Investigation of fermentation profiles, bacterial community structure and bacterial β-carotene synthesis of alfalfa silage treated with propionic acid or its combination with squalene

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Abstract

This study examined the fermentation dynamics, bacterial community composition and bacterial β-carotene synthesis in alfalfa that was ensiled for 3, 15, 45 and 90 days without additives (CON), or with a chemical agent (propionic acid, PA); or a combination of PA and squalene, SQPA). The results showed that silage treated with PA had a lower ($P < 0.01$) pH value than the CON silage in the early ensiling phase (3–15 days). Meanwhile, silage treated with PA had the highest contents of lactic acid, acetic acid and PA after 90 days of ensiling ($P < 0.01$). The β-carotene in alfalfa was lost seriously in the initial ensiling phase (3 days) and epiphytic *Pantoea agglomerans* with the ability to produce β-carotene became extinct. With the extension of ensiling time, the loss of β-carotene was alleviated in all silages. PA and SQPA not only lowered bacterial diversity and simplified bacterial networks but also facilitated the emergence of new β-carotene-producing bacteria. The metabolic function prediction indicated that β-carotene synthesis tended to decrease initially and subsequently increase during ensiling. Furthermore, the variance of enzymes involved in the bacterial synthesis of β-carotene in silages was influenced by PA, SQPA and ensiling time. In summary, the impact of solely adding PA demonstrated superior effects on the fermentation quality of alfalfa silage compared to the effects observed with SQPA. Throughout the ensiling of alfalfa, the succession of different β-carotene-producing bacteria resulted in fluctuations in the levels of β-carotene.

Introduction

β-Carotene (pro-vitamin A), the most active biological form among the carotenoids, is beneficial for both human and livestock health (Lindqvist *et al.*, 2014). Animal products (*e.g.* meat and milk) play a crucial role in diets worldwide, providing many highly bioavailable essential nutrients (van Hooijdonk and Hettinga, 2015). The β-carotene concentrations in animal products depend on their diets (Lindqvist *et al.*, 2014). It makes sense that high β-carotene concentration in feed offers significant advantages not only for livestock health but also for animal products as a β-carotene source for human consumption.

Silage is a primary roughage for ruminants, especially dairy cows, accounting for more than 50% of their rations (Li *et al.*, 2022). Silage making is an anaerobic fermentation process that relied mainly on lactic acid bacteria (LAB). Enormous works have confirmed the loss of β-carotene during silage making (Lv *et al.*, 2020; Zong *et al.*, 2021; Zhang *et al.*, 2022). Some reports have shown contradictory outcomes, indicating an increase in β-carotene concentration after ensiling relative to fresh forage material (Lv *et al.*, 2017; Zhao *et al.*, 2021). Unfortunately, the reason for the diverse findings is unclear. Analogously, Sangija *et al.* (2022) explained that lactic acid fermentation increased β-carotene in African nightshade by disrupting the plant matrix and cell cluster during fermentation, leading to carotenoid liberation in food. Additionally, Garrido-Fernandez *et al.* (2010) demonstrated that *Lactiplantibacillus plantarum* could produce carotenoids since it carried genes for carotenoid biosynthesis. However, the above explanations have yet to be tested in silage.

Alfalfa (*Medicago sativa* L.) is recognized as a preferred legume forage for silage making because of its high nutritional quality (Na *et al.*, 2022). Nevertheless, alfalfa is difficult to ensile well owing to its high buffering capacity (BC) and low water-soluble carbohydrates (WSC). Spontaneous fermentation of alfalfa silage had poor quality due to undesired microbes, such as *Clostridia* (Li *et al.*, 2020). To improve alfalfa silage quality, propionic acid (PA) is considered a high-efficiency additive used in silage due to its antimicrobial activity (Dong *et al.*, 2006), while the antioxidant squalene can be a carbon source for anaerobic or aerobic microbes

(Ghimire *et al.*, 2016), having the potential to alter bacterial community structure and discover functional bacteria. In our previous work, squalene was detected from fermentation products of alfalfa silage. Moreover, while the addition of squalene prevented the degradation of *β*-carotene caused by mixed organic acids simulated in a silage environment, thereby increasing the fluorescence intensity of *β*-carotene, it did not, unfortunately, improve the fermentation quality of alfalfa silage (unpublished data). Considering the advantages of PA and squalene, their combination (SQPA) may have beneficial impacts on both the fermentation quality and *β*-carotene content of alfalfa silage.

Hence, using single-molecule real-time (SMRT) sequencing technology, the purpose of this study was to: (1) investigate the fermentation profiles, bacterial community structure, and *β*-carotene variation in alfalfa silage treated with PA or SQPA during ensiling of 3, 15, 45, and 90 days; (2) clarify whether there were functional bacteria that produced *β*-carotene in alfalfa silage.

Materials and methods

Experimental design and silage preparation

The experiment was conducted using completely randomized blocks, with a 3×4 factorial arrangement of 3 additives (alfalfa forage was ensiled without additives as control, CON; 0.4% PA; 0.4% PA + 1% SQ, SQPA), and four ensiling time points (3, 15, 45 and 90 days), with six replicates. The PA and SQPA were diluted in distilled water and applied on a fresh matter (FM) basis. The CON was sprayed with the same volume of distilled water.

Alfalfa was cultivated in the experimental field of Nanjing Agricultural University (32°86′N, 120°02′E) and was harvested from three fields during its full bloom period in May 2020. Fresh alfalfa material was set up with six repetitions for the determination of biochemical properties. The SQ and PA (analytically pure) were both purchased from Shanghai McLean Biochemical Technology Co., Ltd. The harvested alfalfa was delivered right away to the laboratory and chopped into 1–2 cm lengths by a forage chopper. About 720 g of alfalfa for each treatment was filled in a plastic silo (Volume: 1 litre; Density: 206 kg dry matter (DM)/m^3). According to the aforementioned experimental design, 2.9 g PA, 10.1 g SQPA, and 10.0 g distilled water were uniformly sprayed on fresh alfalfa. To ensure consistent application rates, an additional 7.1 g of distilled water was added to the PA-treated group. The plastic silos were sealed with a cover and tape, and they were covered in dark plastic bags to block out light. All plastic silos were kept in the laboratory cabinet at room temperature (20–38°C). In the corresponding ensiling time, the silo was opened and sampled. Three random samples of each treatment were used to extract microbial DNA for analysing 16S rRNA genes.

