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Citric Acid Influences the Dynamics of the Fermentation Quality, Protease Activity and Microbial Community of Mulberry Leaf Silage

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Abstract: Mulberry (*Morus alba*) leaves has performed well as a high-quality protein supplement for livestock and enriches the edible resources of livestock. However, the harvest of mulberry leaves is seasonal and occurs mainly during the rainy season in southeast China; therefore, humid and sultry weather causes serious losses of mulberry leaf biomass, which pose a challenge for the preservation of mulberry leaves. In this study, we used the silage fermentation method to preserve mulberry leaves and investigated the effects of citric acid on the silage quality of mulberry leaves. Mulberry leaves were ensiled with or without 1% citric acid and 2% citric acid. The chemical composition, protein fraction and microbial community of mulberry leaf silages were analyzed. The results showed that the silage treated with citric acid had a higher dry matter recovery and lactic acid content and a lower acetic acid content, non-protein nitrogen content and ammonia-N content; citric acid also inhibited the activities of carboxypeptidase and aminopeptidase. Moreover, citric acid increased *Lactobacillus* abundance in silages and decreased the abundance of undesired microorganisms, such as *Enterobacter*. In summary, the addition of citric acid improved the fermentation quality of mulberry leaf silages, with 2% citric acid being more effective than 1% citric acid.

Keywords: mulberry leaves; silage; citric acid; fermentation quality

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1. Introduction

Mulberry (*Morus alba*) is a traditional, economical woody plant and is cultivated across more than 780,000 ha in China [1]. The biomass yield of fresh mulberry leaves (ML) ranges from 25 to 30 mt/ha/yr [2]. The crude protein of ML ranges from 15 to 27.6% dry matter (DM) and ML is rich in minerals (2.42–4.71% Ca, 0.23–0.97% P, 1.66–3.25% K) [3]. As it is rich in nutrients, ML has performed well as a high-quality protein supplement for livestock [4]. In addition, ML has many biological benefits because it has antioxidant, anti-inflammatory, antidiabetic and antihyperglycemic properties, etc., and thus could potentially replace antibiotics to improve animal immunity [5,6]. However, the harvest of ML is seasonal and occurs mainly during the rainy season in southeast China, making it difficult to dry fresh ML naturally.

Ensiling is an efficient, low-cost and suitable method of preserving ML quality which can overcome the seasonal imbalance between forage resources and animal production. In a previous study, it was observed that proteolysis was a very serious problem in ML silage [7]. It is known that the activities of plant enzymes and microorganisms are mainly responsible for proteolysis, which can degrade proteins into peptides and further into ammonia-N [8]. However, information regarding the dynamics of proteolysis and the activities of protease and microorganisms in ML silage is lacking. Evaluation of the proteolytic mechanism would be beneficial in providing a theoretical basis for finding suitable proteolytic inhibitors of ML silage.

Citric acid (CA) is an organic acid with a higher pKa than lactic acid, which could directly acidize the fermentation environment [9,10]. CA is easy to obtain by microorganism fermentation. With a pleasant, tart flavor and low cost, CA is widely used in the food and beverage industries and as a complement in animal feed [11]. Compared with formic acid and sulfuric acid, CA might be a milder acidic additive in silage [12]. In previous studies, the addition of CA improved the quality of alfalfa silage and decreased *Clostridium* and *Escherichia* abundance in *Amomum villosum Lour* silages [13,14]. However, little information about CA's influence on the protein fractions and protease activities of silages is available.

Therefore, the goal of this study was to investigate the effect of CA on the fermentation parameters, protein, protease activities and bacterial community of ML silage.

2. Materials and Methods

2.1. Pre-Ensiled Material and Silage Preparation

ML was cultivated and harvested from an experiment field in December 2020 at South China Agricultural University (Guangzhou, China, 113°35′ E, 23°16′ N). CA was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The ML was immediately chopped to a suitable length (1–2 cm) in a clean test bed when it was harvested. The ML was then ensiled with 1% or 2% CA. A control group (CK) involved no addition of CA. In detail, approximately 100 g ML was ensiled in an enclosed bag (20 cm × 30 cm) in triplicate for every treatment. In total, 36 sealed silage bags (3 treatments × 4 ensiling times × 3 repeats) were made and kept at ambient temperature (17–28 °C). After 3, 7, 14 and 30 days of ensiling, to evaluate the fermentation parameters, protease activity and the bacterial community, three silage bags from each treatment were opened at random.

2.2. Microorganism and Fermentation Parameter Analyses

We used the flat colony counting method to count the microbial population of silages according to Yan et al. [15]. A sample (10 g) from each silage bag and sterilized saline water (90 mL) were homogenized in a blender and then serially diluted. The population of lactic acid bacteria was counted in Man Rogosa Sharpe agar and that of coliform bacteria was counted in Violet Red Bile agar, and samples were subsequently incubated at 30 °C for 48 h. Molds and yeasts were cultured and counted in Rose Bengal agar at 28 °C for 48 h and then categorized by colony morphology. All microbial population counts were log10 transformed.

Another sample (10 g) from each silage bag was diluted in sterile distilled water (90 mL) and stored in a freezer at 4 °C for 18 h, then the homogenized sample was filtered. Part of the filtrate was immediately used to measure pH, and the remaining filtrate was used to measure the ammonia-N content and organic acid content. Following Broderick and Kang [16], the phenol-hypochlorite procedure was used to measure the ammonia-N content. The concentration of organic acids was measured using high-performance liquid chromatography (HPLC) (column, Shodex RSpak KC-811S-DVB gel C (8.0 mm × 30 cm; Shimadzu, Tokyo, Japan); oven temperature, 50 °C; mobile phase, 3 mmol/L $HClO_4$; flow rate, 1.0 mL/min; injection volume, 5 μL; detector, SPD-M10AVP) [17].

