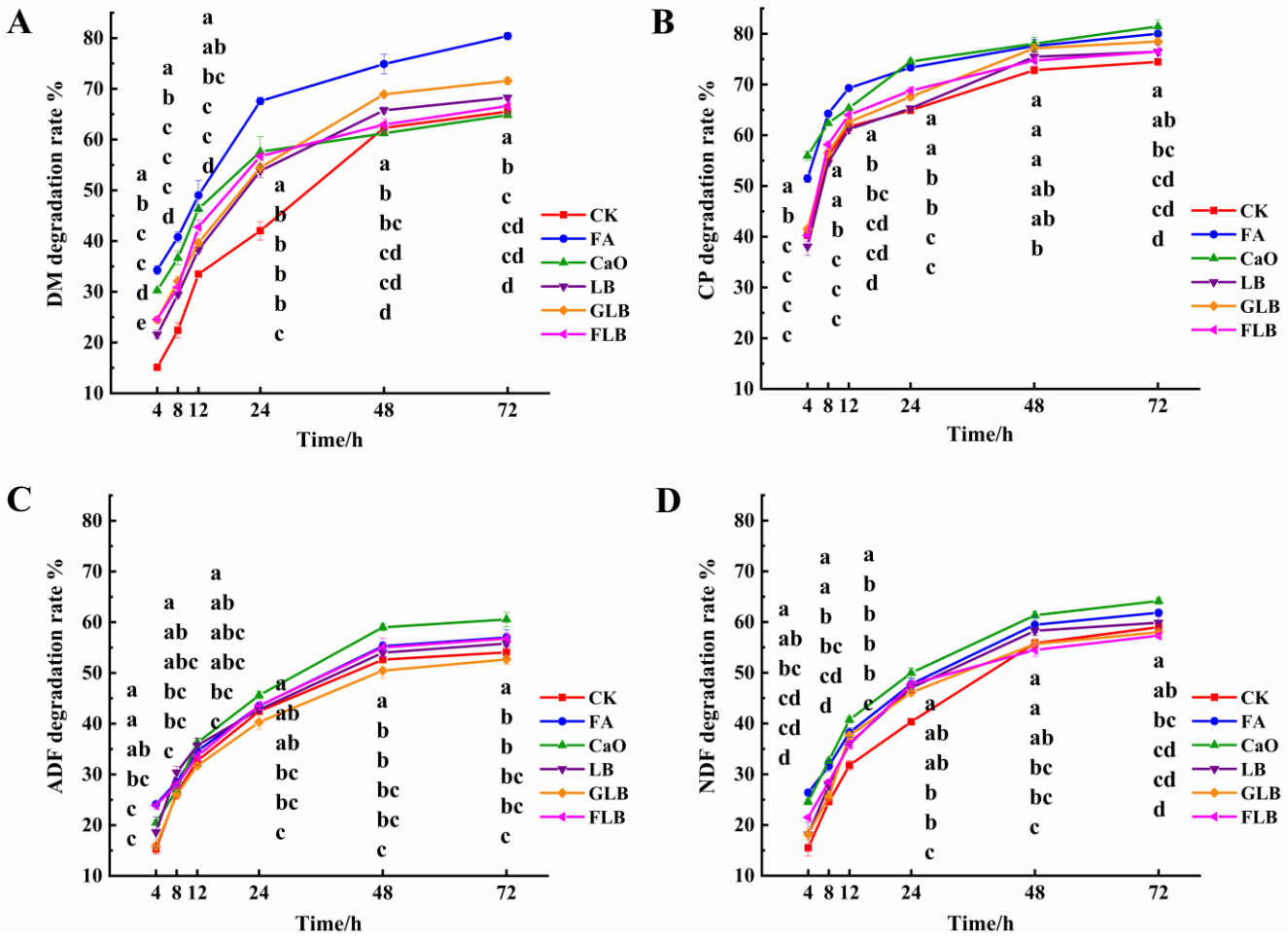



**Figure 7.** The rumen degradations of DM, CP, NDF, and ADF in alfalfa silage. Means with different letters at the same time differ from each other ($p < 0.05$); values are means ± SE; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed (inclusive of residual ash); CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