Microbial count and chemical analyses

The plate counting method was used for the determination of the microbial population. Briefly, 10 g fresh alfalfa was blended with 90 ml sterilized saline solution (8.5 g/l NaCl), and serially diluted. The LAB (anaerobiosis, 37°C, 3 days), aerobic bacteria (aerobiosis, 37°C, 3 days), and yeasts (aerobiosis, 37°C, 3 days) were cultured on deMan Rogosa and Sharp agar, nutrient agar, and potato dextrose agar medium, respectively. All microbial data were transformed to log$_{10}$. The 25 g sample (fresh material or silage) was immersed in 75 ml sterile water and suspended at 4°C overnight. After being filtered through two layers of cheesecloth and a

Whatman filter paper, the filtrate was used for determining pH, ammonia-N and organic acids (lactic, acetic, propionic and butyric acid). The pH was measured by a glass electrode pH meter (Hanna Instruments, Inc., Woonsocket, RI, Italy). The ammonia-N was quantified by the phenol-hypochlorite reaction (Broderick and Kang, 1980). The organic acids were analysed using an HPLC system (Agilent 1260, Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (Carbomix® H-NP5 column, 2.5 mM H$_2$SO$_4$, 0.5 ml/min). The BC of fresh alfalfa was measured by the titration method (Playne and McDonald, 1966). Another sample (above 50 g) was dried in a forced-draft oven and DM content was determined by method 930.15 of the Association of Official Analytical Chemists (AOAC, 1990). The dried sample was ground to pass a 1-mm screen with laboratory knife mills (FW100, Taisite Instrument Co., Ltd., Tianjin, China), and the powder sample was used for the analysis of carbohydrates components and crude protein. The total nitrogen (TN) content was measured by method 984.13 (AOAC, 1990), and crude protein was calculated via the TN content multiplied by 6.25. The contents of neutral detergent fibre (aNDFom, aNDFom was assayed with a heat-stable amylase and expressed exclusive of residual ash), acid detergent fibre (aADFom, aADFom was expressed exclusive of residual ash), and acid detergent lignin (ADL) were determined by a ANKOM fibre analyser (ANKOM-200, ANKOM Technologies, Macedon, NY, USA) following the method described by Van Soest *et al.* (1991). The WSC content was quantified by the anthrone method (Thomas, 1977).

β-carotene analysis

After pre-processing described in our previous study (Liu *et al.*, 2016), *β*-carotene content was determined by Agilent HPLC 1260 (Agilent Technologies, Inc., Germany; column: Inertsil ODS-4, GL Science B.V., Japan; detector: Diode Array Detector, DAD VL, Agilent Technologies, Inc., Germany, 450 nm for detecting *β*-carotene; eluent: 2 ml/min, methanol-acetonitrile 9:1 (v/v); temperature: 45°C).

Measure of enzymes associated with bacterial β-carotene synthesis pathway

The silage samples underwent the following pretreatment: ① The silage samples (freeze-dried and ground, 0.2 g, stem-leaf ratio = 1:3) were added to 1 ml phosphate-buffered saline (pH = 7.4) and mixed thoroughly. ② The mixed solution was centrifuged at 4°C and 3000 × g for 20 min. According to the product manuals provided by Jiangsu Meibiao Biotechnology Co., Ltd (Yancheng, China), enzymes activities, including geranylgeranyl pyrophosphate synthase (EC:2.5.1.29), phytoene synthase (EC:2.5.1.32), phytoene desaturase (EC:1.3.5.5), and lycopene *β*-cyclase (EC:5.5.1.19), were determined by the corresponding ELISA Kits, respectively.

Microbial sequencing and bioinformatic analysis

The PacBio sequencing process was divided into six steps. Briefly, the TIANamp Bacteria DNA isolation kit (DP302-02, Tiangen, Beijing, China) was used to extract microbial community genomic DNA. Then, the bacterial 16S rRNA genes were amplified using the universal bacterial primers (27F and 1492R). The Polymerase Chain Reactions (PCR) were performed in three

samples of each treatment. After electrophoresis, PCR products were purified using the AMPure® PB beads (Pacifc Biosciences, CA, USA) and quantified with Quantus™ Fluorometer (Promega, Madison, WI, USA). Purified products were pooled in equimolar amounts and DNA library was constructed using the SMRTbell® Express Template Prep Kit 2.0 (Pacifc Biosciences, Menlo Park, CA, USA). Purified SMRTbell libraries were sequenced on the Pacbio Sequel II System (Pacifc Biosciences, CA, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Circular consensus sequence (CCS) was extracted and filtered with the SMRTLink 8.0 (minimum full pass = 3, minimum predicted accuracy = 0.99), and sequences outside of a range of < 1400 bp and > 1600 bp were removed. Optimized-CCS were used for further downstream analyses. Operational taxonomic units (OTUs) with 99% similarity cutoff were clustered via USEARCH (version 7.0), and chimeric sequences were identified and removed (Stackebrandt and Goebel, 1994; Edgar, 2013). Each OTU representative sequence was analysed to obtain classification information by RDP Classifier (version 2.13) against the 16S rRNA database (Silva v138) with a confidence threshold of 75% (Wang *et al.*, 2007). The raw sequencing data have been deposited in the NCBI database (accession: PRJNA941960).

The α-diversity analysis (Shannon and Chao 1) was carried out by MOTHUR (version 1.30.2). The principal coordinate analysis (PCoA) was graphically constructed by R software (version 3.3.1). Bacterial Community structures were analysed from genus to species levels by R software. To illuminate the correlations between fermentation indices and bacteria (on species level), spearman's correlation heatmaps of different silages were established by R software (pheatmap package). The co-occurrence networks were statistically calculated by Networkx software (version 2.8) based on Spearman's correlation and were visualized by Gephi software (version 0.9.2). Microbial functions based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were measured by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2; Version 2.2.0), which predicted the function of bacterial community based on the proportion of marker gene sequences in samples (Douglas *et al.*, 2020).

Statistical analyses

The statistical analysis was performed with IBM SPSS 22.0 for Windows statistical software. Initially, all data relating to fermentation quality, chemical composition, and loss rate of β-carotene of alfalfa silage was subjected to Shapiro–Wilk test for normality and Levene test for homogeneity of variance, respectively, to establish the basic assumptions for conducting analysis of variance (ANOVA, GLM). The effects of additives, ensiling time, and their interaction on the fermentation quality, chemical composition, and loss rate of β-carotene of alfalfa silage were analysed using a two-way ANOVA, according to the statistical model below: $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta \times ij) + e_{ij}$, where Y_{ij} = dependent variable; μ = overall mean; α_i = fixed effect of additives; β_j = fixed effect of ensiling time; $(\alpha\beta \times ij)$ = interaction between additives and ensiling time; and e_{ij} = residual error term. In cases where interaction effects were significant, multiple comparisons for means were conducted using the Tukey's test for data with a single fixed effect.

The indices of Shannon and Chao 1 was analysed using a one-way ANOVA for the following model: $Y_{ij} = \mu + \alpha_i + e_{ij}$, where Y_{ij} = dependent variable; μ = overall mean; α_i = fixed effect of treatments; and e_{ij} = residual error term. The difference between groups was compared with Tukey's test for determining the significant ($P < 0.05$).

Beta diversity was assessed using the dispersion level of sample communities based on Bray-Curtis distance, while the dissimilarity among treatments was assessed using the permutational multivariate analysis of variance (PERMANOVA).

The difference of two modules related to β-carotene synthesis (M000364 and M0097) predicted by PICRUSt2 was analysed using a one-way ANOVA for the following model: $Y_{ij} = \mu + \alpha_i + e_{ij}$, where Y_{ij} = dependent variable; μ = overall mean; α_i = fixed effect of additives; and e_{ij} = residual error term. The multiple comparisons of the means were conducted by Tukey's test at the significance level ($P < 0.05$).

Results

Biochemical properties of fresh alfalfa

The chemical and microbiological characteristics of fresh alfalfa are shown in Table 1.