An oven was used to dry the remaining silage at 65 °C for 48 h for measurement of the DM content. For chemical analysis, these dry samples were ground with a grinder (FW100, Taisite Instrument Co., Ltd., Tianjin, China) and passed through a 1.00 mm screen. The powder samples were used to measure protein, fiber and water-soluble carbohydrates. The content of protein fractions, neutral detergent fiber and acid detergent fiber were determined by the method of Wang et al. [2]. Briefly, crude protein and true protein were analyzed using the Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto-Analyzer, FOSS Analytical AB, Hoganas, Sweden). Non-protein nitrogen was calculated from the difference between crude and true protein. Neutral detergent fiber (NDF) and acid detergent fiber content were measured without use of heat-stable amylase and sodium sulfite in an A220 Fiber Analyzer (ANKOM Technology Corp., Macedon, NY, USA). The water-soluble carbohydrate content was analyzed by the anthrone method.

2.3. Protease Activity Analysis

The protease was extracted according to the method of He et al. [8], with some modifications. A sample (10 g) from the silage bag and a 0.1 M sodium phosphate buffer (50 mL, pH 6.0 with 5 mM hyposulfite) were homogenized in a blender, then the homogenate was filtered and the filtrate was centrifuged at 10,000× *g* at 4 °C for 15 min. The supernatant was retained at −80 °C for later use. According to the method of Guo et al. [18], acid protease activity, carboxypeptidase activity and aminopeptidase activity were measured.

2.4. Analysis of Microbial Communities

Following the manufacturer's instructions, microbial DNA was extracted by the HiPure Stool DNA Kits (Magen, Guangzhou, China). The primers 341F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used for amplifying 16S rDNA V3-V4 in a PCR thermal cycler (FlexCycler; Analytik Jena, Jena, Germany). DNA was amplified according to the procedure of Zhang et al. [19]. Illumina NovaSeq 6000 was used to sequence the purified PCR products. The raw reads were assembled according to the procedure set by Liu et al. [20].

2.5. Statistical Analysis

The analyses of the fermentation parameters were performed with SAS version 9.1 (SAS Institute, Cary, NC, USA) to evaluate the influence of treatment and ensiling time. Duncan's multiple range tests were used to measure mean values. $p < 0.05$ was considered significant and $p < 0.01$ was considered extremely significant. In addition, Pearson's correlation analysis between protein fractions and protease activity was conducted with SAS.

3. Results and Discussion

3.1. Characteristics of Pre-Ensiled ML

The characteristics of the pre-ensiled ML are shown in Table 1. The DM content of ML was 374 g/kg fresh matter (FM). The relatively high DM content of ML is comparable with that of tropical legumes, such as alfalfa and stylo [21]. The crude protein content of ML was 139 g/kg DM. It was lower than the 188 g/kg DM reported by Wang et al. [2]; this might be because it was at the mature stage [22]. Neutral detergent fiber is an inferior predictor of digestibility [7]. The low neutral detergent fiber content (440 g/kg DM) indicated that ML probably had better digestive efficiency as a fodder for animals. The water-soluble carbohydrates were a necessary fermentation substrate during the ensiling process and its content of greater than 60 g/kg DM was a guarantee of thorough fermentation [19]. Water-soluble carbohydrate content was 114 g/kg DM in this study, which was enough to support the acid production efficiency of microorganisms. Epiphytic lactic acid bacteria and coliform bacteria counts were 5.72 log10 cfu/g FM and 5.21 log10 cfu/g FM, respectively. Yeasts and molds were below detectable levels (2.00 log10 cfu/g FM). During the ensiling, lactic acid bacteria counts of more than 5.00 log10 cfu/g FM are considered to be a superior predictor of fermentation quality [23]. However, the relatively high coliform bacteria counts might result in poor fermentation, which suggests that fermentation inhibitors were necessary to inhibit undesirable microorganisms.

Table 1. The chemical composition and microbial population of pre-ensiled mulberry leaves.

Items	Mulberry Leaves ± SEM
Dry matter (g/kg FM)	374 ± 12.8
Crude protein (g/kg DM)	139 ± 1.9
Neutral detergent fiber (g/kg DM)	440 ± 35.7
Acid detergent fiber (g/kg DM)	353 ± 24.8
Water soluble carbohydrates (g/kg DM)	114 ± 3.2
Lactic acid bacteria (log_{10} cfu/g FM)	5.72 ± 0.1
Coliform bacteria (log_{10} cfu/g FM)	5.21 ± 0.3

Table 1. *Cont.*

Items	Mulberry Leaves ± SEM
Yeasts (log_{10} cfu/g FM)	<2.00
Molds (log_{10} cfu/g FM)	<2.00

FM, fresh material; DM, dry matter; SEM, standard error of the mean; cfu, colony-forming unit.

3.2. The Fermentation Parameters, Organic Acids and Microbial Population of ML Silages

DM recovery, the dynamics of the pH value, organic acid content and microbial population counts in ML silages are shown in Figure 1. The factor analysis revealed that CA had a significant effect ($p < 0.01$) on DM recovery, pH, lactic acid content, acetic acid content, lactic acid bacteria counts and coliform bacteria counts in ML silages. DM loss is a common issue during every stage of ensiling, which reduces the silage yield [24]. The silages treated with CA showed higher DM recovery than the CK, and the highest DM recovery occurred in the silages treated with 2% CA after 30 days of ensiling. The increase in DM recovery would significantly improve the economic efficiency of ML as an excellent protein supplement for livestock. The pH is the most intuitive fermentation parameter, which directly reflects the quality of silage. The pH value of silages treated with CA was lower than that of the CK during the ensiling process in this study. The pH value of ML silages reached stability in 3 days of ensiling, and the final pH was lower than 4.5 with the addition of 2% CA. It might be that enough CA directly reduced the pH value due to its strong acidity, with a pK1 3.13 [10]. However, the pH value of silage treated with 1% CA was maintained at around 5.0 and the final pH value of the CK remained approximately 6.0, which might be due to the high buffer capacity of ML [25]. The relatively low pH of ML silage ensiled with CA indicated that CA could effectively promote the fermentation of ML.

