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Effect of 3-Phenyllactic Acid and 3-Phenyllactic Acid-Producing Lactic Acid Bacteria on the Characteristics of Alfalfa Silage

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Abstract: In this study, an experiment was performed to evaluate the effect of lactic acid bacteria and 3-phenyllactic acid (PLA) on the fermentation quality and chemical composition of alfalfa silage. Several PLA-tolerant strains were screened from silages and identified. The selected strains (1×10^6 colony forming units/g fresh alfalfa) and PLA (1.0, 2.0, or 3.0 g/kg) were applied to alfalfa before ensiling. After 45 days of storage, the silages were unsealed and subjected to component analysis. Biochemical methods and 16S rDNA gene sequencing were used for the identification of the two strains as *Lactobacillus plantarum*. The characteristics of chemical and fermentation compounds indicated that PLA and the two strains efficiently improved the quality of the alfalfa silage. It can be concluded that the use of the strains and PLA can significantly improve the quality of silage.

Keywords: 3-phenyllactic acid; alfalfa silage; *Lactobacillus plantarum*; strain screening

1. Introduction

Alfalfa silage is a rough feed commonly used for ruminants because it is nutritious. During the process of wilting and storage, some of the proteins in alfalfa silage break down to nonprotein nitrogen (NPN), resulting in poor nitrogen utilization by ruminants [1]. Furthermore, the dietary N that cannot be utilized by ruminants is excreted into the environment [2]. Thus, to meet the N demand of ruminants fed silage-based diets, reduced protein degradation during silage is important.

To obtain high-quality alfalfa silage, a variety of additives are added during the process of making silage, such as lactic acid bacteria, propionic acid, formic acid, cellulase, tannin, and benzoic acid [2–5]. The addition of these additives not only improves the feeding value but also improves the stability of the alfalfa silage.

Researchers isolated lactic acid bacteria from fresh rumen fluid and silages to stimulate the ensiling process [4]. Lactic acid bacteria with high growth rates, fibrolytic activities, and ferulic acid activities were suggested to be efficient in improving the quality of alfalfa silage [4]. Currently, changes in the content of functional composition during alfalfa ensiling have attracted much attention [6,7].

The chemical 3-Phenyllactic acid (PLA) was first identified as an antifungal compound in lactic acid bacteria in 2002 [8]. PLA is a by-product of phenylalanine (Phe) metabolism and has efficient antibacterial action against food-spoilage microorganisms [9,10].

The biosynthesis pathway of PLA is shown in Figure 1. Phe can be generated from either the Phe biosynthesis pathway or protein degradation. Phenylpyruvate is one of the intermediate products of Phe biosynthesis, and it is produced by transamination of Phe [11,12]. Phenylpyruvate can be decarboxylated, reduced, and chemically oxidized, resulting in phenylacetaldehyde, PLA, and benzaldehyde, respectively. PLA is considered a natural environmentally friendly organic acid, and its antimicrobial activity is safe and efficient [10]. Furthermore, it has promising applications as an additive for feed and as an antiseptic agent for food.

Figure 1. Biosynthesis of 3-Phenyllactic acid.

PLA showed positive biofunction when fed to animals. L-PLA could replace 70.1% Phe in the diets of chicks and mice [13]. It was reported that PLA might have positive effects to the immune system of hens and pigs [14,15]. PLA improved the egg production rate, and eggshell breaking strength in laying hens over a short term. It increased immune-related blood cell counts in weanling and growing pigs [15]. Furthermore, the odor of PLA was less pronounced than those of other organic acids. The above results indicate that PLA may be used as an alternative to antibiotics for growth promotion. However, no studies have reported the effect of PLA on plants as a silage additive.

To study the effects of lactic acid bacteria and PLA on the quality of alfalfa silage, the fermentation and nutrition characteristics of silage were analyzed.

2. Materials and Methods

2.1. Lactic Acid Bacteria Screening

Lactic acid bacteria were collected from pickles, alfalfa silage, corn silage, and oat silage. Twenty grams of fresh samples was mixed with 20 g of distilled water for 10 min in a blender. The mixture was centrifuged, the supernatant was spread on solid Man, Rogosa, Sharpe (MRS) medium supplemented with 10 g/L of PLA, and the plates were incubated at 30 °C. A total of 48 colonies was picked and transferred to liquid MRS medium in triplicate. After incubation at 30 °C for 24 h, the OD and the

organic acid were quantified by spectrophotography and HPLC. Two strains (M1 and M3) with similar growth rates and different PLA production efficiencies were chosen.