Chemical composition, fermentation dynamics and microbial counts of alfalfa silage

The interaction of additives and ensiling time had significant ($P < 0.01$) effects on DM, crude protein, pH, organic acids (lactic, acetic, propionic and butyric acid), ammonia-N, LAB and aerobic bacteria, while had non-significant ($P > 0.05$) effects on fiber fractions (aNDFom, aADFom and ADL), WSC and yeasts (Tables 2–4). Throughout the ensiling process, PA- and SQPA-treated silages had a statistically or numerically higher DM content than CON silage. After 3 days of ensiling, PA-treated silage had lower crude protein than CON silage, while its content declined in CON silage from 15 to 90 days. The PA-treated silage had a lower ($P < 0.01$) pH value than that in CON silage in the early ensiling phase (3–15 days). Meanwhile, PA-treated silage had the highest ($P < 0.01$) contents of lactic acid, acetic acid and PA after 90 days of ensiling. Ammonia-N accumulated continuously during the ensiling process. After 90 days of ensiling, the ammonia-N in PA-treated silage was higher ($P < 0.05$) than that in CON and SQPA-treated silages. The PA-treated silages had

Table 1. Chemical and biological composition of fresh alfalfa

Items[a]	Fresh alfalfa ($n = 6$)
pH	5.7 ± 0.1
Water-soluble carbohydrates (g/kg DM)	59.3 ± 1.6
Dry matter (g/kg FM)	286 ± 4.2
Crude protein (g/kg DM)	207 ± 0.6
β-Carotene (mg/kg DM)	162 ± 0.8
Neutral detergent fibre (g/kg DM)	364 ± 2.2
Acid detergent fibre (g/kg DM)	277 ± 4.5
Acid detergent lignin (g/kg DM)	56.7 ± 2.9
Buffering capacity (mEq/kg DM)	281 ± 22.3
Lactic acid bacteria (lg cfu/g FM)	4.3 ± 0.0
Aerobic bacteria (lg cfu/g FM)	7.3 ± 0.2
Yeasts (lg cfu/g FM)	4.5 ± 0.1

[a]DM, dry matter; FM, fresh matter; mEq, milligram equivalent; cfu, colony-forming units.

Table 2. Chemical composition of alfalfa silage treated with propionic acid or a combination of propionic acid and squalene

Items[a]	Additives[b]	Ensiling time (days)				SEM[c]	Significance[d]		
		3	15	45	90		A	D	A × D
Dry matter (g/kg FM)	CON	244[b]	241[b]	236[b]	238[b]	1.9	<0.01	0.03	<0.01
	PA	253[BCb]	250[Cab]	259[BCa]	264[Aa]				
	SQPA	272[Aa]	255[Ba]	254[Ba]	244[Bb]				
Crude protein (g/kg DM)	CON	218[Aa]	202[B]	202[B]	208[B]	1.1	0.01	0.12	<0.01
	PA	202[b]	211	209	209				
	SQPA	206[ab]	202	199	202				
aNDFom (g/kg DM)	CON	356	359	366	357	3.5	0.52	0.57	0.77
	PA	364	337	348	346				
	SQPA	348	340	367	365				
aADFom (g/kg DM)	CON	273	288	294	288	3.0	0.31	0.24	0.39
	PA	287	259	275	277				
	SQPA	266	267	289	290				
ADL (g/kg DM)	CON	60.4	66.0	66.9	63.5	1.17	0.13	0.14	0.38
	PA	57.8	58.2	61.3	57.4				
	SQPA	56.2	56.8	66.4	70.2				

[a]FM, fresh matter; DM, dry matter; aNDFom, neutral detergent fibre; aADFom, acid detergent fibre; ADL, acid detergent lignin.
[b]CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene.
[c]SEM, standard error of the means.
[d]A, effect of additive; D, effect of ensiling time; A × D, interaction of additive and ensiling time.
[a-b]Values with different small letters show significant differences among additives in the same ensiling days ($P < 0.05$).
[A-C]Values with different capital letters show significant differences among ensiling days in the same additives ($P < 0.05$).

the lowest ($P < 0.01$) butyric acid content after 3 days of ensiling. From the perspective of microbe, with the prolonged ensiling time, The LAB counts were on the rise, while aerobic bacteria were exhibiting a downward trend. The LAB counts in all treated silages peaked after 45 days of ensiling, and PA-treated silage had more ($P < 0.01$) LAB counts than that in CON and SQPA-treated silages. The PA-treated silage had lower ($P < 0.01$) aerobic bacteria counts than that in CON silage after 45 and 90 days of ensiling.

Dynamic changes of β-carotene and lipoxygenase in alfalfa silage

The β-carotene loss occurred in alfalfa silage after ensiling, and its dynamic change is presented in Fig. 1. The interaction of additives and ensiling time had significant ($P < 0.01$) effects on β-carotene loss. The lower ($P < 0.01$) β-carotene loss occurred in PA-treated silage for 3 to 45 days of ensiling and in SQPA-treated silage for 3 and 45 days than that in CON silage. The higher ($P < 0.01$) β-carotene loss occurred in PA- and SQPA-treated silages for 90 days than that in CON silage.

Diversity of the bacterial community in alfalfa silage

The bacterial community structure of fresh alfalfa and silages is presented in Fig. 2. The α-diversity analysis (Fig. 2a) showed that Shannon and Chao 1 indices were significantly ($P < 0.01$) higher in fresh alfalfa than that in silages. The abundance heatmap displayed that the top 15 of bacteria belonged to *Weissella*, *Lactobacillus*, *Enterococcus*, *Pantoea*, *Lactococcus* and *Enterobacter* in fresh alfalfa and silages. On 3 days, predominant LAB were *Lactobacillus*, *Weissella*, *Enterococcus* and *Lactococcus* in CON

silage, whereas predominant LAB were only *Weissella* in PA- and SQPA-treated silages. With the extension of ensiling time, *Weissella* was succeeded by *Lactobacillus* in PA- and SQPA-treated silages. Concretely, on species level, as for PA- and SQPA-treated silages, specie *Weissella cibaria* dominated after 3 days of ensiling. Species *L. plantarum*, *Lacticaseibacillus paracasei*, *Apilactobacillus kunkeei*, *Lentilactobacillus parabuchneri* and *Fructilactobacillus kullabergensis* dominated from 15 to 90 days of ensiling.

The β-diversity analysis showed the similarities and differences between bacterial communities (Fig. 3). In every treatment cohort throughout the ensiling process, silage was completely ($P < 0.01$) separated from fresh alfalfa (Figs 3a–d). The PA- and SQPA-treated silages were obviously ($P < 0.01$) separated from CON silage after ensiling for 3 and 15 days (Figs 3a and b). Subsequently, all silages were gathered together (Figs 3c and d).