During the ensiling process, lactic acid and acetic acid were the dominant organic acids in ML silage, and no butyric acid or propionic acid was detected. The addition of CA significantly increased ($p < 0.01$) the lactic acid content and reduced the acetic acid content of ML silages. Similarly, Ke et al. [14] reported that 0.1% CA and 0.5% CA increased the lactic acid content. In the first 7 days of ensiling, the lactic acid accumulated rapidly in the ML silages. After 7 days of ensiling, the lactic acid content decreased in the silages treated with 2% CA, and after 14 days of ensiling in the CK and the silages treated with 1% CA. At the same time, the acetic acid content of the CK and the silages treated with 1% CA increased. The drop in lactic acid content and the rise in acetic acid content indicated that part of the lactic acid was converted into acetic acid, which might be caused by hetero-fermentation of the lactic acid bacteria, such as *Lactobacillus buchneri* [26]. Moreover, the ML silages treated with CA showed a lower ($p < 0.01$) ratio of acetic acid to lactic acid than the CK, which suggested that the addition of CA promoted the accumulation of lactic acid and inhibited the production of acetic acid during the ensiling process.

The dynamics of lactic acid bacteria, coliform bacteria and yeasts are shown in Figure 1. In the first 7 days of ensiling, lactic acid bacteria counts increased rapidly, which could explain the accumulation of lactic acid at the same time. The lactic acid bacteria counts then decreased from 8.0 log10 cfu/g FM to 6.0 log10 cfu/g FM when the ensiling time was prolonged, which was consistent with the report by Wang et al. [2]. The reduction in lactic acid bacteria counts might be explained by the lack of water-soluble carbohydrates as a fermentation substrate [27]. Compared with the CK, lower ($p < 0.01$) coliform bacteria counts were observed in the silages treated with CA during the ensiling process, which could explain the higher DM recovery and the lower acetic acid content in these silages. Moreover, the reduction in coliform bacteria counts of silages treated with CA was consistent with those reported by Mohan and Pohlman [28].

When the ensiling time was prolonged, yeast counts decreased ($p < 0.01$), which might be because the progressively anoxic situation inhibited its growth. Unexpectedly, no yeast was detected in the silages treated with 2% CA during the fermentation process. However, Lv et al. [13] reported that yeast counts were increased in alfalfa and *Amomum villosum* Lour silages with the addition of CA. This discrepancy was probably due to the water-soluble

substances of the ML having a broad spectrum of antimicrobial activity [29]. Moreover, CA and the water-soluble substances of ML might have synergistic effects that inhibit the growth of yeasts. Therefore, these results indicated that more research is needed into the function of CA in controlling fungal growth in ML silage.

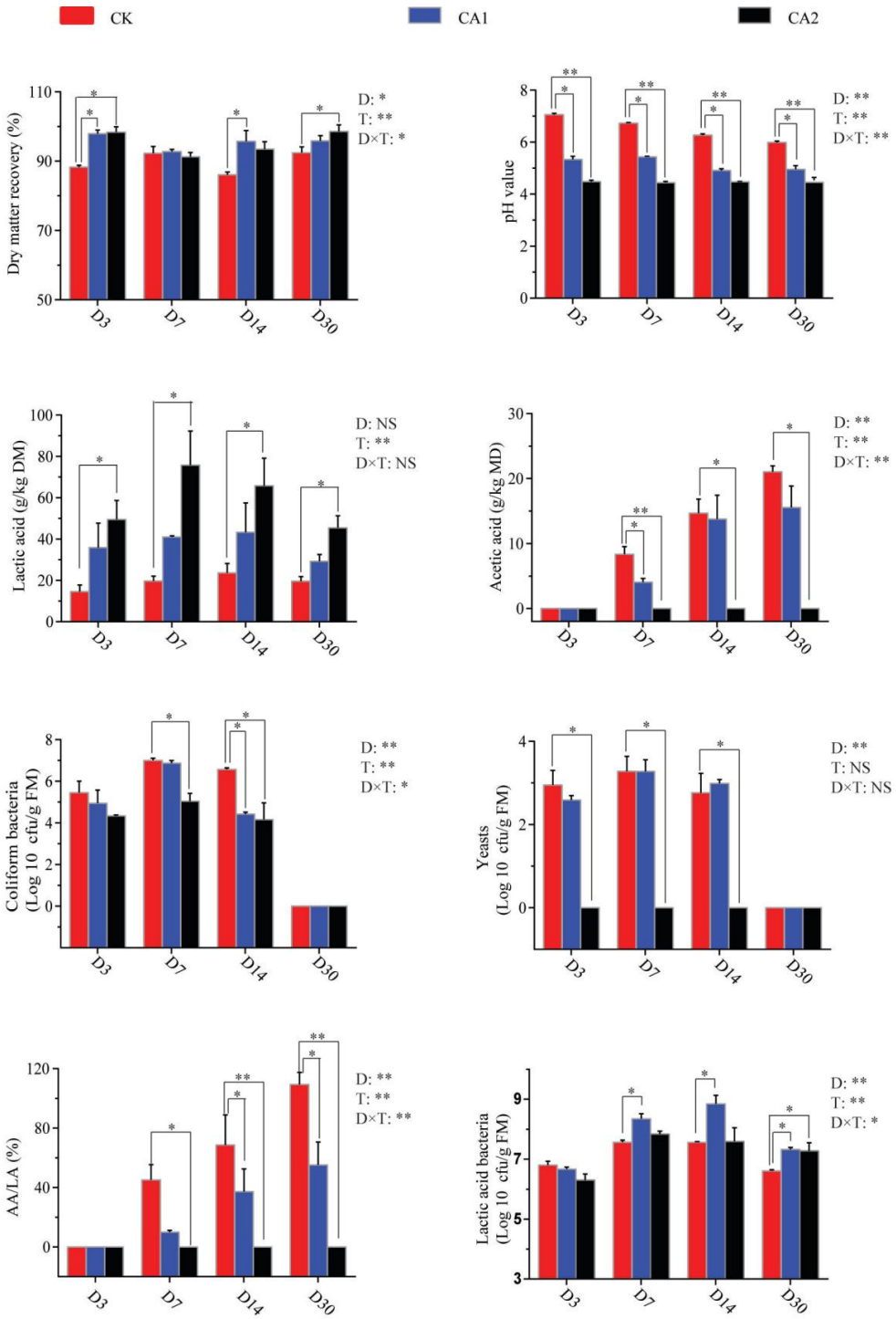

Figure 1. The fermentation parameters and microbial counts of mulberry leaf silages (D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; *, $p < 0.05$; **, $p < 0.01$; NS, non-significance; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling; AA/LA, the ratio of acetic acid to lactic acid; cfu, colony-forming units; FM, pre-ensiled material).