2.2. LAB Identification

The genomic DNA of M1 and M3 was extracted with the DNA Isolation Kit (Tiangen Biotech Co., Ltd., Beijing, China). Genomic DNA was used as a template for 16S rDNA amplification. The following primers were used: 25f (5'-AACTGAAGAGTTTGATCCTGGCTC-3') and 1492r (5'-TACGGCTACCTTGTACGACT-3') [16]. The PCR products were gel-purified and Sanger sequenced by Majorbio Company (Beijing, China). The sequences were aligned in NCBI.

Carbohydrate fermentation was determined in carbohydrate-free MRS medium with filter-sterilized carbohydrates [17]. After inoculating MRS with 1% overnight LAB culture, the medium for carbohydrate fermentation analysis was incubated at 37 °C for 48 h. OD₆₂₀ readings above 0.5 were identified as positive.

2.3. Determination of PLA

Fermented medium was centrifuged at 10,000× g for 20 min, and the supernatant was used for the organic acid analysis. PLA and other organic acids were determined on an Agilent 1100 HPLC system, using the method described by Guo et al. [18].

2.4. Additive Preparation

The strains M1 and M3 were cultured in 3 mL of liquid MRS medium for 20 h, and then 1 mL of broth was transferred to 100 mL of liquid MRS, respectively. The 100 mL of liquid MRS was incubated at 30 °C. After incubating for 24 h, 10 mL of broth from 100 mL of liquid MRS was transferred to 1 L of MRS medium and cultured at 30 °C for 24 h. The cells were collected by centrifugation at 6800× g for 30 min and washed with PBS buffer (pH 8.0). The LAB cells were mixed with non-fat milk solution and then lyophilized. The population of the viable cells per gram was assessed by plate cultivation.

2.5. Silage Making

Alfalfa was harvested during the early blooming period (the first cutting). It was withered to a water content of 67% and chopped into fragments of approximately 0.5 cm; the two factor treatments (3 × 4) were designed as factor A, CK (water), M1 (1 × 10⁶ cfu/g fresh alfalfa), and M3 (1 × 10⁶ cfu/g fresh alfalfa); and factor B, 0, 1, 2, or 3 g PLA/kg fresh alfalfa. The fresh material and additives were mixed thoroughly. A total of 70 g of the mixtures of each treatment was packed into 100 mL plastic jars. Three replicates were made for each treatment. The triplicate silos for each treatment were well sealed and stored at room temperature for 45 days.

2.6. Analytical Methods

For each sample, the materials in the bag were well mixed and divided into 4 parts when the silo was opened. A total of 17.5 g of silage sample was blended with 157.5 g deionized water in a working-blender jar. The extract was centrifuged at 10,000× g and then filtered using 0.22 µm microfiltration membranes. The supernatant was used to value the pH, ammonia nitrogen, and organic acid. Another quarter of silage sample were dried at 65 °C for 48 h, in an oven. Then, the dry sample was ground, screened, and used for chemical composition (dry matter (DM), neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP) and water-soluble carbohydrate (WSC)) analysis by the methods of Guo et al. [18].

2.7. Statistical Analysis

The effects of LAB strains and PLA on the principal characteristics of alfalfa silages were analyzed by two-way analysis of variance. The fixed effects of additives were analyzed by using the same method. The SPSS19.0 software (IBM Corp., Armonk, NY, USA) was employed to evaluate data. The differences between the treatment means and the significance were determined at the 0.05 level of probability and were evaluated by Duncan's multiple range tests. The R soft package was utilized for correlation analysis between fermentation and chemical characteristics of alfalfa silages.

