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Effect of 4-Pentenoic Acid and Malic Acid on Dynamics of Bacterial Community and Fermentation Characteristics in Nettle Silage

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Abstract: [Background] Nettle is a potential non-conventional feed resource due to its high level of crude protein content, and ensiling is better for utilization in the animal industry. Previous integrated analysis (microbiome and metabolome) suggested that 4-pentenoic acid and malic acid in ensiled nettle may inhibit harmful microorganisms within the system. The present study investigated the effects of these two acids on nettle silage quality through the addition of 1% fresh weight of these acids then analyzed the characteristics and bacterial community during 60 days of nettle ensiling. [Results] Addition with 4-pentenoic acid increased the content of crude protein (CP) and acetic acid (AA) when compared with both control and malic acid treated during 30 to 60 days of ensiling ($p<0.05$). Lactic acid (LA) content was highest in malic acid treated (4.21 %, dry matter, DM based) when compared with control and 4-pentenoic acid treated after 7 days of nettle ensiling ($p<0.05$), but lower when compared with 4-pentenoic acid treated after 30 days of nettle ensiling ($p<0.05$), and was not detected in all groups after 60 days of silage. The content of butyric acid (BA) and ammonia (AN) was lowest (2.92%—4.39% of DM and 9.94 % —24.28% of total nitrogen, respectively) in 4-pentenoic treated when compared with both control and malic acid treated during 30 to 60 days of ensiling ($p<0.05$). Both acids treated increased relative abundance of *Weissella* after 30 days of nettle ensiling, especially for 4-pentenoic acid showed the higher capacity. Both acids treated showed trend to inhibited relative abundance of *Clostridium sensu stricto 15* after 30 days of nettle ensiling. *Clostridium sensu stricto 15* showed significant positive correlation with BA and AN ($p<0.05$). [Conclusion] Present study results suggested that addition of 4-pentenoic could improve quality of silage due to reduction level of protein degradation probably resulting from its inhibited activity against to *Clostridium* spp. However, malic acid was less effective than 4-pentenoic acid in suppressing *Clostridium* spp. activity and the associated production of BA and AN, resulting in inferior preservation of CP.

Keywords: nettle; 4-pentenoic acid; malic acid; silage; bacteria

1. Introduction

The rapid expansion of the livestock feed industry in recent years, has caused the critical shortages of conventional protein sources(e.g., corn and soybean meal), which now fail to meet market demand. Developing non-conventional feed sources now constitutes an imperative necessity for ensuring sustainable livestock production systems [1].

Nettles (*Urtica* spp.) are annual or perennial herbaceous plants belonging to the family Urticaceae. Characterized by high nutritional value, robust environmental adaptability, and strong stress resistance, they exhibit widespread global distribution [2]. Furthermore, nettles are rich in essential nutrients including proteins, carbohydrates, lipids, and amino acids, as well as biologically active compounds such as carotenoids terpenoids, α-linolenic acid, 12-linoleic acid, vitamins, cis-9, and polyphenolic compounds [3]. In addition, these plants possess demonstrate antioxidant,

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antibacterial, and anti-inflammatory properties, positioning them as a highly promising alternative feed resource. Recent research demonstrates the significant potential of nettle species as functional feed ingredients for optimizing animal production, enhancing stress resilience, and improving the quality of animal-derived products across livestock and poultry species. Specifically, partial alfalfa (*Medicago sativa*) replacement with Nettles enhanced antioxidant status in dairy cows [4], dietary supplementation ameliorated heat stress in broilers [5], and inclusion improved egg yolk carotenoid deposition in laying hens [6]. Similarly, *Boehmeria nivea* hay substitution boosted rabbit productivity [7]."

Similar to alfalfa, nettles exhibit constrained silage characteristics by several intrinsic factors [8]. Their elevated crude protein content and high buffering capacity, coupled with a concomitantly low water-soluble carbohydrate (WSC) content, result in insufficient fermentable substrate, thereby restricting the proliferation of essential lactic acid bacteria (LAB) during the silage process [9]. Furthermore, the abundant protein serves as a readily available nitrogen source promoting the growth of undesirable Clostridia species. Subsequent proteolysis by *Clostridium* spp. generates ammonia (AN), which neutralizes lactic acid (LA) and impedes the critical decline in system pH [10]. This disruption of the acidification process causes ineffective silage fermentation.

Our previous study showed that malic acid enhances silage *Lactobacillus* growth through specialized metabolic utilization and competitive dominance [3]. *Lactobacillus* spp. express *mleS* and *mic* genes encoding malolactic enzyme and malic enzyme, enabling direct decarboxylation of malic acid to LA via malolactic fermentation (MLF) [3]. Concurrently, malic acid is funneled into pyruvate metabolism (*pdhB*, *ldh* upregulation) and glycolysis, sustaining energy production and biosynthetic precursors [11]. Additionally, naturally occurring antimicrobial compounds within nettles, such as 4-pentenoic acid, formic acid, and phenolic compounds, exhibit broad-spectrum inhibitory activity against microbial growth. 4-Pentenoic acid may act as a potential spoilage factor in nettle silage, as it demonstrated inhibitory effects against beneficial LAB (e.g., *Pediococcus pentosaceus*) in vitro through impairing fatty acid synthesis and cell membrane integrity, which could contribute to the observed quality deterioration [11]. Whereas prior microbiome analyses identified 4-pentenoic acid and malic acid as key fermentation inhibitors in nettle silage [3,11], their targeted application as antibacterial additives against *Clostridium* remains unexplored. To address this knowledge gap, we added these two acids in nettle silage in order to determined how these acids are involved in the fermentation process in the present study.