3.3. The Protein Fraction of Ml Silages

The dynamics of the protein fractions of ML silages are shown in Table 2. Proteolysis is a common phenomenon in silage and is the main factor affecting the protein quality of silage [7]. In the first stage, under the action of plant proteolytic enzymes, most of the proteins are degraded into non-protein nitrogen, such as free amino acids, oligopeptides and dipeptides, then these hydrolysis products are further converted to ammonia-N by microorganism activities [7]. When the ensiling time was prolonged, the content of non-protein nitrogen increased ($p < 0.01$) from 122 g/kg TN to 380 g/kg TN, which indicated extensive proteolysis in the ML silage. The increase in non-protein nitrogen indicated a decline in the nutritional value of silage proteins, considering that the utilization efficiency of non-protein nitrogen in ruminants is lower than that of true protein [30]. Thus, inhibitors, which could inhibit protease and microbial activities, were necessary. After 30 days of ensiling, the highest non-protein nitrogen content was observed in the CK, whereas 2% CA decreased ($p < 0.05$) non-protein nitrogen content. Similarly, the addition of CA in alfalfa silages showed a quite different non-protein nitrogen content which decreased with the addition of 0.5% CA after 60 days of ensiling [31]. Moreover, compared with the CK, the non-protein nitrogen content varied less and had a lower content (310 vs. 380 g/kg DM) in the silages treated with 2% CA, implying that the proteins could be preserved better and more stably in silages treated with 2% CA.

Table 2. The protein fractions of mulberry leaf silages.

Item	Treatments	Ensiling Days				SEM	*p*-Value		
		3	7	14	30		D	T	D × T
Crude protein (g/kg DM)	CK	134 ^{A,b}	147 ^{A,a}	154 ^{A,a}	153 ^{A,a}	8.57	0.000	0.000	0.011
	CA1	136 ^{A,a}	138 ^{A,B,a}	137 ^{B,a}	146 ^{A,B,a}				
	CA2	133 ^{A,b,c}	130 ^{B,c}	143 ^{B,a}	139 ^{B,a,b}				
True protein (g/kg TN)	CK	878 ^{A,a}	792 ^{A,b}	679 ^{B,c}	620 ^{B,d}	80.1	0.000	0.679	0.000
	CA1	816 ^{B,a}	748 ^{B,b}	752 ^{A,b}	625 ^{A,B,c}				
	CA2	808 ^{B,a}	764 ^{A,B,b}	678 ^{B,c}	690 ^{A,c}				
Non-protein nitrogen (g/kg TN)	CK	122 ^{B,d}	208 ^{B,c}	321 ^{A,b}	380 ^{A,a}	80.1	0.000	0.679	0.000
	CA1	184 ^{A,c}	252 ^{A,b}	248 ^{B,b}	375 ^{A,B,a}				
	CA2	196 ^{A,c}	236 ^{A,b}	322 ^{A,a}	310 ^{B,a}				
Ammonia-N (g/kg TN)	CK	4.40 ^{A}	7.38 ^{A}	14.6 ^{A}	18.9 ^{A}	5.15	0.000	0.000	0.000
	CA1	3.71 ^{B}	4.57 ^{B}	7.58 ^{B}	10.8 ^{B}				
	CA2	1.76 ^{B}	2.45 ^{C}	3.95 ^{C}	6.07 ^{C}				

D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; DM, dry matter; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; TN, total nitrogen. Different lowercase letters $^{(a–c)}$ in the same row indicate significant differences at $p < 0.05$; different uppercase letters $^{(A–C)}$ in the same column indicate significant differences at $p < 0.05$.

Ammonia-N, reflecting the degree of deamination of amino acids and peptide, is a more precise index compared with non-protein nitrogen. Consistent with the non-protein nitrogen content, the content of ammonia-N increased ($p < 0.01$) when the ensiling time was prolonged, which was related to the deamination of amino acids and peptides caused by microbial activities [8]. During ensiling, the content of ammonia-N decreased ($p < 0.01$) in the silages treated with CA, indicating that CA inhibited the deamination of microorganisms. This inhibition could suggest that the amount of free amino acids, oligopeptides and dipeptides in non-protein nitrogen increased with the addition of CA, thereby improving the nutritive value of non-protein nitrogen. Similarly, Ke et al. [31] observed that ammonia-N decreased from 132 g/kg TN to 31.7 g/kg TN with the addition of 0.5% CA.

Compared with the CK, the relatively low content of ammonia-N in the silages treated with CA might be due to direct acidification and the antimicrobial properties of CA inhibiting the growth of undesirable microorganisms, such as *Clostridium* and *Enterobacter*, which is in accordance with the decrease in coliform bacteria counts [13]. Moreover, the

silages treated with 2% CA showed the lowest ammonia-N content during the ensiling process. The above results indicate that CA improved the silage quality of ML.

3.4. The Protease Activities of ML Silage

The protease activities of ML silage are shown in Table 3. Carboxypeptidase, aminopeptidase and acid protease were the exopeptidases mainly responsible for the first stages of proteolysis [8]. Carboxypeptidase activity increased ($p < 0.01$) when the ensiling time was prolonged. The increased activity of carboxypeptidase may be related to the decrease in pH towards 5.4, at which point carboxypeptidase was optimally active [32]. However, carboxypeptidase activity remained stable in the silage treated with 2% CA during the ensiling process. However, carboxypeptidase activity was consistently higher than 60.0 units/h/DM at 30 days of ensiling in all treatments, which could partly account for the relatively high non-protein nitrogen content of ML silage (Table 2). Aminopeptidase had low tolerance to pH and the optimal activity of aminopeptidase was observed at pH 7.0 [18]. Aminopeptidase activity gradually decreased ($p < 0.01$) in this study, which was consistent with the change in pH value (Figure 1). Moreover, aminopeptidase activity sharply decreased ($p < 0.01$) with the addition of CA, which might be due to the direct acidification of CA. Similarly, Yuan et al. [32] and He et al. [8] observed that formic acid decreased aminopeptidase activity by rapid acidification of the silage. Furthermore, aminopeptidase can release free amino acid residues to provide an additional easily assimilated carbon source for lactic acid bacteria via deamination, accumulating extracellular ammonia-N [8]. Thus, the CA-inhibited aminopeptidase activity might partially account for the decrease in ammonia-N (Table 2).