Correlation between bacterial flora and fermentation products as well as their internal network in alfalfa silage

The correlation analysis between bacterial community and fermentation products in different silages is shown in Fig. 4. In CON silage, notable ($P < 0.05$, $1 > |r| > 0.6$) correlations were observed between LAB species (*A. kunkeei* and *F. kullabergensis*) and pH, WSC, butyric acid and acetic acid. Significant ($P < 0.05$, $1 > |r| > 0.6$) correlations were found between *L. plantarum* and butyric acid and lactic acid. In PA-treated silage, *Levilactobacillus brevis* and *L. plantarum* showed a significant ($P < 0.01$, $1 > |r| > 0.6$) correlation with pH. Significant ($P < 0.05$, $1 > |r| > 0.6$) correlations were found between LAB species (*A. kunkeei* and *F. kullabergensis*) and WSC and acetic acid. In SQPA-treated silage, *Lactobacillus helsingborgensis*, *Lactobacillus apis*, *F. kullabergensis*, *A. kunkeei* and

Table 3. Fermentation features of alfalfa silage treated with propionic acid or a combination of propionic acid and squalene

Items[a]	Additives[b]	Ensiling time (days)				SEM[c]	Significance[d]		
		3	15	45	90		A	D	A × D
pH	CON	5.0^{Aa}	4.7^{Ba}	4.7^{Ba}	4.5^{Cb}	0.03	<0.01	<0.01	<0.01
	PA	4.8^{Ab}	4.5^{Cb}	4.6^{Bb}	4.6^{Ba}				
	SQPA	4.9^{Aab}	4.6^{Bab}	4.6^{Bb}	4.4^{Cc}				
Lactic acid (g/kg DM)	CON	47.8^{ab}	52.1	53.2^{a}	48.1^{b}	1.03	0.83	0.15	<0.01
	PA	43.4^{Bb}	45.5^{B}	49.4^{Bab}	63.6^{Aa}				
	SQPA	57.7^{Aa}	49.5^{B}	45.5^{Bb}	45.8^{Bb}				
Acetic acid (g/kg DM)	CON	48.8^{a}	49.2^{a}	56.2^{a}	55.3^{b}	3.01	<0.01	<0.01	<0.01
	PA	15.1^{Cb}	37.1^{Bb}	49.5^{Bab}	91.4^{Aa}				
	SQPA	32.0^{Cab}	34.4^{BCb}	44.5^{ABb}	53.6^{Ab}				
Propionic acid (g/kg DM)	CON	16.5^{Ab}	5.8^{Bb}	5.9^{Bb}	6.5^{Bc}	1.04	<0.01	<0.01	<0.01
	PA	16.5^{Bb}	14.6^{Ba}	17.1^{Ba}	24.5^{Aa}				
	SQPA	25.2^{Aa}	15.5^{Ba}	15.8^{Ba}	15.4^{Bb}				
Butyric acid (g/kg DM)	CON	3.8^{Aa}	0.0^{B}	0.0^{B}	0.0^{B}	0.02	0.04	<0.01	<0.01
	PA	0.0^{b}	0.0	0.0	0.7				
	SQPA	3.4^{Aa}	0.0^{B}	0.5^{B}	0.0^{B}				
Ammonia-N (g/kg TN)	CON	26.1^{C}	52.5^{B}	68.7^{B}	96.6^{Aa}	3.77	0.27	<0.01	0.01
	PA	27.5^{C}	46.2^{B}	77.3^{A}	73.8^{Ab}				
	SQPA	25.5^{B}	66.2^{A}	65.3^{A}	70.2^{Ab}				
WSC (g/kg DM)	CON	43.4	22.8	25.0	14.2	1.66	0.90	<0.01	0.23
	PA	41.9	28.0	19.6	18.3				
	SQPA	33.3	25.9	22.4	20.5				

[a]DM, dry matter; TN, total nitrogen; WSC, water-soluble carbohydrates.
[b]CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene.
[c]SEM, standard error of the means.
[d]A, effect of additive; D, effect of ensiling time; A × D, interaction of additive and ensiling time.
[a-c]Values with different small letters show significant differences among additives in the same ensiling days ($P < 0.05$).
[A-C]Values with different capital letters show significant differences among ensiling days in the same additives ($P < 0.05$).

Bombilactobacillus mellis had significant ($P < 0.05$, $1 > |r| > 0.6$) correlations with pH, lactic acid, WSC and acetic acid. Besides, *Bifidobacterium asteroids* had significant ($P < 0.05$, $1 > |r| > 0.6$) correlations with WSC and acetic acid. *Lacticaseibacillus paracasei* and *L. parabuchneri* had significant ($P < 0.05$, $1 > |r| > 0.6$) correlations with lactic acid.

As exhibited in Fig. 5, distinct co-occurrence patterns were observed in the bacterial communities of various treated silages. The node and edge numbers of the network reflected the network complexity. The node and edge numbers underwent irregular changes with ensiling time. Overall, the edges of PA- and SQPA-treated silages were numerically less than those of CON silage. Additionally, the ratio of negative and positive (N/P) represented the network stability. The value of N/P in PA-treated silage was numerically lower than that in CON silage.

Bacterial metabolic function prediction and analysis of enzymes associated with bacterial β-carotene synthesis pathway in alfalfa silage

Utilizing the KEGG pathways database with PICRUSt2, predictive bacterial function profiles are shown in Fig. 6a. According to the findings, the metabolism of cofactors and vitamins was among the top 10 of all metabolic pathways in alfalfa silage. Further results showed that (Fig. 6b), regarding bacteria, two modules related to β-carotene synthesis, C10-C20 isoprenoid biosynthesis (M00364) and β-carotene biosynthesis (M00097), were altered during ensiling. Ensiling time had significant ($P < 0.01$) effects on both change of M00364 and M00097. The modules of M00364 and M00097 drastically reduced in the initial stage of ensiling, followed by a descent remission as the ensiling time extended. After ensiling for 90 days, M00364 and M00097 showed a positive increase.

Using PCoA (Fig. 6c), the variances of enzymes (EC:2.5.1.29, EC:2.5.1.32, EC:1.3.5.5, EC:5.5.1.19) involved in the bacterial synthesis of β-carotene in different silages were characterized. The results exhibited significant differences in enzyme activities in different treated silages (PERMANOVA, $R^2 = 0.54$, $P < 0.01$). Additionally, ensiling time resulted in varying degrees of alterations ($P < 0.01$) in enzyme activities.

Discussion
Fermentability of pre-ensiled alfalfa

In this study, fresh alfalfa possessed appropriate BC, which was far below the value from some existing reports (Turan and Onenc,

Table 4. Microbial counts of alfalfa silage treated with propionic acid or a combination of propionic acid and squalene

Items[a]	Additives[b]	Ensiling time (days)				SEM[c]	Significance[d]		
		3	15	45	90		A	D	A × D
LAB (log$_{10}$ cfu/g FM)	CON	7.9[B]	8.1[Bb]	9.1[A]	8.6[AB]	0.11	<0.01	<0.01	<0.01
	PA	8.1[B]	9.7[Aa]	9.7[A]	8.5[B]				
	SQPA	8.4	8.4[b]	8.9	8.7				
Aerobic bacteria (log$_{10}$ cfu/g FM)	CON	6.7[A]	6.1[B]	5.5[Ca]	5.0[Da]	0.06	<0.01	<0.01	<0.01
	PA	6.6[A]	6.2[A]	4.0[Bb]	4.3[Bb]				
	SQPA	6.8[A]	6.3[A]	5.3[Ba]	5.3[Ba]				
Yeasts (log$_{10}$ cfu/g FM)	CON	<2.0	<2.0	<2.0	<2.0	0.06	0.89	<0.01	0.43
	PA	<2.0	<2.0	<2.0	<2.0				
	SQPA	2.2	<2.0	<2.0	<2.0				

[a]LAB, lactic acid bacteria; FM, fresh matter; cfu, colony-forming units.
[b]CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene.
[c]SEM, standard error of the means.
[d]A, effect of additive; D, effect of ensiling time; A × D, interaction of additive and ensiling time.
[a-b]Values with different small letters show significant differences among additives in the same ensiling days ($P < 0.05$).
[A-D]Values with different capital letters show significant differences among ensiling days in the same additives ($P < 0.05$).