2. Materials and Methods

2.1. Silage Prepartion

Nettle (*Urtica cannabina*) was harvested on 10th September 2023 from the wild in Shawan County, central mountains of Tianshan, Xinjiang, China (E 84°58′—86°24′; N 43°26′—45°20′). After air-drying to 394.2g·kg^{-1} fresh weight, plants were cut into 2 cm segments using a fodder chopper. Approximately 1.0 kg of homogenized material was vacuum-sealed in polyethylene bags (23 cm× 30 cm) fitted with one-way valves. Established two treatments, addition with 1% of fresh weight for malic acid (MA, BioUltra grade, Macklin Biochemical Co., Ltd., Shanghai, China; Product No. M815619) and 4-pentenoic acid (4PA, ≥ 97 % purity, Aladdin Biochemical Technology Co., Ltd., Shanghai, China; Product No. P112354), respectively, based on our previous research demonstrating the efficacy of 1% of fresh weight in modulating microbial activity and fermentation in nettle silage, particularly for suppressing undesirable Clostridial [11]. Control groups were also prepared. Each treatment comprised nine replicates, totaling 45 sample bags stored at 24 ℃ for 60 days.

2.2. Characteristics Analysis of Nettle Silage

After 7, 30 and 60 days of ensiling, 200 g silage samples were dried at 65℃ for 48 h and ground to pass through a 1.0-mm sieve, and then used to determine DM content. Total nitrogen (TN) was determined using an automatic Kjeldahl nitrogen analyzer (K9840, Hanon Co. Ltd, Shandong,

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China), and crude protein was calculated according to the method of the Association of Official Agricultural Chemists (AOAC). WSC content was determined according to methods describe above [12].

A 20-g silage sample from each treatment was combined with 180 mL of deionized water to assess fermentation characteristics. The mixture (1:9 for v/v) was filtered through four layers of cheesecloth. The pH was immediately measured with a portable pH meter (PHS-3C, Instrument and Electrical Science Instrument Co., Ltd., Shanghai, China), and the supernatant was retained for ammonia and organic acid analyses following established protocols [3,13]. Organic acids (actic acid, LA; acetic acid, AA; propionic acid, PA; butyric acid, BA) were quantified via HPLC employing a C18 column (150 × 4.6 mm, 94 FMF-5559-EONU, FLM Scientific Instrument Co., Ltd., Guangzhou, China). The Na_2HPO_4 (1 mM, pH 2.7) and methanol were used for mobile phase A and B, respectively, with a flow rate of 0.6 mL·min^{-1}. Separation used a 20 μL injection volume at 50 ℃ with the gradient: 0 min, 100 % A; 5—40 min, 98 % A.

2.3. Sequencing Analysis of Bacterial Community in Nettle Silage

Total DNA was extracted from triplicate samples per treatment group after 7, 30, and 60 ensiling days using the commercial DNA Kit (FastDNA® Spin Kit for Soil, MP Biomedicals, USA). Primers (338F: ACTCCTACGGGAGGCAGCAG; 806R: GGACTACHVGGGTWTCTAAT) targeting the V3—V4 regions of 16S rDNA were used for PCR amplification. The amplicons were extracted, purified, and the raw sequences analyzed [3]. The sequences were uploaded to the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/): PRJNA1233857 (accessed on March 13, 2025) for addition with 4-pentenoic acid and PRJNA1236718 (accessed on March 16, 2025) for addition with malic acid into nettle silage, respectively.

2.4. Statistical Analysis

Data from the three silage groups underwent one-way ANOVA in SPSS 22 (SPSS Inc., Chicago, IL, USA), with Tukey's test ($p < 0.05$) determining treatment differences. Microbiota bioinformatics utilized the Majorbio Cloud platform (https://cloud.majorbio.com).

3. Results

3.1. Effect of 4-Pentenoic Acid and Malic on Characteristics of Nettle Silage

Table 1. Characteristics during nettle ensiling after addition with relevant organic acids % DM

Index	Time/day	Groups			SEM	*p* value
		CK	MA	4PA		
DM (% fresh weight)	7	37.72	37.23	36.58	1.245	0.675
	30	35.45 ^{b}	36.66 ^{a}	35.39 ^{b}	0.038	0.027
	60	36.33	36.78	35.08	0.871	0.211
CP% DM	7	16.23 ^{a}	15.89 ^{b}	16.32 ^{a}	0.039	<0.001
	30	15.27 ^{c}	15.60 ^{b}	15.96 ^{a}	0.067	<0.001
	60	13.27 ^{c}	13.72 ^{b}	14.83 ^{a}	0.062	<0.001
WSC% DM	7	2.92	3.30	2.95	0.230	0.263
	30	0.68	0.73	0.71	0.045	0.606
	60	0.69	0.74	0.68	0.041	0.386
pH	7	7.65 ^{c}	7.80 ^{b}	7.98 ^{a}	0.024	<0.001
	30	8.35 ^{a}	8.15 ^{b}	8.11 ^{b}	0.064	0.019
	60	8.47 ^{a}	8.24 ^{a}	8.10 ^{b}	0.096	0.023
LA/(g·kg^{-1} DM)	7	2.68 ^{b}	4.21 ^{a}	3.86 ^{b}	0.425	0.026
	30	0.016 ^{c}	0.17 ^{b}	0.40 ^{a}	0.048	<0.001
	60	ND	ND	ND	-	-