Table 3. The protease activities of mulberry leaf silages.

Item	Treatments	Ensiling Days				SEM	*p*-Value		
		3	7	14	30		D	T	D×T
Carboxypeptidase (units/h/DM)	CK	70.9 ^{A,b}	66.9 ^{A,b}	80.8 ^{A,a}	83.1 ^{A,a}	7.33	0.003	0.000	0.021
	CA1	69.2 ^{A,a}	74.8 ^{B,a}	76.5 ^{A,a}	80.8 ^{A,a}				
	CA2	66.9 ^{A,a}	66.9 ^{A,a}	66.2 ^{B,a}	65.9 ^{B,a}				
Aminopeptidase (units/h/DM)	CK	86.5 ^{A,a}	83.4 ^{A,a}	23.9 ^{A,b}	17.4 ^{A,b}	28.2	0.000	0.000	0.000
	CA1	59.6 ^{B,a}	65.0 ^{A,a}	18.8 ^{A,B,b}	14.7 ^{A,b}				
	CA2	23.6 ^{C,a}	17.7 ^{B,a,b}	14.75 ^{C,b}	13.5 ^{A,b}				
Acid protease (units/h/DM)	CK	51.3 ^{A,a}	47.1 ^{B,a}	50.5 ^{A,a}	36.6 ^{A,b}	6.76	0.000	0.018	0.360
	CA1	55.2 ^{A,a}	53.9 ^{A,a}	53.5 ^{A,a}	41.3 ^{A,b}				
	CA2	49.7 ^{A,a,b}	55.2 ^{A,a}	54.4 ^{A,a,b}	45.8 ^{A,b}				

D, ensiling days; T, treatments; D×T, the interaction of ensiling days and treatments; DM, dry matter; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid. Different lowercase letters $^{(a,b)}$ in the same row indicate significant differences at $p < 0.05$. Different uppercase letters $^{(A–C)}$ in the same column indicate significant differences at $p < 0.05$.

In contrast to carboxypeptidase and aminopeptidase, acid protease activity was slightly increased ($p < 0.05$) in the silages treated with CA during the ensiling process. Although the pH in the silages treated with CA decreased, the pH still approached the optimal level for acid protease (4.50 in alfalfa). Thus, CA enhanced the acid protease activity. Although acid protease activity was lower than carboxypeptidase activity, it varied from 47.1 to 55.2 units/h/DM, which also promoted the hydrolysis of proteins. Overall, the relatively high activity of carboxypeptidase and the increased activity of acid protease in the silages treated with CA suggested that more CA should be added to ML silage to adjust the pH below 4.5, which might better reduce the degree of protein hydrolysis and improve the silage quality of ML.

To further study the relationship between protease and protein fractions, Pearson's correlations were conducted on the data for protein fractions and protease activity. As shown in Table 4, carboxypeptidase activity was negatively correlated ($p < 0.01$) with true protein content but positively correlated ($p < 0.01$) with non-protein nitrogen and ammonia-N content,

which might partly explain the proteolysis in ML silage. The results indicated that CA inhibited ($p < 0.01$) carboxypeptidase activity could effectively inhibit extensive proteolysis in ML silage during the ensiling process, with 2% CA showing the best outcome.

Table 4. Pearson's correlations between protease activities and the protein fractions of mulberry leaf silages.

Item	True Protein	Non-Protein Nitrogen	Ammonia-N	Carboxypeptidase	Aminopeptidase	Acid Protease
True protein	1					
Non-protein nitrogen	−1.00 0.00	1				
Ammonia-N	−0.65 0.06	0.65 0.06	1			
Carboxypeptidase	−0.81 <0.01	0.81 <0.01	0.80 <0.01	1		
Aminopeptidase	−0.51 0.16	0.51 0.16	0.56 0.11	0.62 0.08	1	
Acid protease	0.56 0.11	−0.56 0.11	−0.74 0.02	−0.53 0.14	0.06 0.87	1

Values at the top for each category denote the correlation coefficients; values at the bottom denote the *p*-value of the correlation.

3.5. Bacterial Community of ML Silage

3.5.1. The Dynamic Variation and Alpha Diversity of the Bacterial Community

The dynamic variation of the bacterial community was illustrated by principal component analysis (Figure 2). The results of the principal coordinate analysis was based on UniFrac (unweighted) distances. In the CK, the bacterial community showed the clearest distinctions when the ensiling time was prolonged. Moreover, after 7 days of ensiling, it was obvious that the silages treated with CA were separate from the CK, which suggested that the microbial community changed during the ensiling process. The distinctiveness of the bacterial communities among all treatments might account for the better fermentation that occurred in the ML silages treated with CA.