**Table 4.** Ruminal degradation kinetics of DM, CP, NDF, and ADF of alfalfa silage.

| Items | | a, % | b, % | a + b, % | c, $h^{-1}$ | ED, % | $R^2$ |
|---|---|---|---|---|---|---|---|
| DM | CK | 6.4 ± 1.41 $^{d}$ | 63.8 ± 1.43 $^{a}$ | 70.2 ± 0.02 $^{c}$ | 0.04 ± 0.00 $^{c}$ | 45.0 ± 0.54 $^{e}$ | 0.98 |
| | FA | 21.0 ± 1.70 $^{a}$ | 59.8 ± 1.53 $^{ab}$ | 80.8 ± 0.17 $^{a}$ | 0.06 ± 0.00 $^{b}$ | 62.1 ± 0.65 $^{a}$ | 0.98 |
| | CaO | 16.9 ± 1.80 $^{ab}$ | 47.2 ± 1.67 $^{c}$ | 64.0 ± 0.13 $^{d}$ | 0.08 ± 0.01 $^{a}$ | 52.4 ± 0.54 $^{bc}$ | 0.97 |
| | LB | 8.9 ± 0.77 $^{d}$ | 60.9 ± 0.69 $^{ab}$ | 69.8 ± 0.08 $^{c}$ | 0.05 ± 0.00 $^{b}$ | 50.6 ± 0.30 $^{d}$ | 0.99 |
| | GLB | 13.6 ± 0.66 $^{bc}$ | 60.7 ± 0.60 $^{ab}$ | 74.3 ± 0.06 $^{b}$ | 0.05 ± 0.00 $^{bc}$ | 53.1 ± 0.27 $^{b}$ | 0.99 |
| | FLB | 9.7 ± 1.53 $^{cd}$ | 56.7 ± 1.41 $^{b}$ | 66.4 ± 0.12 $^{d}$ | 0.07 ± 0.00 $^{a}$ | 51.2 ± 0.50 $^{cd}$ | 0.98 |
| CP | CK | 21.6 ± 3.04 $^{cd}$ | 50.6 ± 2.89 $^{bc}$ | 72.2 ± 0.15 $^{e}$ | 0.13 ± 0.01 $^{ab}$ | 63.7 ± 0.63 $^{c}$ | 0.95 |
| | FA | 33.9 ± 2.31 $^{b}$ | 44.0 ± 2.22 $^{c}$ | 79.2 ± 1.28 $^{b}$ | 0.13 ± 0.01 $^{a}$ | 70.9 ± 0.45 $^{a}$ | 0.96 |
| | CaO | 49.2 ± 1.64 $^{a}$ | 31.7 ± 1.49 $^{d}$ | 80.9 ± 0.15 $^{a}$ | 0.06 ± 0.01 $^{d}$ | 71.8 ± 0.58 $^{a}$ | 0.98 |
| | LB | 21.5 ± 3.07 $^{cd}$ | 53.2 ± 2.92 $^{ab}$ | 74.7 ± 0.16 $^{d}$ | 0.11 ± 0.01 $^{b}$ | 64.4 ± 0.72 $^{bc}$ | 0.94 |
| | GLB | 28.0 ± 2.31 $^{bc}$ | 49.1 ± 2.18 $^{bc}$ | 77.1 ± 0.13 $^{c}$ | 0.09 ± 0.01 $^{c}$ | 66.6 ± 0.59 $^{b}$ | 0.96 |
| | FLB | 13.2 ± 3.41 $^{d}$ | 60.9 ± 3.28 $^{a}$ | 74.2 ± 0.13 $^{d}$ | 0.15 ± 0.01 $^{a}$ | 65.5 ± 0.59 $^{bc}$ | 0.96 |
| ADF | CK | 4.9 ± 1.10 $^{c}$ | 49.4 ± 1.00 $^{ab}$ | 54.3 ± 0.09 $^{d}$ | 0.07 ± 0.00 $^{a}$ | 40.6 ± 0.37 $^{c}$ | 0.99 |
| | FA | 17.1 ± 0.75 $^{a}$ | 42.6 ± 0.71 $^{c}$ | 59.7 ± 0.04 $^{b}$ | 0.04 ± 0.00 $^{b}$ | 43.7 ± 0.30 $^{b}$ | 0.99 |
| | CaO | 12.2 ± 1.30 $^{ab}$ | 50.9 ± 1.19 $^{a}$ | 63.1 ± 0.11 $^{a}$ | 0.05 ± 0.00 $^{b}$ | 45.2 ± 0.53 $^{a}$ | 0.98 |
| | LB | 10.7 ± 1.81 $^{b}$ | 45.2 ± 1.64 $^{bc}$ | 0.06 ± 0.01 $^{a}$ | 0.06 ± 0.01 $^{a}$ | 42.7 ± 0.65 $^{b}$ | 0.96 |
| | GLB | 7.5 ± 1.20 $^{bc}$ | 45.4 ± 1.09 $^{bc}$ | 0.06 ± 0.00 $^{a}$ | 0.06 ± 0.00 $^{a}$ | 40.1 ± 0.42 $^{c}$ | 0.98 |
| | FLB | 16.6 ± 0.93 $^{a}$ | 43.1 ± 0.89 $^{c}$ | 0.04 ± 0.00 $^{b}$ | 0.04 ± 0.00 $^{b}$ | 43.4 ± 0.38 $^{b}$ | 0.98 |
| NDF | CK | 8.3 ± 1.03 $^{bc}$ | 53.3 ± 0.97 $^{a}$ | 61.6 ± 0.06 $^{b}$ | 0.04 ± 0.00 $^{b}$ | 41.9 ± 0.42 $^{c}$ | 0.99 |
| | FA | 18.8 ± 0.83 $^{a}$ | 45.3 ± 0.77 $^{c}$ | 64.6 ± 0.50 $^{a}$ | 0.04 ± 0.00 $^{b}$ | 47.6 ± 0.34 $^{a}$ | 0.99 |
| | CaO | 15.0 ± 0.87 $^{a}$ | 48.8 ± 0.78 $^{bc}$ | 63.8 ± 0.09 $^{a}$ | 0.04 ± 0.00 $^{b}$ | 48.8 ± 0.33 $^{a}$ | 0.99 |
| | LB | 6.8 ± 1.28 $^{bc}$ | 53.8 ± 1.16 $^{a}$ | 60.6 ± 0.12 $^{c}$ | 0.06 ± 0.00 $^{a}$ | 44.9 ± 0.46 $^{b}$ | 0.98 |
| | GLB | 5.6 ± 1.32 $^{c}$ | 52.3 ± 1.21 $^{ab}$ | 57.9 ± 0.11 $^{d}$ | 0.07 ± 0.00 $^{a}$ | 43.7 ± 0.44 $^{b}$ | 0.98 |
| | FLB | 10.7 ± 0.80 $^{b}$ | 46.8 ± 0.73 $^{c}$ | 57.4 ± 0.07 $^{d}$ | 0.06 ± 0.00 $^{a}$ | 44.1 ± 0.28 $^{b}$ | 0.99 |