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	7	1.74	1.74	1.77	0.251	0.990
AA/(g·kg^{-1} DM)	30	0.94 ^{b}	0.93 ^{b}	1.57 ^{a}	0.070	<0.001
	60	0.63 ^{b}	0.50 ^{b}	1.37 ^{a}	0.166	0.004
	7	0.054	0.068	0.076	0.016	0.413
PA/(g·kg^{-1} DM)	30	0.082	0.078	0.050	0.019	0.215
	60	0.19 ^{a}	0.083 ^{b}	0.065 ^{b}	0.026	0.005
	7	ND	ND	ND	-	-
BA/(g·kg^{-1} DM)	30	4.99 ^{a}	5.59 ^{a}	2.92 ^{b}	0.287	<0.001
	60	8.31 ^{a}	7.56 ^{a}	4.79 ^{b}	0.367	<0.001
	7	9.81	14.30	9.94	2.280	0.160
AN (% of TN) DM	30	12.90 ^{a}	12.47 ^{a}	10.86 ^{b}	0.190	<0.001
	60	32.79 ^{b}	39.33 ^{a}	24.28 ^{c}	1.150	<0.001

Note: CK: control group; MA: addition with 1% (fresh weight) malic acid; 4PA: addition with 1 % (fresh weight) 4-pentenoic acid. Different lowercase letters on the same row indicate significant differences ($p<0.05$). DM, dry matter basis; CP, crude protein; WSC, water-soluble carbohydrate; LA, lactic acid; AA, acetic Acid; PA, propanoic acid; BA, butyric acid; AN, ammonia. Data are presented as the means ± standard error. ND: not detected. SEM: standard error of the mean.

The characteristics of nettle after wilting were 39.42 % fresh weight of dry matter, 16.89 % DM of crude protein, 7.50 % DM of WSC and 8.04 of pH, respectively. As show in table 1, DM was highest in MA treated than control and 4PA after 30 days of nettle ensiling ($p < 0.05$). CP was lowest in malic acid treated than control and 4-pentenoic acid after 7 days of nettle ensiling ($p < 0.05$); highest in 4PA treated, followed by control during 30—60 days of nettle ensiling. 4PA treated showed highest pH compared with control and MA treated after 7 days of nettle ensiling ($p < 0.05$), while it showed lowest pH after 30—60 days of ensiling ($p < 0.05$).

As shown in **Table1**, DM was highest in MA treated when compared with control and 4PA treated after 30 days of ensiling ($p<0.05$). The content of CP was higher in 4PA treated than MA treated after 7 days of ensiling ($p<0.05$), and showed highest content in 4PA treated than other two groups during 30—60 days of ensiling ($p<0.05$). The content of WSC showed none difference between control and each two treated groups during entire ensiling process ($p>0.05$). The pH value (7.98) was highest in 4PA treated after 7 days of ensiling, but lower during 30—60 days of ensiling when compared with control and MA treated ($p<0.05$). The content of LA was highest in MA treated after 7 days of ensiling when compared with control and 4PA treated ($p<0.05$), but lower after 30 days of ensiling when compared with 4PA treated ($p<0.05$), and lowest in control after 30 days of ensiling when compared with other two treated groups ($p<0.05$). The content of AA was highest in 4PA treated during 30—60 days of ensiling when compared with control and MA treated ($p<0.05$). The content of PA was highest in control when compared with two treated groups after 60 days of ensiling ($p<0.05$). The content of BA was lowest in 4PA treated during 30—60 days of ensiling when compared with control and MA treated ($p<0.05$). Ammonia was lower in 4PA treated compared with control and MA treated ($p<0.05$) after 30 days of ensiling, lowest in 4PA treated compared with control and MA treated ($p<0.05$) after 60 days of ensiling.

3.2. Effect of 4-Pentenoic Acid and Malic on Bacteria Community of Nettle Silage

As shown in Figure 1 A and B, both chao 1 and ace index showed none difference between each treated groups and control ($p>0.05$). Shannon index was highest in MA treated after 60 days of nettle ensiling when compared with 7 days ($p<0.05$, Figure 1 C).

As shown in Figure 2, there are clear distance between control and other acids treated groups after 30 days of nettle ensiling.

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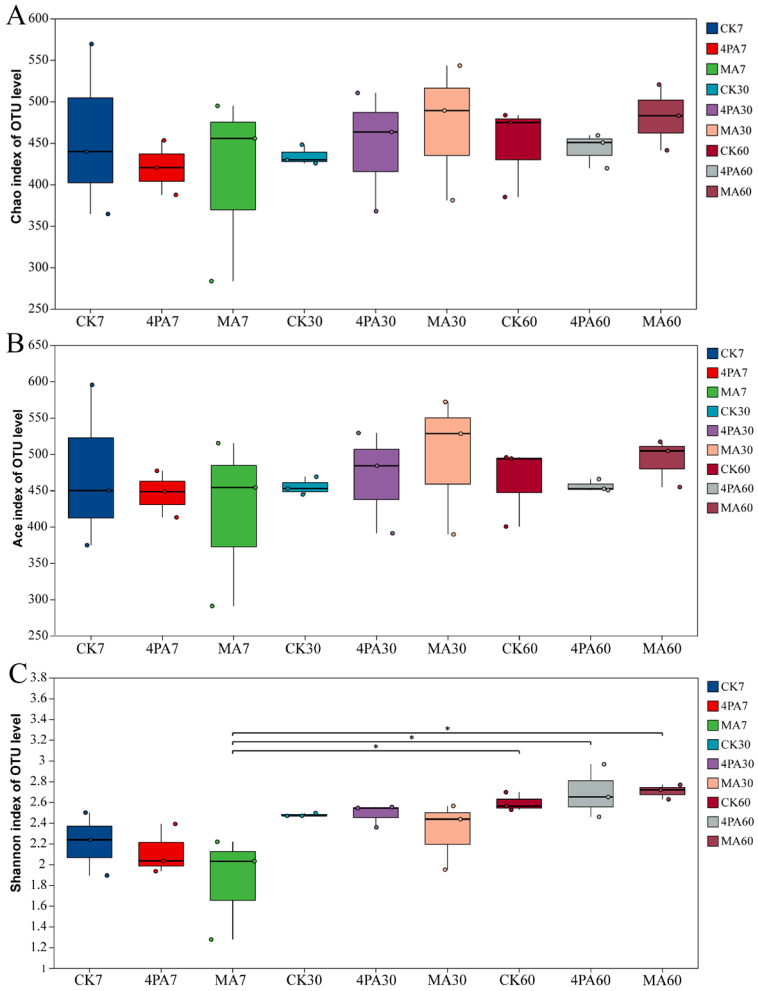