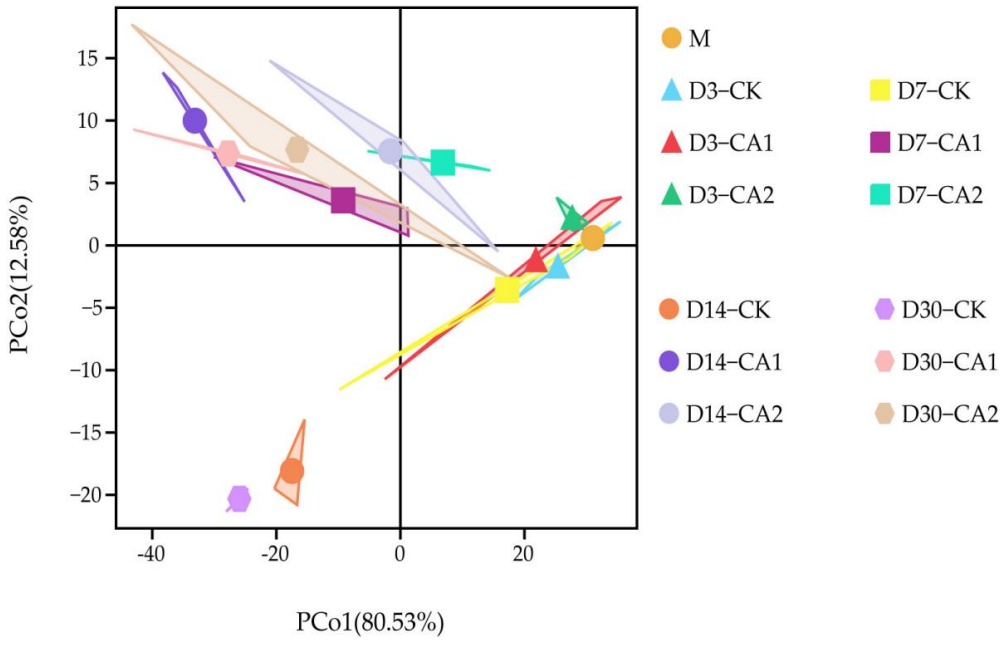

Figure 2. Principal component analysis of the bacterial community for mulberry leaf silages (M, pre-ensiled material; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling).

The alpha diversity of the bacterial community in ML silage is shown in Table 5. The coverage values of all samples were approximately 0.99, which indicated that most of the bacterial community was captured by the adequately large sequencing data. The Sobs, Ace and Chao1 indices decreased, but the Simpson index increased with a longer ensiling time, which indicated that the bacterial richness of ML silage decreased and dominant bacterial community richness increased. Compared with the CK, the silage treated with CA generally showed a lower Shannon index at the same stage of ensiling, which indicated that CA decreased the diversity of the bacterial community of ML silage.

Table 5. Alpha diversity of the bacterial community for mulberry leaf silages.

Items	Treatments	Ensiling Days			
		3	7	14	30
Sobs	CK	1605	1339	1384	1373
	CA1	1477	1402	1102	1305
	CA2	1376	1499	1301	1337
Ace	CK	2512	2242	2263	2151
	CA1	2384	2348	1951	2097
	CA2	2159	2417	2152	2241
Chao1	CK	2483	2268	2204	2115
	CA1	2308	2294	1964	2082
	CA2	2095	2453	2160	2168
Shannon	CK	3.00	3.18	5.02	5.22
	CA1	2.97	4.32	4.36	4.62
	CA2	2.62	3.59	3.68	4.13
Simpson	CK	0.50	0.56	0.89	0.93
	CA1	0.51	0.79	0.86	0.86
	CA2	0.45	0.66	0.71	0.76
Coverage	CK	0.99	0.99	0.99	0.99
	CA1	0.99	0.99	0.99	0.99
	CA2	0.99	0.99	0.99	0.99

CK, the control; CA1, 1% citric acid; CA2, 2% citric acid.

3.5.2. The Changes in the Dynamics of Relative Abundance among Bacterial Communities

The changes in the dynamics of relative abundance among bacterial communities by phylum are shown in Figure 3. The relative abundance of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (up to 69%, 11% and 10%, respectively) indicated that they were the dominant phylum in pre-ensiled ML; similar results were observed by Liu et al. [20]. With the prolonged ensiling time, the abundance of *Proteobacteria* and *Bacteroidetes* gradually decreased. After 30 days of ensiling, *Firmicutes* became the most abundant phylum in ML silages treated with CA, and *Proteobacteria* was still the most abundant phylum in the CK. Compared with the CK, the ML silages treated with CA showed a greater *Firmicutes* abundance but lower *Proteobacteria* abundance during all the stages of ensiling. *Proteobacteria* decreased in the silages treated with CA, which might be because the CA could enhance the permeability of the outer membrane of Gram-negative bacteria and inhibit the growth of *Proteobacteria* [33].

The dynamics of relative abundance among bacterial communities by genus are shown in Figure 4. The relative abundance of bacterial communities was very low in the pre-ensiled ML. *Methylobacterium* (4.01%) and *Sphingomonas* (1.03%) were the dominant genera in pre-ensiled ML, which was consistent with the results for pre-ensiled alfalfa reported by Ni et al. [34]. After 30 days of ensiling, *Lactobacillus* (24.9%), *Kosakonia* (9.67%), *Enterobacter* (8.96%), *Methylobacterium* (8.89%) and *Enterococcus* (6.26%) were dominant in the CK, whereas *Lactobacillus* was the only dominant genus in the silages treated with CA. *Lactobacillus* is usually the dominant genus in silage due to its more active and rapid growth when the environmental pH declines. Some *Lactobacillus* species are usually screened as inoculants due to their rapid growth, which enhances the fermentation quality, such as *Lactobacillus plantarum* and *Lactobacillus casei* [17,26]. The relative abundance of *Lactobacillus* increased from 0.4 to 24.9, 60.7 and 47.3% in the CK and the silages treated with 1% CA and 2% CA after 30 days of ensiling, respectively. In the

same fermentation period, the silages treated with CA showed a higher *Lactobacillus* abundance, which was consistent with the higher accumulation of lactic acid content (Figure 1). Moreover, *Lactobacillus* abundance decreased in the silages treated with 1% CA after 14 days of ensiling, which might be due to the rapid depletion of fermentation substrates, which could not support the growth of *Lactobacillus*. The rapid depletion of fermentation substrate abundance might be responsible for the conversion of lactic acid to acetic acid in the silages treated with 1% CA because lactic acid becomes an alternative fermentation substrate for *Lactobacillus*. Similarly, Yan et al. [15] observed that relatively high *Lactobacillus* abundance corresponds to an elevation in acetic acid and a reduction in lactic acid. This conversion might be because different species of *Lactobacillus* have different metabolic patterns and functions. Similar to *Lactobacillus*, *Enterococcus* are desirable bacteria in the initial fermentation process, with the function of producing lactic acid to create a weak acid environment, which contributes to *Lactobacillus* fermentation. In the silages treated with CA, the relative abundance of *Enterococcus* decreased (Figure 5), which might be due to its low tolerance to the acidic environment caused by CA [17,23].