3. Results

3.1. Strain Screening and Characteristics of the Strains

Most PLA-producing strains were isolated from pickles [9]. As a newly found antifungal compound, PLA showed inhibitory effects toward yeast and mold species, such as *Penicillium*, *Aspergillus*, *Candida*, *Rhodotorula*, and *Rhizopus* species [10–12,19]. PLA was also detected in silages, and its presence might relate to LAB [20]. Xu et al. reported that the content of PLA in silage was probably produced by *L. acetotolerans* and positively correlated with *L. plantarum* [20]. It was speculated that the antifungal activity of LAB strains was positively related to the metabolic content of PLA [12].

In the current study, the LAB strains were collected from silages and pickles. The PLA-tolerant bacteria were obtained on solid MRS supplemented with PLA. Then, the PLA-tolerant LAB strains were screened in order to obtain strains with the ability to produce PLA. In this study, eight strains (X1–X6, X8, and X9) from alfalfa silage, six strains (Y1–Y6) from pickled watermelon, nine strains (P1–P9) from corn silage, nine strains (S1–S9) from oat silage, two strains (C1 and C2) from pickled carob, five strains (M1–M5) from pickled cucumber, three strains (A2, A4, and A5) from pickled radish, and six strains (N1–N6) from pickled Chinese cabbage were selected. Among the 48 strains obtained, 42 could grow rapidly in liquid MRS, and 39 could reduce the pH of the medium to less than 4.5.

Most of the candidate strains showed vigorous growth in liquid MRS medium and produced different organic acid contents (Figure 2). Based on the OD₆₂₀ and the pH in liquid MRS medium, eleven strains with similar growth rates and abilities to produce organic acid were selected for further analysis. The organic acid in the supernatant was analyzed by HPLC. As shown in Table 1, the selected strains showed differences in their ability to produce PLA. Nine strains produced over 0.050 mg/mL of PLA. Most of the strains selected from homemade pickles produced a higher content of PLA than the LAB from silages, which revealed that silage and homemade pickles were potential resources for screening for PLA-producing strains. Two of the eleven strains were chosen based on their outstanding lactic acid- and PLA-producing abilities. The identities of the two strains designated M1 and M3 were determined by analyzing the 16S rDNA, which was similar to the 15S rDNA of *L. plantarum*. The 16S rDNA of M1 and M3 showed 100% and 99% identity to *L. plantarum* strain KCCP 11,446 (Accession No. MN453623.1) and *L. plantarum* strain gp1 (Accession No. KM495856.1), respectively. The screened strains, M1 and M3, both exhibited a high growth rate, and lactic acid productivity thus showed potential use for silage making. Then, the carbohydrate fermentation was performed. The two strains had similar carbohydrate-fermentation abilities (Table 2), similar abilities to produce lactic acid, and different abilities to convert PLA from glucose (Table 1).

Figure 2. The growth (A) and pH (B) of the strains incubated in MRS medium.

Table 1. The acid production of lactobacillus bacteria cultivated in MRS medium.

Strain	Lactic Acid (mg/mL ²)	Acetic Acid (mg/mL)	Propionic Acid (mg/mL)	Butyric Acid (mg/mL)	Phenyllactic Acid (mg/mL)
X5	10.82 ± 0.08de	2.87 ± 0.02bc	2.19 ± 0.00a	ND	0.054 ± 0.001cd
X6	10.65 ± 0.06ef	2.87 ± 0.00bc	2.17 ± 0.01ab	ND	0.053 ± 0.001cd
X9	10.50 ± 0.24f	2.86 ± 0.02cd	2.15 ± 0.04bc	ND	0.051 ± 0.000d
Y2	10.50 ± 0.18f	2.87 ± 0.01bc	2.18 ± 0.02ab	ND	0.058 ± 0.001b
Y3	10.45 ± 0.14f	2.89 ± 0.01ab	2.12 ± 0.01cd	ND	0.052 ± 0.001cd
Y6	10.97 ± 0.22bcde	2.89 ± 0.01ab	2.17 ± 0.01ab	ND	0.052 ± 0.001cd
M1	11.28 ± 0.08ab	2.80 ± 0.01f	1.97 ± 0.02f	ND	0.066 ± 0.000a
M2	10.90 ± 0.37cde	2.86 ± 0.02cd	2.02 ± 0.03e	ND	0.052 ± 0.001cd
M3	11.33 ± 0.12a	2.84 ± 0.01e	2.13 ± 0.01cd	ND	0.055 ± 0.000c
M5	11.02 ± 0.06abcd	2.84 ± 0.01 de	2.13 ± 0.01cd	ND	0.048 ± 0.001d
A4	11.22 ± 0.07abc	2.91 ± 0.01a	2.09 ± 0.01	ND	0.050 ± 0.001d

Values with different small row (a–f) show significant differences among acid content in the same column.