Figure 1. Alpha diversity of bacterial community after addition with malic and 4-pentenoic acid during nettle ensiling. (A) chao 1 index; (B) ace index; (C) Shannon index. CK7, 4PA7 and MA7: control group, addition with 4-pentenoic acid and addition with malic acid after 7 days of nettle ensiling, respectively. CK30, 4PA30 and MA30: control group, addition with 4-pentenoic acid and addition with malic acid after 30 days of nettle ensiling, respectively. CK60, 4PA60 and MA60: control group, addition with 4-pentenoic acid and addition with malic acid after 60 days of nettle ensiling, respectively. "*" means p<0.05. Same as below.

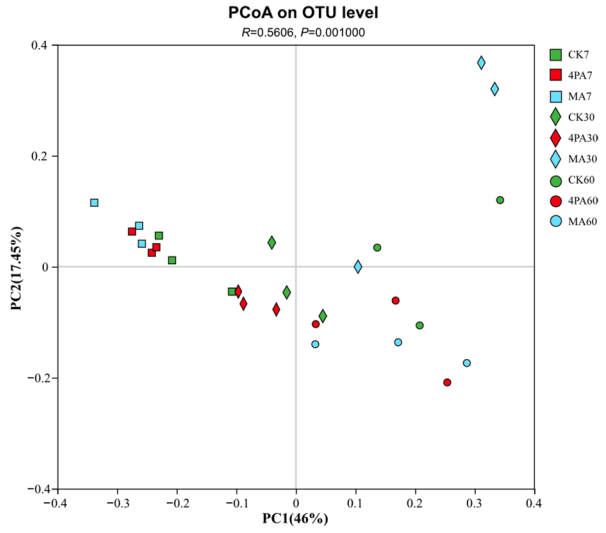

Figure 2. Principal-coordinate analysis (PCoA) plots based on weighted UniFrac distance for bacterial community and treatment (R=0.5606, p=0.00100). CK7, 4PA7 and MA7: control group, addition with 4-pentenoic

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acid and addition with malic acid after 7 days of nettle ensiling, respectively. CK30, 4PA30 and MA30: control group, addition with 4-pentenoic acid and addition with malic acid after 30 days of nettle ensiling, respectively. CK60, 4PA60 and MA60: control group, addition with 4-pentenoic acid and addition with malic acid after 60 days of nettle ensiling, respectively.

As shown in Figure 3 A, Firmicutes were the most dominant bacteria among control and each treated groups, followed by Cyanobacteria and Proteobacteria during 60 days of nettle ensiling, and none significant difference was observed among each groups (p>0.05).

As shown in Figure 3 B, *Aerococcus* (50.62 %—61.52 %) was the dominant bacteria both control and two acids treated groups after 7 days of nettle ensiling, followed by *Enterococcus* (5.66 %—12.32 %) and *Jeotgalibaca* (2.51 %—9.25 %), no significant difference was observed for these bacteria among each groups (p>0.05). *Aerococcus* (20.56 %—41.07%) was dominant bacteria in both control and two acids treated groups after 30 days of nettle ensiling, followed by *Irregularibacter* (2.50 %—39.78 %) and *Atopostipes* (8.04 %—11.67 %), none difference was observed for these bacteria among each groups (p>0.05). *Desemzia* showed the significant highest relative abundance in 4PA treated when compared with control and MA treated, and control was higher than malic treated after 30 days of nettle ensiling (relative abundant were 3.50 %, 2.43 % and 4.16% for control, MA treated and 4-pentenoic acid, respectively, p<0.05). *Weissella* showed significant highest relative abundant in 4PA treated when compared with control and MA treated, and MA treated was higher than control after 30 days of nettle ensiling (relative abundant were 0.64 %, 1.41 % and 2.55 % for control, MA treated and 4-pentenoic acid, respectively, p<0.05). Aerococcus (22.94 %—24.62 %) was dominant bacteria in both control and two acids treated groups after 60 days of nettle ensiling, followed by *Atopostipes* (15.53 %—22.45 %) and *Irregularibacter* (9.37 %—21.30 %), none difference was observed for these bacteria among each groups (p>0.05). *Tissierella* showed significant highest relative abundant in control than other two treated groups, MA treated was higher than 4PA treated after 60 days of nettle ensiling (relative abundant were 6.85 %, 2.30 % and 0.08 % for control, MA treated and 4-pentenoic acid, respectively, p<0.05).