2018; Yang *et al.*, 2020), but was close to the result stated by Wang *et al.* (2021*a*) (317 mEq/kg DM). Low BC reduces the resistance to pH decrease, which was conducive to fermentation. Moreover, the WSC (>50 g/kg DM) content met the requirement for acceptable silage (Li *et al.*, 2019). In addition to chemical characteristics, the microbial composition is crucial for fermentability. Epiphytic microflora is responsible for silage fermentation, of which LAB are the most concerned becoming a significant factor in predicting the adequacy of fermentation (Lin *et al.*, 1992). According to

Cai (1999), the minimum amount of epiphytic LAB to obtain well-preserved silage was 5 log cfu/g FM. In addition to LAB, epiphytic microflora includes many undesirable microbes, which can bring serious fermentation loss (Wróbel *et al.*, 2023). Therefore, insufficient epiphytic LAB and a high number of aerobic bacteria were unfavourable for spontaneous fermentation (Cai *et al.*, 1998).

Chemical composition and fermentation profiles of alfalfa silage

With regards to non-carbohydrate components, the WSC was fermented well by LAB during ensiling, leading to no significant difference in residual WSC among all treated silages (Huo *et al.*, 2022). As for structural carbohydrates, the contents of aNDFom, aADFom and ADL were not significantly affected by PA and SQPA, which was in accordance with the study conducted by Zhang *et al.* (2019). Fibre degradation depends on enzymatic or strong acid hydrolysis (Huisden *et al.*, 2009). It could be speculated that fibre degradation was limited by insufficient enzyme activity and acidity in this study. The high production of lactic acid and rapid decline in pH value in all treated silages after 3 days of ensiling indicated strong lactic acid fermentation (Wang *et al.*, 2021*a*). The addition of PA directly formed an acidic environment, which was conducive to the growth of LAB in the early stage (Kim *et al.*, 2015). This was consistent with the outcome of this study, statistically or numerically more LAB counts in PA- and SQPA-treated silages than that in CON silage after 3 and 15 days of ensiling. Additionally, the high accumulation of acetic acid during ensiling was related to low DM content (244–272 g/kg DM). Excessive acetic acid (>4–6%) is often detected in extremely wet (>70% moisture) silages (Kung *et al.*, 2018). Interestingly, compared with the CON, the accumulation rate of acetic acid was slower in PA-treated silage during the early to mid-stage of fermentation (3–45 days), but faster during the later stage. Adding organic acid as an additive created conditions for subsequent growth of heterofermentative LAB in the later stage of fermentation
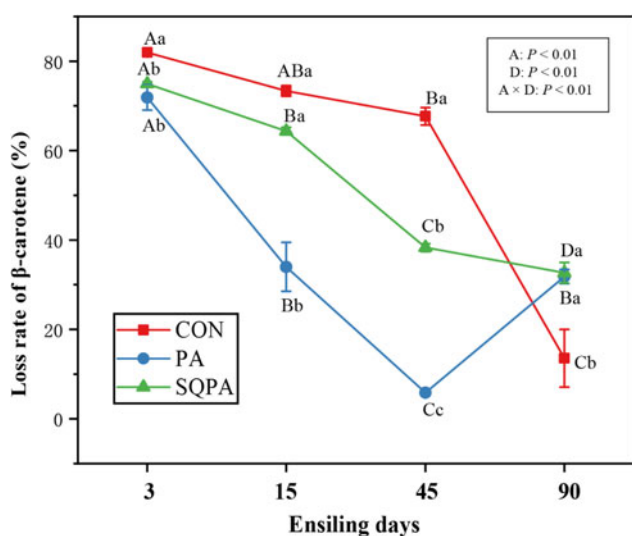
Figure 1. The dynamic of loss rate of β-carotene in alfalfa silage treated with propionic acid or a combination of propionic acid and squalene. Loss rate of β-carotene (%) = (initial content − final content)/initial content × 100%; initial content refers to the β-carotene in fresh alfalfa; final content refers to the β-carotene after the corresponding ensiling time. CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene; A, effect of additive; D, effect of ensiling time; A × D, interaction of additive and ensiling time; Values with different small letters show significant differences among additives in the same ensiling days ($P < 0.05$); Values with different capital letters show significant differences among ensiling days in the same additives ($P < 0.05$).
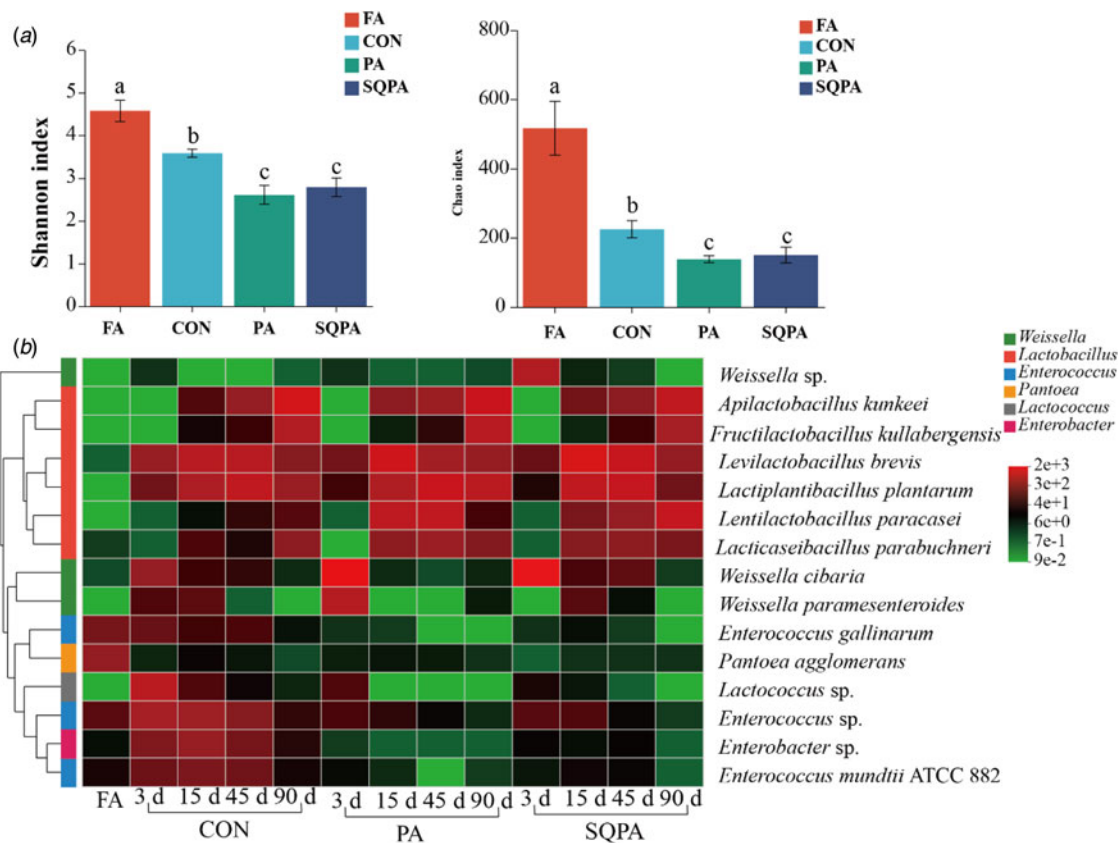
Figure 2. The α-diversity (a) and abundance heatmap (top 15, b) of fresh alfalfa and after ensiling. FA, fresh alfalfa; CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene; 3, 15, 45 and 90, alfalfa was ensiled for 3, 15, 45, and 90 days; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Tyrolová *et al.*, 2017). Due to multiple uncontrollable factors, the change of PA in different treated silages was hard to explain with the current evidence available. As a volatile substance, PA loss is inevitable during the fermentation process. Meanwhile, extra PA was generated during the fermentation process. Theoretically, PA should be higher in silages where it was added. Nevertheless, these uncertainties can impact the final outcome. Kim *et al.* (2015) found that PA was higher in silages treated with PA at early stages of fermentation (2–7 days), but did not persist to the end (100 days). Excessive proteolysis frequently occurs in poor-quality alfalfa silage. It is caused by the multiplication of clostridia, leading to the formation of ammonia-N and butyric acid (Kung *et al.*, 2018). In this study, the concentrations of ammonia-N and butyric acid detected in all silages were less than the threshold of 150 g/kg TN and 5 g/kg DM, respectively, indicating well-conserved legume silage (Kung *et al.*, 2018). After 90 days of ensiling, PA- and SQPA-treated silages reduced the formation of ammonia-N. This was attributed to the rapid reduction of pH value due to the great production of lactic acid, suppressing the decarboxylation or deamination of amino acids by undesirable clostridia (Oliveira *et al.*, 2017). Adding PA increased the lactic acid content, indicating that silage quality was improved, which is consistent with the findings of Jia *et al.* (2021). On the other hand, the combination of squalene and PA weakened the effect of PA in improving the fermentation quality. The reason was that SQPA promoted the growth of undesirable *Enterobacter* sp. (Fig. 2b).