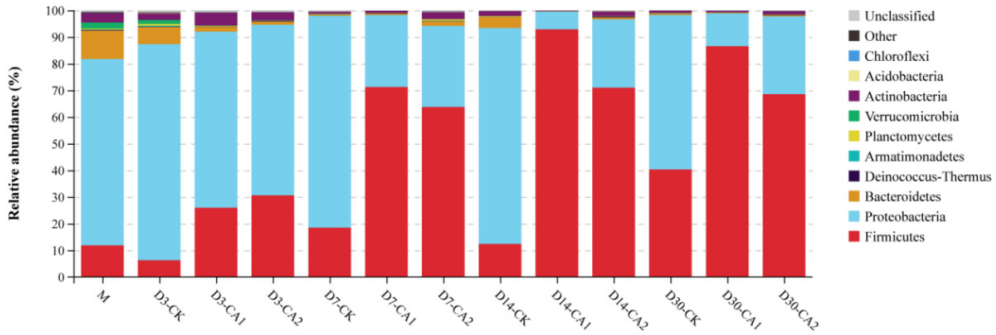

Figure 3. Bacterial community and relative abundance by phylum for mulberry leaf silages (M, pre-ensiled material; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling).

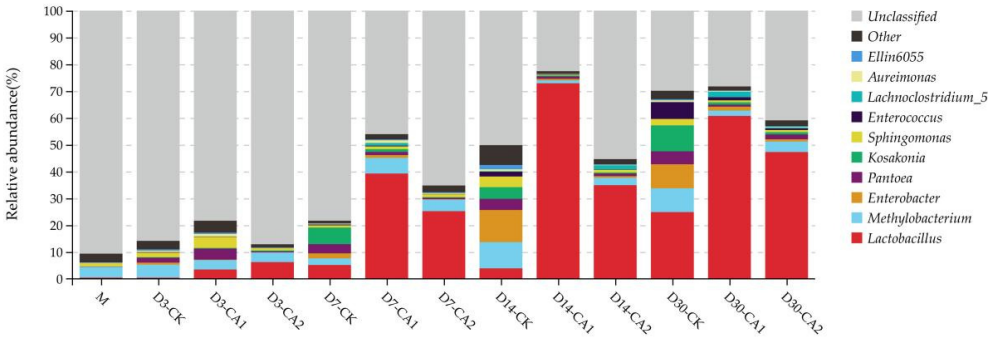

Figure 4. Bacterial community and relative abundance by genus for mulberry leaf silages (M, pre-ensiled material; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling).

It is known that *Enterobacter* is an undesirable facultative anaerobe in the fermentation process, being the principal competitor of the lactic acid bacteria for water-soluble carbohydrates under anaerobic conditions; its principal fermentation product is acetic acid [26]. Yang et al. [35] reported that *Enterobacter* was positively correlated with pH and negatively correlated with lactic acid. The high relative abundance of *Enterobacter* could explain the relatively high pH (5.99) and acetic acid content (11.0 g/kg DM) in CK after 30 days of ensiling. Moreover, *Enterobacter* can deaminate and decarboxylate amino acids, thereby enhancing ammonia-N production [36]. The relative abundance of *Enterobacter* decreased from 8.96 to 0.83% with the addition of CA after 30 days of ensiling in this study (Figure 5), which might account for the lower acetic acid and ammonia-N content in the silages treated with CA. *Methylobacterium* are facultative methylotrophic bacteria and are commonly symbiotic with plants. The relative abundance of

Methylobacterium decreased from 9.78 to 1.12% and 8.89 to 2.00% in the silages treated with CA after 14 days and 30 days of ensiling, respectively, because it is strictly aerobic and neutrophilic (Figure 5) [37]. *Methylobacterium* are considered to be undesirable bacteria in silage because it positively correlates with levels of ammonia-N and negatively correlates with lactic acid [38,39]. The roles of *Sphingomonas*, *Pantoea* and *Kosakonia* in silage are still unclear. Fitzgerald et al. [40] observed that *Sphingomonas* is involved in nitrification under low dissolved oxygen conditions, which might resist the production of ammonia-N. The relative abundance of *Sphingomonas* decreased from 2.38 to 0.72% in the silages treated with CA after 30 days of ensiling. Ogunade et al. [39] speculated that *Pantoea* could preserve protein through their negative correlation with ammonia-N content. However, Li et al. [41] inferred that *Pantoea* might compete for the fermentation substrate with lactic acid bacteria and cause an accumulation of butyric acid in silage because *Pantoea* is separate from the *Enterobacter* genus. Furthermore, Guan et al. [42] reported that *Pantoea* was more active in high-moisture environments and positively correlated with butyric acid. However, no butyric acid was detected in the current study, which might be due to the low relative abundance of *Pantoea* (0.08%) in pre-ensiled ML. Similar to *Pantoea*, *Kosakonia* was separated from the *Enterobacter* genus. He et al. [43] reported that *Kosakonia* might reduce the ammonia-N content in silage due to its potential nitrogen fixing. The relative abundance of *Pantoea* and *Kosakonia* showed a similarly increasing trend in the CK during ensiling and was lower in the silages treated with CA in the current study. The relative abundance of *Pantoea* and *Kosakonia* decreased from 4.82 to 0.75% and 9.68 to 0.86% after 30 days of ensiling, respectively (Figure 5). The potential capacity of *Pantoea*, *Kosakonia* and *Sphingomonas* to reduce ammonia-N might explain the relatively low ammonia-N in the CK after 30 days of ensiling (Table 2). However, the conventional functions of *Pantoea* and *Kosakonia* in silage are complicated; therefore, more research should be conducted to evaluate their function. Moreover, *Roseomonas* (Gram-negative bacteria) can split urea and assimilate arabinose and glucose, suggesting that it might compete for water-soluble carbohydrates with lactic acid bacteria and promote the production of ammonia-N [44]. The relative abundance of *Roseomonas* in the silages treated with CA decreased from 0.05 to 0.03%, from 0.10 to 0.03%, from 0.66 to 0.01% and from 0.20 to 0.03% after 3, 7, 14 and 30 days of ensiling in the current study, respectively.