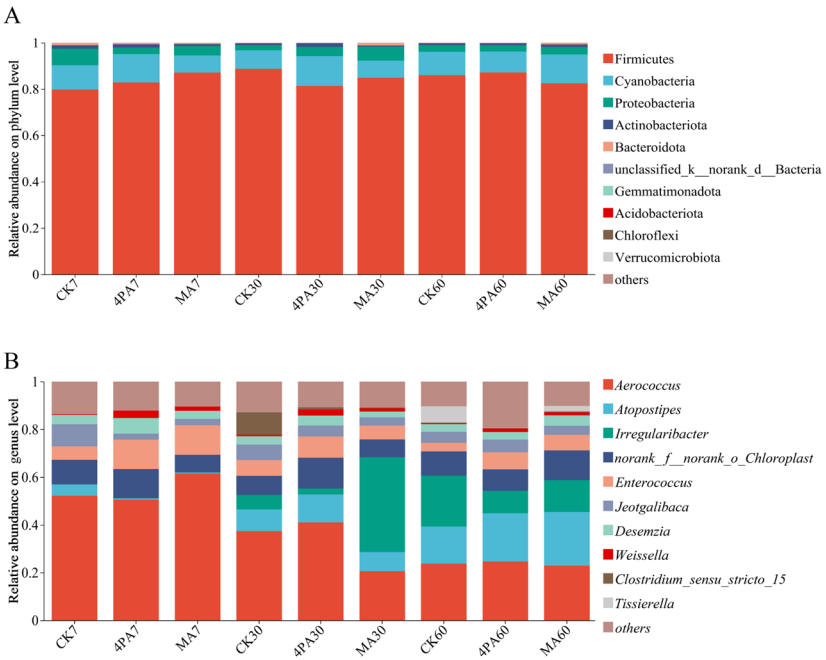

Figure 3. Bacterial community analysis on phylum (A) and genus level (B) after addition with malic and 4-pentenoic acid during nettle ensiling. CK7, 4PA7 and MA7: control group, addition with 4-pentenoic acid and addition with malic acid after 7 days of nettle ensiling, respectively. CK30, 4PA30 and MA30: control group, addition with 4-pentenoic acid and addition with malic acid after 30 days of nettle ensiling, respectively. CK60,

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4PA60 and MA60: control group, addition with 4-pentenoic acid and addition with malic acid after 60 days of nettle ensiling, respectively.

As shown in Figure 4, *Jeotgalibaca*, *Lactococcus* and *Aerococcus* were the most significant difference bacteria in control, 4-pentenoic acid and MA treated groups, respectively, after 7 days of nettle ensiling (p<0.05). *Facklamia* and *Rubellimicrobium* were the most significant difference bacteria in 4-pentenoic acid and MA treated groups, respectively, after 30 days of nettle ensiling (p<0.05). *Atopostipes* and *Tissierella* were the most significant difference bacteria in control and MA treated groups, respectively, after 60 days of nettle ensiling (p<0.05).