The PA, SQPA, and ensiling time influenced β-carotene loss in alfalfa silage

In this study, with the extension of fermentation, the *β*-carotene loss was alleviated, especially PA-treated silage for 45 days and CON silage for 90 days. There are two explanations for this phenomenon. The first explanation is that the extraction of *β*-carotene derives from stable lipoprotein complexes due to fermentation. Another explanation is that *β*-carotene is synthesized by LAB. Fermentation disrupts the plant matrix and cell cluster, contributing to carotenoid liberation and bioaccessibility (Sangija *et al.*, 2022). Furthermore, some LAB are known to synthesize carotenoids, including *L. plantarum*, *Enterococcus gilvus* and *Lactobacillus gasseri* (Garrido-Fernandez *et al.*, 2010; Hagi *et al.*, 2015; Xu *et al.*, 2020). Therefore, silage could be a candidate resource bank for *β*-carotene-synthesizing LAB.

The PA and SQPA simplified the microflora and altered the keystone taxon

Anaerobic fermentation resulted in the stability of microflora in alfalfa silage. This was because dominant species constrained species diversity (Polley *et al.*, 2007). In the initial ensiling phase (3 days), PA and SQPA simplified the microbial community composition by promoting the growth of *Weissella* (*W. cibaria*), supported by lower diversity indices (Chao 1 and Shannon, Fig. 2a). This deduced that resistance to low pH of *Weissella* was stronger than other cocci LAB, such as *Enterococcus* and *Lactococcus*.
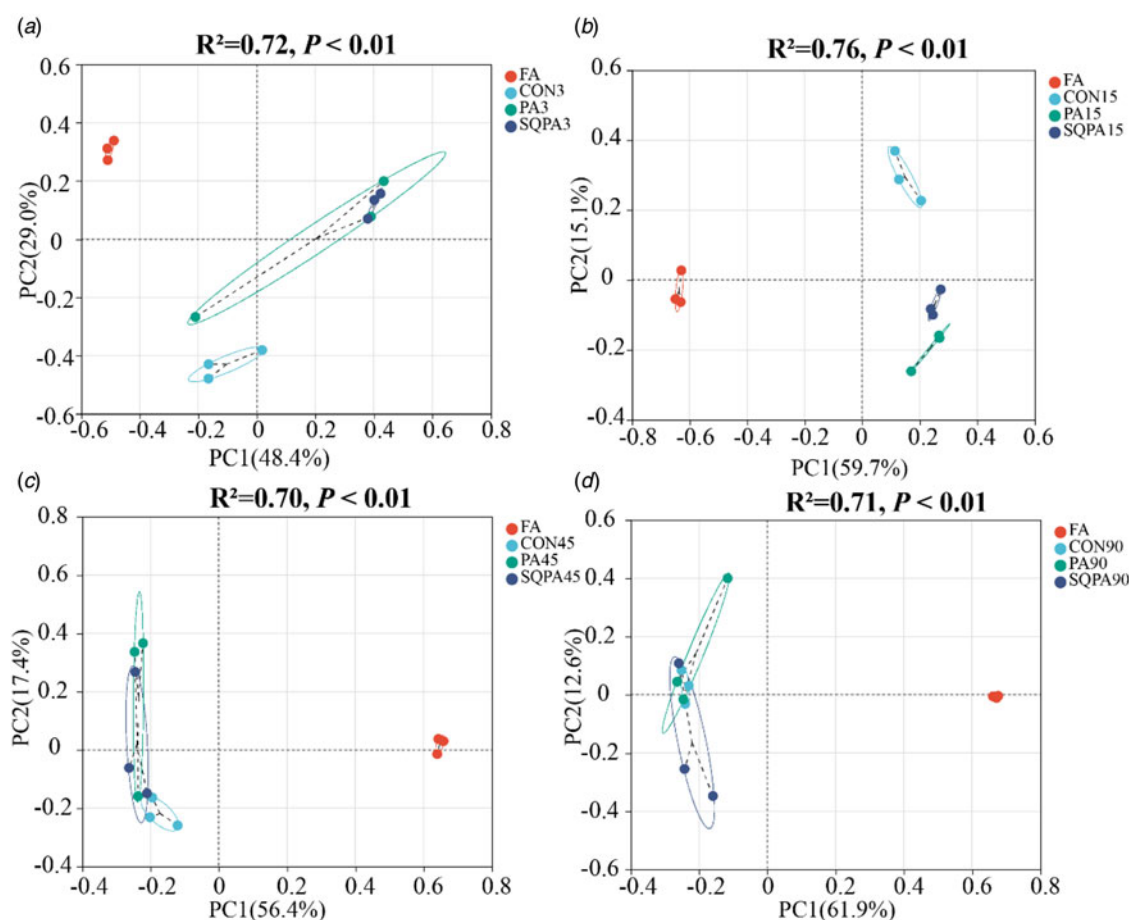
Figure 3. The β-diversity based on principal coordinates analysis of bacterial communities in fresh alfalfa and alfalfa ensiled with different treatments for 3 days (a), 15 days (b), 45 days (c), and 90 days (d), respectively. CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene; 3, 15, 45 and 90, alfalfa was ensiled for 3, 15, 45, and 90 days.