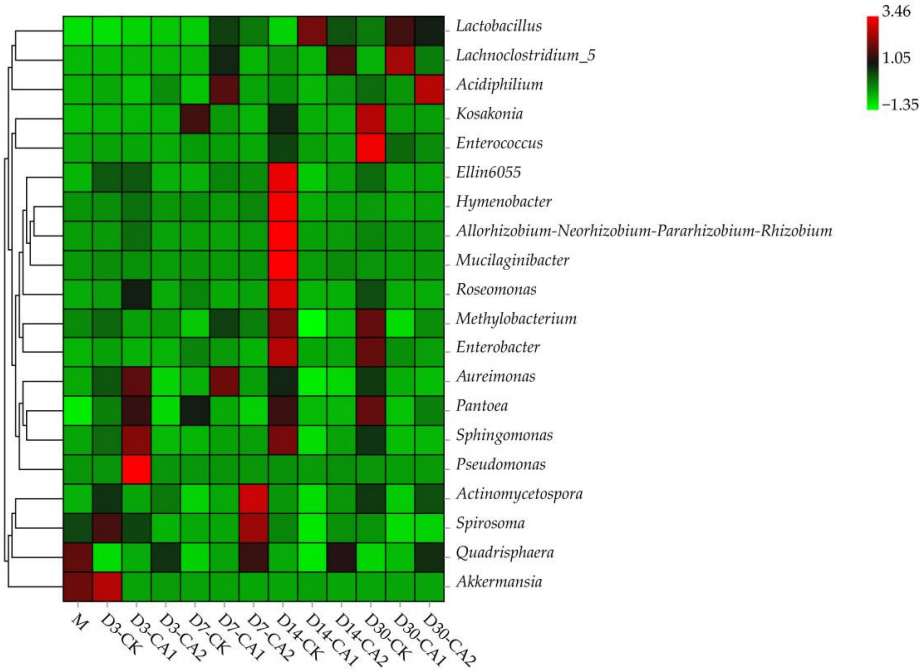

Figure 5. Heatmap of prominent bacterial genera (20 most abundant genera) for mulberry leaf silages (M, pre-ensiled material; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling).

3.5.3. 16S rDNA Gene-Predicted Functional Profiles

The 16S rDNA gene-predicted functional profiles are shown in Figure 6. CA inhibited the metabolism of energy, carbohydrate, folding, sorting and degradation, and membrane transport, which might be attributed to CA decreasing the pH value and inhibiting microbial activities directly. Moreover, CA inhibited the amino acid metabolism. Thus, the decrease in ammonia-N in the silages treated with CA might be because CA reduced the amino acid metabolism of *Enterobacter*, consistent with the decrease in *Enterobacter* abundance. All conversions of the abovementioned bacteria communities suggest that CA could enhance lactic acid bacteria fermentation by inhibiting the undesirable microorganisms in ML silage. The reduction in undesirable microorganisms and the increase in lactic acid bacteria might account for the lower pH, the increased DM recovery and the reduction in ammonia-N content in the silage treated with CA. Overall, CA had a positive effect on the fermentation quality and silage quality of ML.

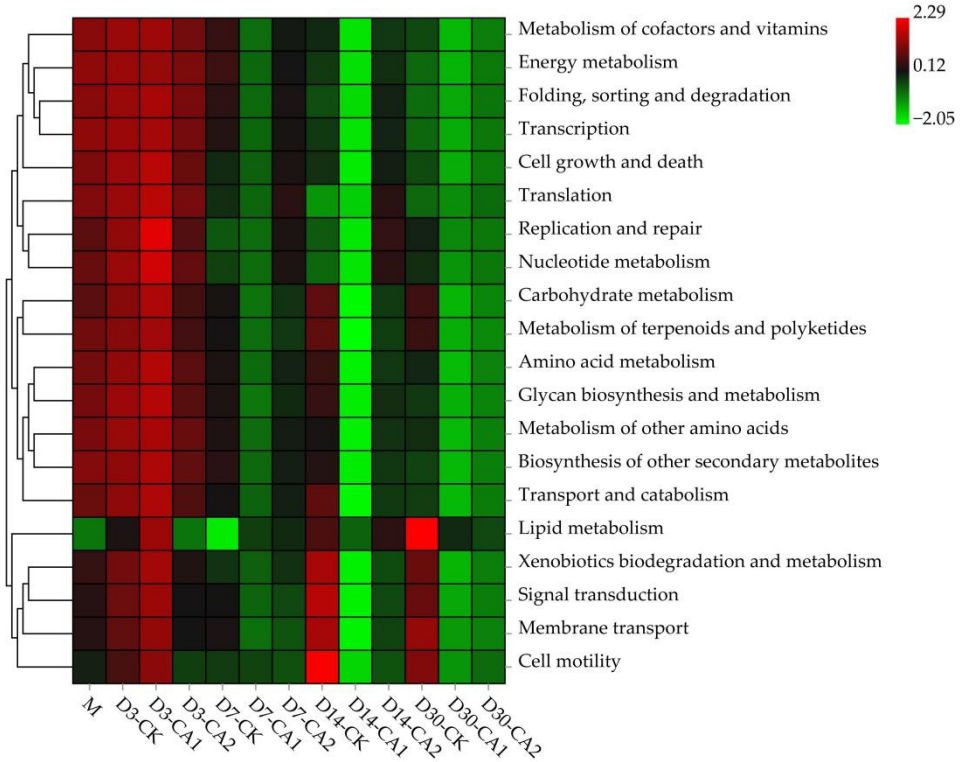

Figure 6. Heatmap of 16S rDNA gene-predicted functional profiles obtained with PICRUSt2 (M, pre-ensiled material; CK, the control; CA1, 1% citric acid; CA2, 2% citric acid; D3, 3 days of ensiling; D7, 7 days of ensiling; D14, 14 days of ensiling; D30, 30 days of ensiling).

4. Conclusions

The study reveal that CA was useful for improving the fermentation quality and nutrition of ML silage. The content of acetic acid, non-protein nitrogen and ammonia-N, and coliform bacteria counts decreased in the silage treated with CA. The abundance of *Enterobacter*, *Methylobacterium*, *Sphingomonas*, *Pantoea*, *Kosakonia* and *Roseomonas* decreased, whereas *Lactobacillus* abundance increased when CA was added. Thus, the addition of CA could effectively preserve the quality of ML silage, and 2% CA was the most effective.

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