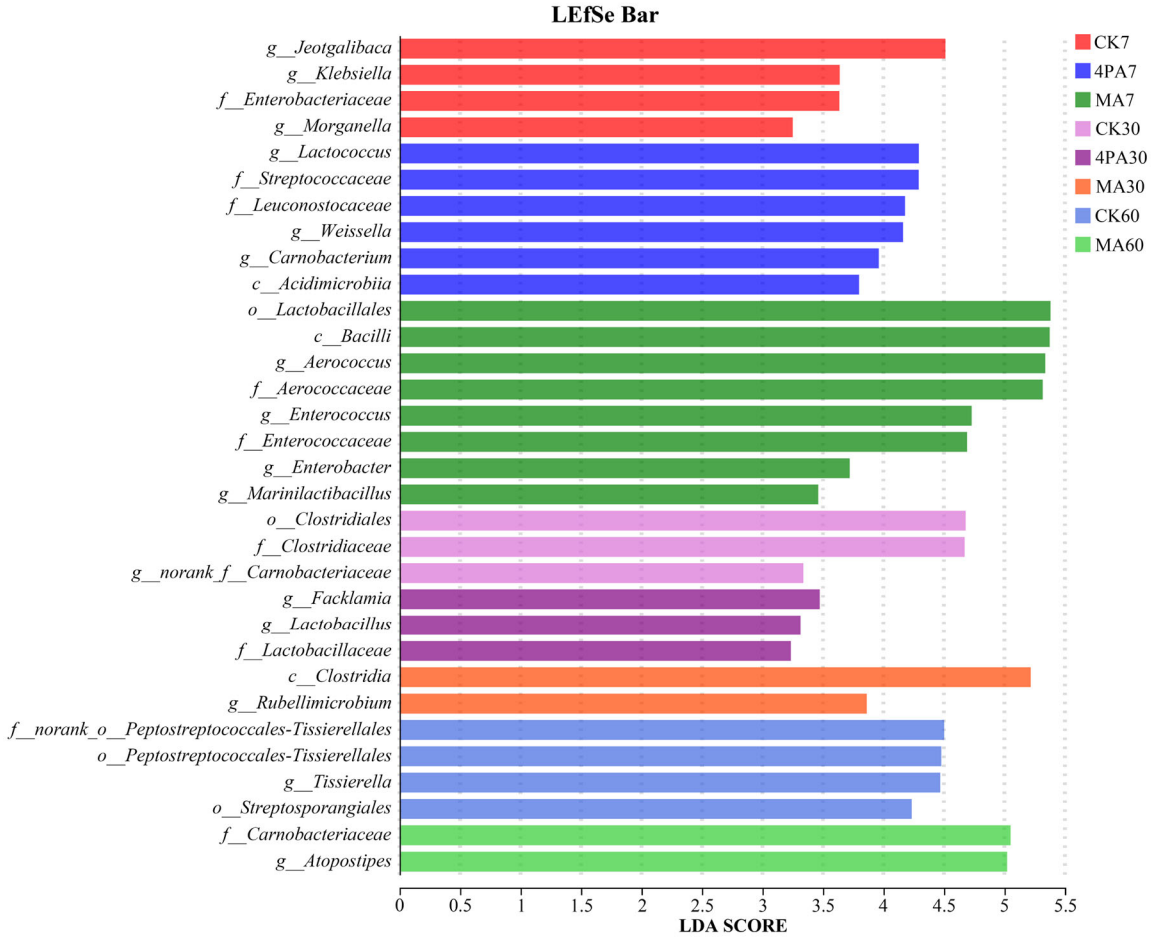

Figure 4. Comparison of microbial variations using LEfSe (LDA effect size) analysis of nettle silage after addition with malic and 4-pentenoic acid. CK7, 4PA7 and MA7: control group, addition with 4-pentenoic acid and addition with malic acid after 7 days of nettle ensiling, respectively. CK30, 4PA30 and MA30: control group, addition with 4-pentenoic acid and addition with malic acid after 30 days of nettle ensiling, respectively. CK60, 4PA60 and MA60: control group, addition with 4-pentenoic acid and addition with malic acid after 60 days of nettle ensiling, respectively.

3.3. Correlation Analysis Between Bacteria Community and Fermentation Characteristics in Nettle Silage

As shown in Figure 5, *Tissierella*, *Anaerosporobacter*, *Irregularibacter*, *Tepidimicrobium*, *Atopostipes* and *Clostridium sensu stricto 15* showed significant positively correlation with ammonia nitrogen, BA and pH; while *Aerococcus*, *Lactococcus*, *Carnobacterium* and *Enterococcus* showed significant negative correlation with each characteristics (p<0.05). *Tissierella*, *Anaerosporobacter*, *Irregularibacter*, *Tepidimicrobium*, *Atopostipes* and *Clostridium sensu stricto 15* showed significant negatively correlation

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with WSC, CP, LA and AA; while *Aerococcus*, *Lactococcus*, *Carnobacterium* and *Enterococcus* showed significant positive correlation with each characteristics (p<0.05).

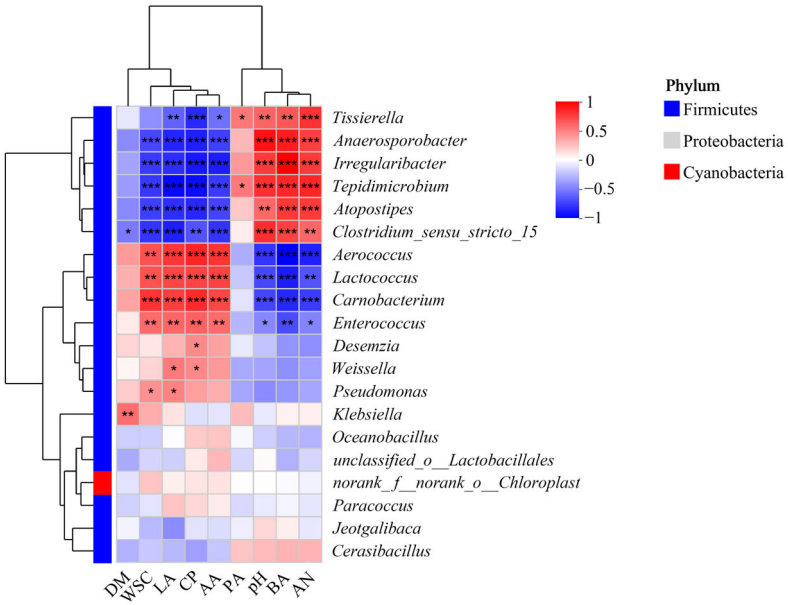

Figure 5. Heatmap of correlation analysis between bacteria and characteristic in nettle silage. Red/blue indicate positive/negative correlations. "*" means p<0.05; "**" means 0.01<p<0.05; "***" means p<0.001. Note: DM, dry matter; WSC, water-soluble carbohydrate; LA, lactic acid; CP, crude protein; AA, acetic Acid; PA, propanoic acid; BA, butyric acid; AN, ammonia; "*" indicates significant correlation (P<0.05); "**" indicates highly significant correlation (P<0.01).

4. Discussion

4.1. Effect of 4-Pentenoic Acid and Malic on Characteristics of Nettle Silage

While our prior research [14] focused on optimizing nettle ensiling using LAB inoculants. This experimental design builds directly on previous work, which identified 4PA and MA as key limiting factors in nettle silage fermentation. Critically, 4PA actively inhibits LAB activity [3, 11]; the current study extends these findings by evaluating plant-derived organic acids (specifically 4PA and MA) as targeted antimicrobial additives against spoilage microorganisms, particularly *Clostridium* spp. This experimental design builds directly on previous work, which identified 4PA and MA as key limiting factors in nettle silage fermentation. Critically, 4PA actively inhibits LAB activity [11]; Consequently, this work represents a mechanistically justified progression toward understanding acid-mediated preservation strategies in high-protein silages.

DM content was highest attributed to malic acid after 30 days of ensiling, but this effect was disappeared as the silage fermentation time was prolonged. After 60 days of ensiling, 4PA treatment reduced crude protein loss by 43% compared to control (CK: 21.4% DM loss vs. 4PA: 12.2% DM loss) and by 39.0% versus malic acid (MA) treatment (2.85% DM loss), demonstrating superior preservation of protein integrity. The persistently high pH (>8.0 after 60 days) and complete disappearance of LA in all groups (Table 1) align with established characteristics of failed nettle ensiling [3]. These phenomena were caused by clostridial dominance—*Clostridium sensu stricto 15* degrades proteins into AN, neutralizing organic acids and maintaining high pH. Accumulation of LA mainly responds for pH reduction in silage [15]. As present study showed that LA content was decreased until disappeared after 60 days of ensiling, which respond to pH level. LAB mainly convert WSC into organic acids such as lactic acid and acetic acid [16]. Data revealed that content of WSC was stabilized during 30—60 days of nettle ensiling, indicate that LAB fermentation process probably