Weissella gender are thought to be early colonizers and then they are outcompeted by acid-tolerant *Lactobacillus* gender due to the pH drop as fermentation progresses (Ogunade *et al.*, 2018). *Enterococcus* and *Weissella* species constitute a significant portion of the microbial flora typically found on the surfaces of various forage plants (Cai, 1999). Due to the lower acid tolerance, the high abundance of *Enterococcus* in CON in initial ensiling phase indicated that spontaneous fermentation did not achieve a rapid decrease in pH. *Enterobacter* species are usually undesirable bacteria, which can compete with LAB for the available sugars and can also break down proteins (Wróbel *et al.*, 2023). Hence, with the extension of fermentation, the succession from *W. cibaria* to *L. plantarum*, *L. brevis*, *L. paracasei*, *A. kunkeei*, *L. parabuchneri* and *F. kullabergensis* occurred in PA- and SQPA-treated silages (Fig. 2b). The β-diversity analysis revealed that PA and SQPA influenced the differences of bacterial flora in the early ensiling phase (3 and 15 days). The PA could directly acidify the anaerobic environment and inhibit the growth of undesirable microbes in the early stage of ensiling and accelerated the succession of the bacterial community. As mentioned above, *W. cibaria* swiftly took over as the dominating bacterial species after 3 days of ensiling. It could not be ignored that microflora separation occurred in the early ensiling stages (3 and 15 days) while aggregation happened in the mid-to-late ensiling stages (45 and 90 days) among all silages. This implied that distinct

treatment groups possessed varying microflora in the early ensiling stages, while convergence in microflora is observed in the mid-to-late ensiling stages. Silage additives started acting early in the fermentation process, yet well-fermented silage harboured homogeneous microflora in the stable stage of late fermentation. Likely, Eliasson *et al.* (2023) stated that inoculation resulted in well fermented silages comprising a homogenous microbiota.

The performance of bacteria impacted the fermentation quality. In this study, *Lactobacillus* was the key bacteria for encouraging fermentation. Notably, in PA- and SQPA-treated silages, heatmaps showed that different species belonging to the *Lactobacillus* played a pivotal role in improving fermentation quality. Xu *et al.* (2021) pointed out that keystone taxon in different silages were entirely distinct. The construction of bacterial networks is commonly employed to illustrate the micro-ecology in silage, wherein the fermentation quality of silage can be highlighted by the complexity of the network. Bai *et al.* (2021) stated that bacterial correlation structures in silage with a high fermentation quality were more straightforward with lower network complexity. Thus, from the perspective of the microbes, adding PA and SQPA simplified the bacterial network, improving fermentation quality. Likewise, compared with SQPA, PA further simplified the bacterial network. Namely, PA-treated silage had better fermentation quality. Besides, Bai *et al.* (2022) indicated that fermentation quality was negatively correlated with bacterial
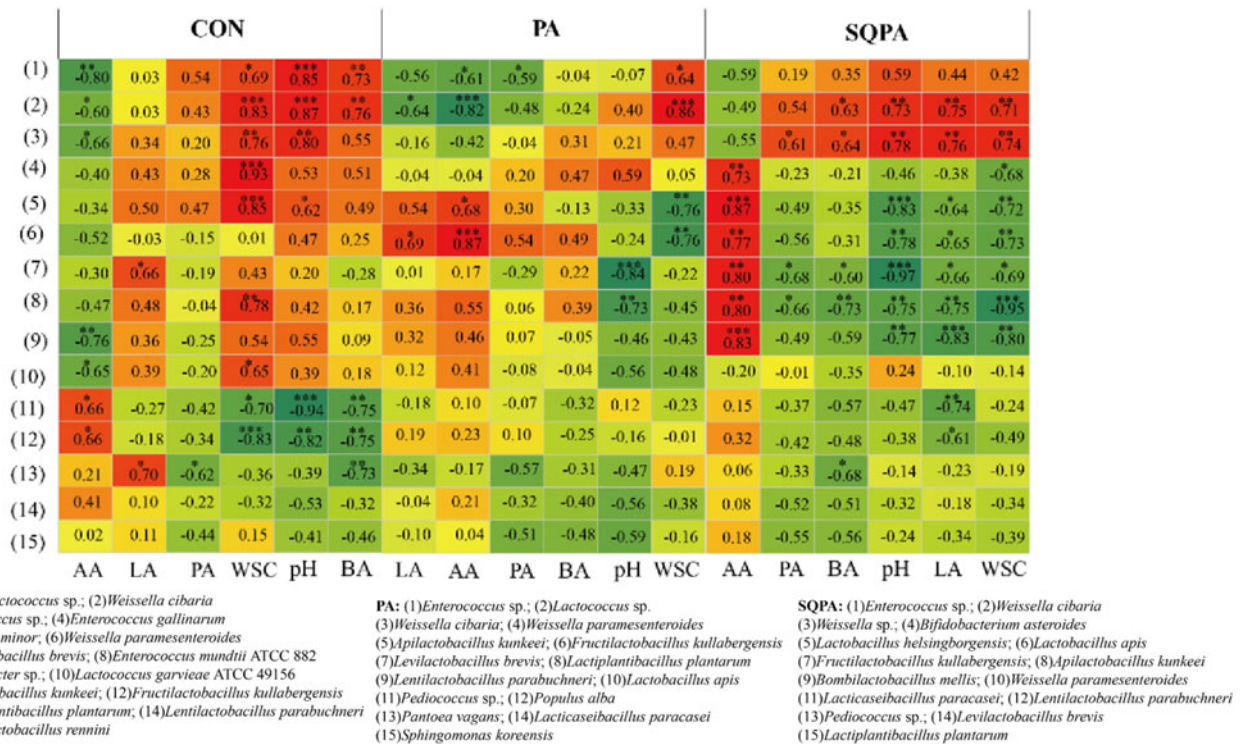
Figure 4. Correlation analysis between bacterial communities (top 15 species) and fermentation characteristics in different alfalfa silages. CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene; AA, acetic acid; LA, lactic acid; PA, propionic acid; BA, butyric acid; WSC, water-soluble carbohydrates; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

community stability. This was in line with this study, substantiated by a lower N/P in PA-treated silage.