Means with different letters within a column differ from each other ($p < 0.05$); values are means ± SE; a, rapid degradation part (%); b, slow degradation part (%); c, degradation rate of slow degradation part; ED, effective degradation rate (%); DM, dry matter; CP, crude protein; NDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed (inclusive of residual ash); CK, control, no additive; FA, 0.6% formic acid; CaO, 3% calcium oxide and 3% urea; LB, $1 \times 10^6$ cfu/g *L. buchneri*; GLB, 2% glucose and $1 \times 10^6$ cfu/g *L. buchneri*; FLB, 2% fucoidan and $1 \times 10^6$ cfu/g *L. buchneri*.

The degradabilities of ADF and NDF in the CaO treatment were greater ($p < 0.05$) than in the other groups at 72 h, and the EDs of DM, ADF, and NDF in this treatment were higher than in all other treatments. This was due, most likely, to the disruption of the connection between polyester and cellulose by the alkali treatment, which enabled the solubilization and utilization of structural carbohydrates by microorganisms and proteases in the rumen [51]. In the FA treatment, the EDs of ADF and NDF were greater ($p < 0.05$) than in CK, which might be due to the production of ferulic acid esterase by *Bacillus* (see Section 3.3.2).

## 4. Conclusions

After 60 days of ensiling alfalfa, the FA, CaO, LB, GLB, and FLB treatments altered the bacterial communities, improved fermentation quality, and decreased nutrient loss. The addition of LB increased the relative abundance of *Lactobacillus*. The GLB treatment reduced the NDF content and $NH_3$-N:TN ratio in the 60 day silage and increased the relative abundance of LAB and the content of lactic acid. The FA treatment lowered the pH and reduced the degradations of DM and CP, whereas the CaO treatment increased the pH, decreased the NDF and ADF content, and enhanced the degradations of NDF and ADF. Both these treatments inhibited the relative abundance of *Enterobacter*. In conclusion, FA, LB, GLB, and FLB treatments improved the quality of alfalfa silage, and the CaO treatment increased the ruminal degradation of the silage.

**Author Contributions:** Conceptualization, F.Y. and J.Z.; methodology, L.Z., W.L. and Q.F.; software, L.Z., W.L. and Q.F.; validation, L.Z., W.L. and Q.F.; formal analysis, L.Z., W.L. and Q.F.; investigation, L.Z., F.Y., J.Z., Q.F., W.L., J.L., Y.L. (Yan Li), Y.Z. and Y.L. (Yijia Liu); resources, F.Y. and Y.Q.; data curation, L.Z., W.L. and Q.F.; writing—original draft preparation, W.L., L.Z., Q.F. and J.L.; writing—review and editing, F.Y., J.Z. and A.A.D.; visualization, A.A.D.; supervision, F.Y. and J.Z.; project administration, F.Y. and J.Z.; funding acquisition, F.Y. and J.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was jointly funded by the National Natural Science Foundation of China (42075116 and 32101418), the Research Funds for Distinguished Young Scientists in Fujian Agriculture and Forestry University (xjq201817), and the Fujian Provincial Subsidy Project for Science and Technology Special Commissioner (2022S2071).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We thank Jun Lei, Sutao Li, Xuelin Han, Juan Zhang, Qianfu Gan, and others for their work associated with silage and animal experiments. We are grateful to the two reviewers for their constructive suggestions.

**Conflicts of Interest:** The authors declare no conflict of interest.

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