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stopped during this period. However, both ammonia and BA were increased with ensiling time prolonged, indicate that Clostridial fermentation occurred during nettle ensiling. There are two types of *Clostridium* fermentation that cause silage to spoil and deteriorate, the type of glycolysis such as *C. butyricum* that generates hydrogen, carbon dioxide and BA through the fermentation of sugar and LA, and the type of protein decomposition such as *C. sporogenes* that produces ammonium nitrogen and amines by breaking down free amino acids and proteins [17, 18]. WSC content was decreased 72.26 % after 30 days of nettle ensiling and stabilized with ensiling time prolonged, same as LA content except it disappear with ensiling time prolonged to 60 days. The results probably suggest that these glycolysis type of *Clostridium* spp. prefer utilized LA as carbon source during nettle ensiling. *C. sporogenes* indicated strong capacity to degradation protein, especially when WSC is limited. As present study demonstrated that content of ammonia increased 3-fold after 60 days of ensiling when compared with 7 days of ensiling, indicate that activity of protein decomposition type of *Clostridium* was extremely higher during ensiling.

4-pentenoic acid is not a conventional organic acid additive of silage, it mainly found in nettle silage [3]. As a plant-specialized metabolite, 4-pentenoic acid uniquely targets proteolytic Clostridial in nettle silage—unlike conventional additives like malic acid. Little know about inhibit activity of 4-pentenoic acid against to bacteria, only one study observed that 4-pentenoic acid showed certainly level of capacity to inhibited relative abundance of *C. botulinum* [19]. Our previous studies identified 4-pentenoic acid as an endogenous metabolite in naturally fermented nettle silage, exhibits differential antibacterial activity: it strongly inhibits the beneficial lactic acid bacterium *Pediococcus pentosaceus* (MIC = 16 mg·mL^{-1}) by disrupting cell membrane integrity and suppressing fatty acid biosynthesis (*fabG*) and acid-tolerance proteins (*accA*). Conversely, 4PA indirectly suppresses harmful bacteria (e.g., *Clostridium sensu stricto 15*) by promoting antagonistic LAB genera (e.g., *Enterococcus*), evidenced by significantly reduced AN production (p<0.05) [3,11]. The present study supplemented 4-pentenoic acid at 1% fresh weight—a concentration exceeding typical endogenous levels (0.2—0.5 mg·g^{-1} DM) observed in natural fermentation. Results confirmed that exogenous 4-pentenoic acid significantly suppressed proteolytic *Clostridium sensu stricto 15*, reducing BA and AN production by 42.35% and 25.95%, respectively, after 60 days of ensiling. This demonstrates 4-pentenoic acid's capacity to inhibit relative abundance of *Clostridium spp*, and particularly glycolytic types, though the pharmacological dosage used here may not reflect its physiological function in natural systems.

Malic acid effectively suppresses harmful microorganisms (e.g., Clostridial and molds) through rapid acidification, without significantly inhibiting beneficial LAB, while synergistically enhancing silage quality when combined with homofermentative LAB. As a conventional organic acid additive in silage production, studies demonstrate that malic acid supplementation at 0.6%—1% (fresh weight basis) reduces AN content in silages with high CP levels (16.86%—22.20% of dry matter, DM) [20-22]. Unexpectedly, in nettle silage (CP levels is 16.89% of DM), MA treatment increased AN production at the late stage of ensiling (day 60). This discrepancy likely stems from complex inhibitory interactions among endogenous biologically active compounds and microorganisms, necessitating further mechanistic investigation.

4.2. Effect of 4-Pentenoic Acid and Malic On Bacteria Community of Nettle Silage

Aerococcus belong to *Lactobacillales*, often consider as "more peripheral" LAB, usually observed in fermented food [23]. Only a few studies have found this bacterium exists in silage [24-26]. As present study results demonstrated that, *Aerococcus* was most dominant bacteria during entire nettle ensiling process, and acids treated revealed none impact for it. Same result was observed in paper mulberry silage as *Aerococcus* was dominant during ensiling, but dropped 55.79 % activity when inoculation with some strain of *Lactobacillus* [24]. However, relative abundant of this bacteria were usually below 10 % in alfalfa and pennisetum, even below 1 % after *Lactobacillus* was inoculated [25,26]. Clearly, *Lactobacillus* had capacity to inhibited *Aerococcus* activity. Thus, high level relative abundance of *Aerococcus* probably due to both nettle and paper mulberry contain lots of bioactivity

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compounds (polyphenol, flavonoids, organic acids, etc.) such as morin and naringin which impact *Lactobacillus* activity [3,27].

Both acids treated increased relative abundance of *Weissella* after 30 days of nettle ensiling, especially for 4-pentenoic acid showed the higher capacity. *Weissella* is obligate heterofermentative LAB which initiating early fermentation of silage, and activity could highly reduction when pH decline (usually < 5.0) with ensiling prolonged [28]. One study observed that malic acid decreased *Weissella* activity in silage system, it was mainly due to pH rapidly declined reach to 4.0 in *Moringa oleifera* leaves (MOL) silages [29]. Present study indicated that pH was over 8, indicate 4-pentenoic acid and malic acid probably was the mainly reason for this bacteria growth, specific mechanism of this need further study. Furthermore, both acids treated inhibited relative abundance of *Tissierella* after 60 days of nettle ensiling, especially for 4-pentenoic acid showed the higher capacity. *Tissierella* could growth at pH range from 6.5 to 8.5 (optimum pH of 8.3) in sludge fermentation and produce butyric acid [30].