Table 2. Carbohydrate fermentation of *L. plantarum* strain.

Carbohydrate	<i>L. Plantarum</i> M1	<i>L. Plantarum</i> M3
Arabinose	+	+
Cellobiose	+	+
Fructose	+	+
Galactose	+	+
Glucose	+	+
Lactose	+	+
Maltose	+	+
Mannose	+	+
Raffinose	+	+
Rhamnose	—	—
Sucrose	+	+
Xylose	+	+

The symbols represent the growth of the strains on the solid medium containing carbohydrate, +, normal; —, no growth.

3.2. Chemical Composition of Fresh Alfalfa

The chemical composition of fresh alfalfa was tested after it was wilted. The WSC, CP, NDF, and ADF content of the alfalfa were 5.65%, 20.58%, 39.42%, and 29.29% DM, respectively. The $\text{NH}_3\text{-N/TN}$ was 3.09%.

3.3. The Fermentation Characteristics of Alfalfa Silage

The fermentation characteristics and PLA content of alfalfa silage are shown in Table 3. As indicated in Table 3, after 45 days of ensiling, the silages treated with PLA had lower pH values.

Table 3. Effect of lactobacillus bacteria and phenyllactic acid on the fermentation characters of alfalfa silages.

Strains ¹	Initial Content of PLA	pH	Lactic Acid (% DM ²)	Acetic Acid (% DM)	Propionic Acid (% DM)	Butyric Acid (% DM)	Phenyllactic Acid (% DM)
CK	0%	4.65 ± 0.07a	4.17 ± 0.02d	2.75 ± 0.19d	0.64 ± 0.03c	ND ³	0.04 ± 0.00b
	0.1%	4.54 ± 0.04b	4.36 ± 0.09cd	3.04 ± 0.04bc	0.64 ± 0.06c	ND	0.08 ± 0.00a
	0.2%	4.43 ± 0.14c	5.16 ± 0.38ab	2.94 ± 0.20bcd	0.55 ± 0.02c	ND	0.05 ± 0.00a
	0.3%	4.00 ± 0.03g	5.85 ± 0.16a	2.84 ± 0.06cd	0.83 ± 0.06b	ND	0.07 ± 0.01a
M1	0%	4.13 ± 0.03e	5.14 ± 0.05ab	3.33 ± 0.06a	0.91 ± 0.12b	ND	0.03 ± 0.00b
	0.1%	4.06 ± 0.01efg	4.95 ± 0.63bc	2.98 ± 0.21bcd	0.89 ± 0.07b	ND	0.04 ± 0.01a
	0.2%	4.03 ± 0.02fg	4.96 ± 0.18bc	2.77 ± 0.08d	0.93 ± 0.01b	ND	0.05 ± 0.00a
	0.3%	3.99 ± 0.02g	5.86 ± 0.05a	3.17 ± 0.15ab	1.12 ± 0.06a	ND	0.05 ± 0.00a
M3	0%	4.29 ± 0.01d	4.14 ± 0.17d	3.16 ± 0.09ab	1.08 ± 0.10a	ND	0.03 ± 0.01b
	0.1%	4.10 ± 0.02ef	5.88 ± 0.06a	3.06 ± 0.03bc	1.05 ± 0.00a	ND	0.02 ± 0.00b
	0.2%	3.99 ± 0.04g	5.68 ± 0.15ab	2.85 ± 0.03	1.10 ± 0.02a	ND	0.03 ± 0.01b
	0.3%	4.06 ± 0.02efg	5.71 ± 0.10ab	3.12 ± 0.10ab	1.10 ± 0.02a	ND	0.04 ± 0.00b
Significant	Strains	*	**	**	**		**
	C _{PLA} ⁴	**	**	**	**		**
	I	**	**	**	*		**

Means with different small row (a–f) show significant differences ($p < 0.05$) among ensiling days in the same column. ** Significant at $p < 0.01$; * significant at $p < 0.05$; NS, not significant at $p < 0.05$; ¹ CK, no additive control; M1, *Lactobacillus plantarum* M1; M3, *Lactobacillus plantarum* M3; PLA, 3-phenyllactic acid; ² DM, dry matter; ³ ND, not detected; ⁴ C_{PLA}, initial content of PLA; I, interaction between treatments and initial content of PLA. Values with different small row (a–f) show significant differences among acid content in the same column.