Prolonging ensiling time promoted bacterial β-carotene synthesis

The functional prediction could be used to evaluate the effect of bacterial communities on metabolic pathways that underlie silage production (Bai *et al.*, 2021). The metabolism of cofactors and vitamins was one of the top 10 microbial metabolic processes of silage. The modules (M00364 and M00096) involving β-carotene synthesis declined in the initial ensiling phase (3 days), indicating the limitation of bacterial β-carotene synthesis. This was in line with the fact that the anaerobic fermentation process weakened the β-carotene related microbes on the surface of the grass. Among these bacteria, *Pantoea agglomerans* carried the gene *crtEBIY* for β-carotene synthesis and was a β-carotene producer (Wang *et al.*, 2021b). With the extension of ensiling time, bacterial community
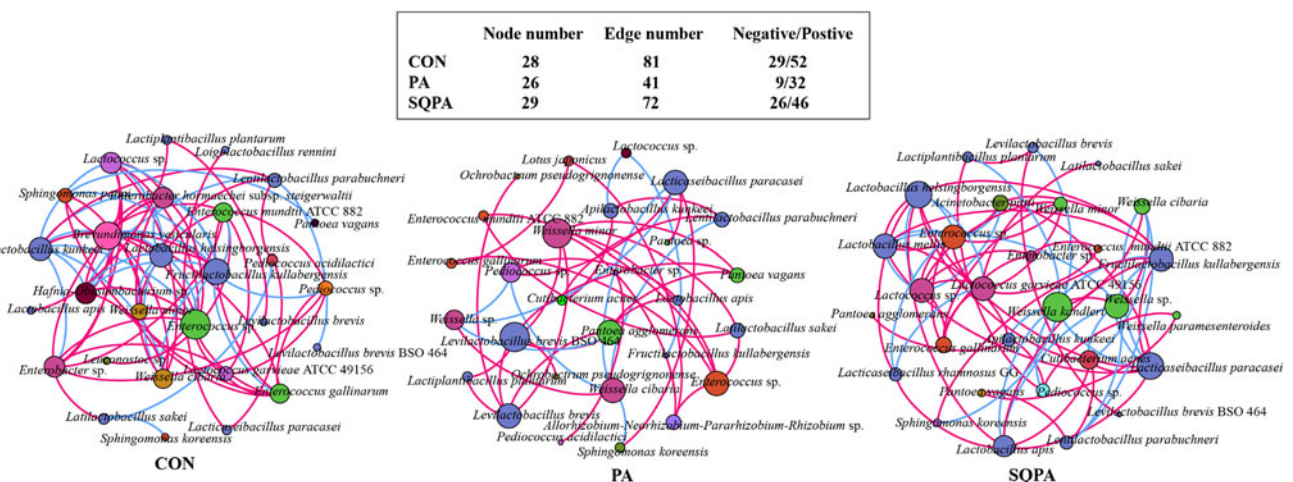
Figure 5. The bacterial co-occurrence networks (on the species level) in different alfalfa silages. Nodes represent individual species; edges represent significant Spearman correlations ($|\rho| > 0.5$, $P < 0.05$). The size of each node is proportional to the number of connected edges, and the nodes are labelled by species and coloured by genus. The colour of the edges corresponds to a positive (red) or negative (blue) relationship; CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene.
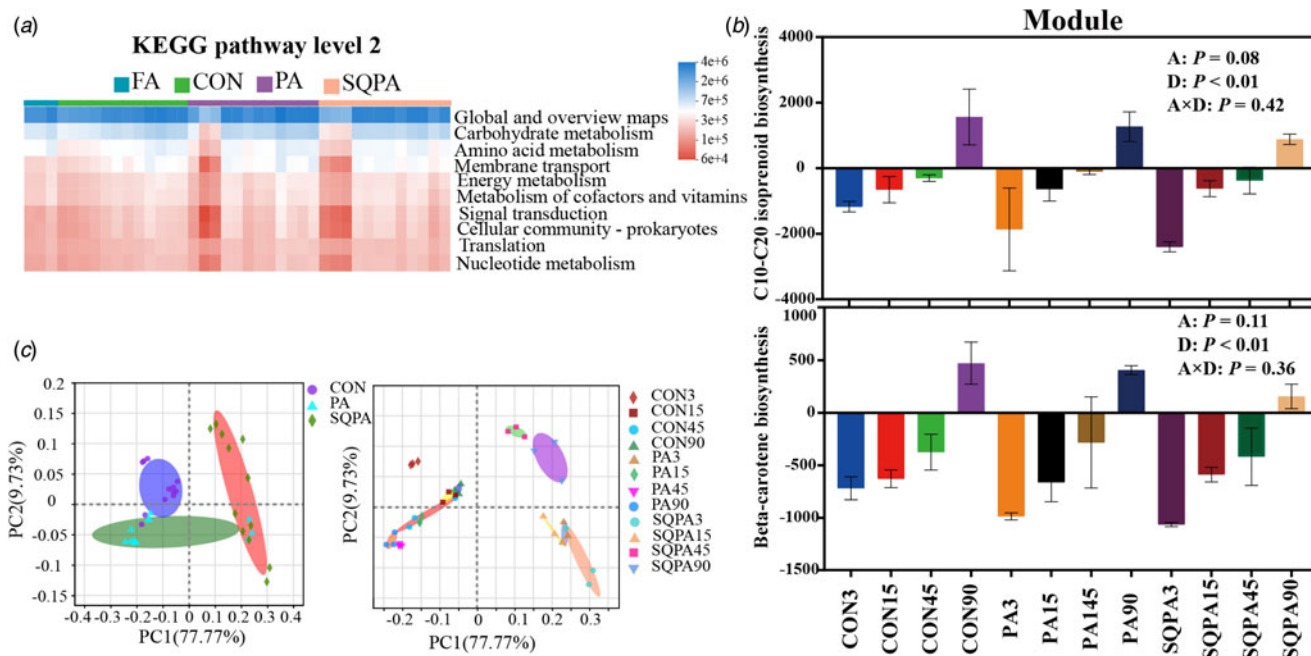
Figure 6. The bacterial metabolic function prediction in fresh alfalfa and silages were analysed by PICRUSt2 (a), the change of C10-C20 isoprenoid biosynthesis and β-carotene biosynthesis before and after ensiling (b), and principal coordinates analysis of enzymes (geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene β-cyclase) associated with bacterial β-carotene synthesis pathway in different alfalfa silages (c). Change (C10-C20 isoprenoid biosynthesis or β-carotene biosynthesis) = relative abundance (after ensiling) – relative abundance (before ensiling). CON, alfalfa ensiled without additive; PA, alfalfa ensiled with propionic acid; SQPA, alfalfa ensiled with a combination of propionic acid and squalene; 3, 15, 45 and 90, alfalfa was ensiled for 3, 15, 45 and 90 days; A, effect of additive; D, effect of ensiling time; A × D, interaction of additive and ensiling time.

succession occurred and novel β-carotene producers might emerge. This was confirmed by the recovery of bacterial β-carotene synthesis. This could explain the phenomenon reported by Zhao *et al.* (2021), who observed an increase in β-carotene in rye silage after 60 days of ensiling. Although the PA and SQPA did not have a significant effect on M00364 and M00096, based on PCoA, it was demonstrated that PA and SQPA could affect the bacterial synthesis of enzymes involved in the β-carotene pathway. Similarly, Lee *et al.* (2018) found that red pepper fermented with *L. parabuchneri* can change carotenoids markedly during the fermentation process. Moreover, many parameters (carbon source, temperature and etc) can affect the biotechnological synthesis of β-carotene (Gupta *et al.*, 2022). The use of additives altered the conditions for bacterial synthesis of β-carotene. These findings implied that further investigation into the microbial viewpoint on β-carotene regulation in silage was promising.

Conclusion

Adding PA alone had a better effect than SQPA on improving fermentation quality. The bacterial β-carotene synthesis occurred during alfalfa ensiling and prolonging the ensiling time benefited its synthesis. The PA and SQPA mainly exerted roles in bacterial β-carotene synthesis in the initial and middle stage. Future studies will concentrate on screening and purifying β-carotene-producing bacteria, thereby contributing to animal nutrition and productivity.

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Ethical standards. This article does not contain any studies with human participants or animals performed by any of the authors.

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