Additionally, present study indicated that 4PA treated showed trend to inhibited relative abundance of *Clostridium sensu stricto 15* after 30 days of nettle ensiling. It is the first time to found that 4-pentenoic acid probably showed the capacity to inhibited *Clostridium* in silage. Same studies were found that addition with malic acid (5 $g \cdot kg^{-1}$ of FM) into alfalfa and cassava silage could decreased relative abundances of *Clostridium* (BA and AN were significantly decreased.) [21,22].

Regarding the dual effects on LAB and Clostridia, both acids exhibited distinct patterns. MA transiently stimulated LAB activity early in ensiling (e.g., increased LA by 57.1% vs. control at day 7; Table 1), likely via its metabolic utilization by *Lactobacillus* [3,11]. However, this LAB-promoting effect diminished at day 30, coinciding with resurgence of *Clostridium sensu stricto 15* (Figure 3B) and elevated BA/AN in MA silages. In contrast, 4PA initially suppressed LAB (e.g., reduced LA by 8.3% vs. MA at day 7), consistent with its inhibitory role against *Pediococcus* in nettle silage [3,11]. Crucially, 4PA demonstrated superior and sustained inhibition of *Clostridium sensu stricto 15* , correlating with 42.4% lower BA and 26.0% lower AN vs. MA at day 60 . This indicates that while both acids may partially inhibit LAB, 4PA's selective and potent anti-clostridial activity (evidenced by its direct suppression of C. botulinum in vitro [11,19]) overrides its early LAB suppression, ultimately preserving protein integrity and fermentation quality. MA treated's weaker clostridial inhibition likely stems from its primary role as a metabolic substrate rather than a dedicated antimicrobial agent.

Notably, based on LEfSe analysis, present study showed that *Jeotgalibaca* were potential important bacteria involved in early-fermentation stage (7 days) of nettle silage, however, this result changed to *Lactococcus* and *Aerococcus* when addition with malic acid and 4-pentenoic acid, respectively. *Facklamia* and *Rubellimicrobium* were potential important bacteria involved in middle-fermentation stage (30 days) of nettle silage when addition with 4-pentenoic acid and malic acid, respectively. *Atopostipes* was potential important bacteria involved in later-fermentation stage (60 days) of nettle silage, however, this result changed to *Tissierella* when addition with malic acid. Overall, the results suggested that both acids inhibited harmful microorganisms (such as *Clostridium*) throughout the entire fermentation period, with 4-pentenoic acid showing superior efficacy.

4.3. Correlation Analysis Between Bacteria Community and Fermentation Characteristics in Nettle Silage

Clostridium sensu stricto 15 was positive correlated with BA and AN accumulation, which explains the substantial protein degradation (evidenced by elevated AN content in present study) and BA production in control and malic acid-treated silages after 30—60 days of ensiling. This genus likely drove proteolysis and saccharolytic activity, converting proteins and residual organic acids into ammonia and butyrate, thereby elevating pH and compromising silage quality [31].

In contrast, genera such as *Aerococcus*, *Lactococcus*, *Carnobacterium*, and *Enterococcus* demonstrated negative correlations with BA, AN, and pH , while positively correlating with WSC, CP, LA, and AA. Although *Aerococcus* dominated during entire ensiling process of nettle, its result in preserving CP and WSC (positive correlation) suggests potential metabolic activity that limited protein degradation. While *Aerococcus* showed positive correlation with CP, its persistent dominance

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in high-pH silage suggests environmental tolerance rather than proteolytic suppression [27]. Notably, *Lactococcus* and *Enterococcus*—typically associated with early-stage LAB fermentation—showed positive links to LA and AA but were insufficient to counteract clostridial bacteria activity in control groups [24].

Critically, the suppression of *Clostridium sensu stricto 15* and *Tissierella* by organic acid treatments underpins their efficacy in improving silage quality. The 4-pentenoic acid treatment most strongly inhibited these genera (decreased relative abundance of *Tissierella* for 98.80%, CK 6.85% vs. 4PA 0.08%) , corresponding to its decreased production of 42.35 % and 25.95% for BA and AN, respectively, after 60 days of ensiling . This microbial inhibition activity in silage probably aligns with the mechanism of 4-pentenoic acid showed antibacterial activity in vitro [11]. Malic acid also reduced *Tissierella* (decreased for 66.42%) but was less effective against *Clostridium sensu stricto 15*, consistent with its weaker suppression of BA and AN production.

The inverse correlation between *Weissella* and clostridial genera further supports *Weissella* as a beneficial heterofermenter in this high-pH system [32]. Enhanced *Weissella* activity in 4-pentenoic acid treatment silages (2.55% vs. 0.64% in control after 30 days of ensiling) likely contributed to AA accumulation (1.37% DM vs. 0.63% DM in control), partially offsetting the lack of LA acidification [33].

5. Conclusions

This study confirms that 4-pentenoic acid (addition with 1% FW) effectively enhances nettle silage quality by specifically inhibiting proteolytic *Clostridium sensu stricto 15*, thereby reducing BA and AN production, while preserving crude protein. Concurrently, it promotes *Weissella* activity and AA accumulation, partially compensating for LA deficiency. Compared to malic acid, 4-pentenoic acid demonstrates superior efficacy in controlling clostridial spoilage, establishing it as a novel promising plant-derived preservative for high-protein silages.

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Abbreviations

The following abbreviations are used in this manuscript:

AA	Acetic acid
AN	Ammonia-N
BA	Butyric acid
CP	Crude protein
DM	Dry matter

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LA	Lactic acid
LAB	Lactic acid bacteria
MA	malic acid
PA	Propionic acid
WSC	Water-soluble carbohydrate
4PA	4-pentenoic acid

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