The effect of PLA on the content of LA, AA, PA, and PLA in silage was significant ($p < 0.01$). BA was not detectable in the silage. The pH values decreased when PLA was used as an additive. The two selected strains or PLA significantly ($p < 0.01$) increased the content of lactic acid in silage compared with the control. The higher PLA concentrations in the control group than in the control group might be derived from the Phe generated by protein degradation.

The content of O₂ in the early ensiling period promotes the growth of fungi, and the fungi compete with the growth of LAB, resulting in DM loss or even failure in ensiling. During the process of alfalfa ensiling, the initial populations of fungi might have little effect on fermentation based on the observation that the LAB fermented rapidly. The inoculant PLA might inhibit the growth of fungi in early ensiling and have no effect on LAB fermentation. Thus, the silages treated with PLA showed significantly lower ($p < 0.01$) pH values than the control after 45 days of ensiling.

3.4. The Nutrient Characteristics of Alfalfa Silage

The contents of nutrient substances are shown in Table 4. These results indicated that the PLA and LAB strains had higher crude protein contents ($p < 0.05$). The NDF, ADF, and ammonia nitrogen contents in silage and ensiled for 45 days were obviously lower ($p < 0.05$) than those in CK.

Table 4. Effect of lactobacillus bacteria and phenyllactic acid on the nutrition characteristics and concentrations of structural carbohydrates in alfalfa silages.

Strains ¹	Initial Content of PLA	Items ²					
		DM (% FM ³)	CP (%DM)	WSC (%DM)	NDF (%DM)	ADF (%DM)	Ammonia Nitrogen (% TN ⁴)
CK	0‰	25.64 ± 0.94b	20.04 ± 0.27d	1.95 ± 0.41b	44.65 ± 0.34a	34.41 ± 0.29ab	11.28 ± 0.75a
	1.0‰	24.26 ± 0.17c	20.37 ± 0.18bcd	2.19 ± 0.44ab	44.62 ± 0.48a	34.64 ± 0.37ab	11.01 ± 0.39a
	2.0‰	23.76 ± 0.50c	20.58 ± 0.38bcd	2.06 ± 0.13ab	44.20 ± 0.51a	33.32 ± 0.81cd	10.39 ± 0.96ab
	3.0‰	26.24 ± 0.12b	20.49 ± 0.14bcd	2.11 ± 0.09b	43.11 ± 0.12b	33.92 ± 0.45bc	7.58 ± 0.10e
M1	0‰	24.16 ± 0.29c	20.38 ± 0.17bcd	2.37 ± 0.27ab	42.57 ± 0.19b	32.52 ± 0.54ef	8.94 ± 0.67cd
	1.0‰	26.46 ± 0.44b	20.77 ± 0.41bc	2.28 ± 0.16ab	42.36 ± 0.48b	32.11 ± 0.11de	7.75 ± 0.64de
	2.0‰	26.53 ± 0.16b	20.88 ± 0.26b	2.72 ± 0.51a	42.53 ± 0.13b	31.96 ± 0.24fg	7.16 ± 1.29e
	3.0‰	26.13 ± 0.64b	21.62 ± 0.32a	2.15 ± 0.14ab	41.05 ± 0.31c	31.08 ± 0.25h	6.82 ± 0.64e
M3	0‰	28.38 ± 0.36a	20.19 ± 0.14cd	1.88 ± 0.28b	42.61 ± 0.49b	31.69 ± 0.38gh	9.33 ± 0.37ab
	1.0‰	27.81 ± 0.20a	20.43 ± 0.57bcd	2.21 ± 0.22ab	40.09 ± 0.79c	31.00 ± 0.28h	8.05 ± 0.20de
	2.0‰	28.01 ± 0.63a	20.53 ± 0.26bcd	1.95 ± 0.42b	41.51 ± 0.37c	31.54 ± 0.35gh	7.47 ± 0.47e
	3.0‰	27.51 ± 0.72a	20.59 ± 0.37bcd	2.16 ± 0.24ab	40.97 ± 0.07c	32.10 ± 0.25fg	7.11 ± 0.15e
Significant	Strains	NS	**	*	**	**	**
	C _{PLA} ⁵	NS	**	NS	**	**	**
	I ⁶	NS	NS	NS	NS	**	*

Means with different small row (a–f) show significant differences ($p < 0.05$) among ensiling days in the same column. ** Significant at $p < 0.01$; * significant at $p < 0.05$; NS, not significant at $p < 0.05$. ¹ CK, no additive control; M1, *Lactobacillus plantarum* M1; M3, *Lactobacillus plantarum* M3; PLA, 3-phenyllactic acid; ² DM, dry matter; WSC, water soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; ND, not detected; ³ FM, fresh material; ⁴ TN, total nitrogen; ⁵ C_{PLA}, initial content of PLA; ⁶ I, interaction between treatments and initial content of PLA. Values with different small row (a–f) show significant differences among acid content in the same column.

3.5. Correlation Analysis between Silage Fermentation and Treatments

To investigate the relationship between chemical and fermentation characteristics, Spearman-related analysis was performed to determine the correlation of the variables. The results (Figure 3) showed that the PLA content was positively correlated with pH, NH₃-N, NDF, and ADF and negatively correlated with LA, AA, and PA contents during ensiling. The LA content was positively correlated with AA, PA, and CP and negatively correlated with the PLA, DM, pH, NH₃-N, NDF, and ADF contents during ensiling. It was obvious that the LA, AA, and PA were mostly generated by LAB species in the sealed silos. These results suggested that PLA in the alfalfa silage was not solely generated by LAB species during ensiling. The content of PLA in the alfalfa silage treated by M1 was higher than that in the silage treated by M3, suggesting that the PLA content was positively related to the PLA productivity of LAB additives. However, other microorganisms might biotransfer PLA.

Figure 3. Spearman correlation between the fermentation indices in alfalfa silage after 45 days of ensiling. LA, lactic acid; AA, acetic acid; PA, propionic acid; PLA, phenyllactic acid; DM, dry matter; NH₃-N, ammonia nitrogen; CP, crude protein; WSC, water soluble carbohydrate; NDF, neutral detergent fibre; ADF, acid detergent fibre. The color means the significant correlation ($p < 0.05$); the deeper color of the square, the more significant the correlation.

4. Discussion

Homofermentative and heterofermentative LAB are often used as silage inoculants, to produce lactic acid and reduce pH rapidly, which create unfavorable conditions for the growth of mold, *Clostridium*, *Acetobacter*, and other microbes. For a long time, the growth rate and the ability to reduce the pH of the medium were two rules for selecting LAB as inoculants [21,22]. However, currently, biofunctional LAB are more favorable [4,23]. The metabolite analysis indicated that some inoculants produced more amino acids, organic acids, and phenolic acids in the silage samples than in the blank control-treated silage [23]. In the reports of Jin et al., FAE-producing *L. buchneri* increased the NDF digestibility of small-grain silage in situ [24]. Li et al. isolated two bacterial strains that demonstrated cellulolytic potential from Tibetan yak rumen and found that the strains increased the WSC contents of *Pennisetum sinense* silage [25].

The alfalfa proteins gradually degraded during ensiling. Others have used various strategies to reduce the proteolysis of alfalfa [1]. Tannins have been used because they reversibly bind proteins [1]. Formic acid can effectively prevent proteolysis by decreasing pH and inhibiting proteases [3]. Antimicrobial formaldehyde can form crosslinks between protein chains, thus reducing protein degradability [3]. LAB and previously fermented juice were used for the accumulation of lactic acid at early stages of ensiling [5].

A phenolic-related antibiotic (PLA) from *L. plantarum* was identified by Arasu [26]. In this work, several LAB strains were selected, and their PLA productivities were analyzed. *L. plantarum* M1 produced more PLA in MRS medium and alfalfa silage than *L. plantarum* M3. Then, the two strains

and PLA showed the ability to improve the quality of alfalfa silage, especially in decreasing the content of $\text{NH}_3\text{-N}$. The $\text{NH}_3\text{-N}$ is an important index of protein degradation.

Xu et al. found PLA and other metabolites with bacteriostatic activity in whole-crop corn silage, but the treatments had no significant effect on the metabolites [20]. The PLA content was recorded in frozen, grass-dominated crop silages inoculated with LAB [27]. The concentration of PLA first increased and then decreased as the incubation time increased. However, until now, limited studies have been reported on PLA. Most of the reports analyzed gramineae silage [27], and the PLA was positively related to the abundance of *L. acetotolerans* in whole-crop corn silage [20]. In this work, the PLA content was related to the content of $\text{NH}_3\text{-N}$, indicating that the PLA produced during alfalfa ensiling was different from that produced during corn ensiling.

The phenethylamine was biotransformed from Phe by L-phenylalanine carboxy-lyase. The relative concentration of phenethylamine in the $\text{NH}_3\text{-N}$ -rich silage was higher than that in other silage. The Phe may be formed in two ways, as indicated in Figure 1: one is the phenylalanine biosynthesis, and the other is by protein degradation. It was reported that *Clostridium sporogenes* could ferment L-phenylalanine to ammonia, CO_2 , PLA and 3-phenylpropionate [28]. Based on the relationship between the $\text{NH}_3\text{-N}$ and PLA contents at the end of silage, the activities of *Clostridium* during ensiling promoted the production of PLA from Phe generated during proteolysis. Moreover, the content of PLA in MRS medium was higher than that in alfalfa silages, which may be ascribed to the higher availability of amino acids (especially Phe) in the MRS medium compared with that in silages. A similar phenomenon occurred in PLA production by the same strain in MRS broth and in pickles [9]. Other studies have increased PLA production by adding Phe to mustard pickles and synthetic media [9,29]. The PLA was biotransformed from Phe in the raw materials. The difference in the PLA content could be attributed to different amino acid contents in MRS and in alfalfa silage. The Phe was biosynthesized to PLA with a relatively low conversion rate because the rate of transamination was limited; Phe was consumed by other reaction, and LAB provided an insufficient supply of electron acceptors.

Compared with ordinary preservatives, low PLA content improved the quality of alfalfa silage. In practice, ordinary organic acids may cause corrosion of machinery. In this study, the content of PLA was significantly lower than that of ordinary preservatives. In addition, the lactate content remarkably increased with increasing PLA content, indicating that the growth and metabolic activity of LAB strains were unaffected. The content of CP and $\text{NH}_3\text{-N}$ were significantly affected by the PLA and LAB strains. The lowest $\text{NH}_3\text{-N}$, lowest ADF, and highest CP content were obtained in the alfalfa silage treated with M1 and 0.3% PLA, indicating that the biofunctional compound and its producing strains could be used as additives of alfalfa silage, to decrease protein degradation and increase the feed value.

On the other hand, PLA might function as a supplement in farm-animal feed. Phe cannot be synthesized by animals. The additional Phe by infusing increased the yield, and protein content of milk. D-Phe and L-PLA have similar efficacy when used to supplement a Phe-deficient diet or when fed as a source of dietary Phe [11]. PLA could be bioconverted to Phe by bacteria and fungi and used as a supplementary essential amino acid by animals. Thus, PLA might act as an amino acid supplement and antifungal compound when used in silage.

The selected strains exhibited high PLA productivity and low $\text{NH}_3\text{-N}$ content when used as additives in alfalfa silage, indicating that, in addition to lactic acid and acetic acid, the PLA generated by LAB strains was also important for the quality of alfalfa silage. Currently, the functional compounds generated by LAB strains are important for improving aerobic stability [22] and the silage quality, thus providing new ideas for screening for silage additive.

5. Conclusions

The results indicate that PLA improves the quality of alfalfa silage. The chemical additive PLA effectively prevents crude protein in silage from degrading, even at low concentrations. LAB strains that produced high concentrations of PLA could also prevent protein degradation during the ensiling period. The results in this work provide a new way to explore silage additive.

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