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RESEARCH ARTICLE

Hexanoic acid addition helps to clarify the possible mechanisms of the increased β-carotene content during alfalfa fermentation

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Highlights

- Hexanoic acid modulated β-carotene metabolism during alfalfa ensiling.
- *Lactobacillus* harboring *crtNM* operon contributed to β-carotene synthesis.
- *Lactobacillus kullabergensis* and *Lactobacillus senioris* were key positive regulators of β-carotene.

Abstract

The objectives of this study were to evaluate the effect of hexanoic acid (HA) supplementations (0, as the control, CON; 0.05%, HA1; 0.1%, HA2; 0.2%, HA3) on β-carotene, and ascertain the way and key factors of HA influencing β-carotene content of alfalfa (*Medicago sativa* L.) after ensiled in an oxygen-free and dark conditions for 10, 40, and 80 d (from May to August, 2021). This was achieved by examining the dynamic change of β-carotene, activities of β-carotene-related enzymes, and bacterial community succession of ensiled alfalfa, using operon *crtNM* identification, *crtE* gene quantitation, and single-molecule real-time sequencing technology. The results revealed that when compared with the fresh material, terminal alfalfa silage treated with different level of HA supplementations (0, 0.05, 0.1, 0.2%; fresh weight basis) increased β-carotene content up to 2.86, 85.8, 159, and 133%, accordingly. Meanwhile, alfalfa silage treated with higher levels of HA (0.1 and 0.2%) showed superior effects compared to those treated with lower levels of supplementation (0 and 0.05%). HA supplementation specifically facilitated the increase abundance of *Lactobacillus kullabergensis* and the emergence of *L. senioris*. Multiple linear regression models inferred that *L. kullabergensis*, *L. apis*, *L. saniviri*, *L. senioris*, peroxidase, phytoene desaturase, and lycopene β-cyclase positively regulated β-carotene. Conversely, *Lactobacillus rennini* and *L. brevis* adjusted β-carotene, negatively. Positive regulations of the above bacterial species and enzymes had a stronger role in increasing β-carotene than *L. rennini* and *L. brevis*. In conclusion, the β-carotene increase of ensiled alfalfa may be regulated by HA supplementation *via* multiple positive factors, including 4 special *Lactobacillus* species (*L. kullabergensis*, *L. apis*, *L. saniviri*, and *L. senioris*), and 3 vegetative β-carotene-related enzymes (peroxidase, phytoene desaturase, and lycopene β-cyclase).

Keywords: hexanoic acid, alfalfa silage, β-carotene, *Lactobacillus* species

1. Introduction

β-Carotene, an essential precursor of vitamin A, has various biological functions and applications. Due to its anti-cancer, anti-disease, and immune enhancement properties, improving immunity and preventing tumors, thrombosis, atherosclerosis, and antiaging (Mata-Gómez *et al*. 2014; Wang L *et al*. 2021), it plays a crucial role in maintaining human health in the pharmaceutical industry. In animal husbandry, β-carotene used to prevent nyctalopia (night blindness) and xeropthalmia (abnormal dryness), as well as to enhance cow health, immune response, mammary gland health, and reproductive performance (Johansson *et al*. 2014; Gul *et al*. 2015; Oliveira

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et al. 2015). Moreover, β-carotene served as the nutritional and functional component in dairy products, such as an increased yield of milk with high lactose and fat content and the enhancement of antioxidant capacity (Wang *et al*. 2017; Myrtsi *et al*. 2023). In the food industry, β-carotene was used as the edible pigment to create colored food products (Bogacz-Radomska and Harasym 2018). Human health and animal wellness have driven the generation of a β-carotene rich diet.

Alfalfa (*Medicago sativa* L.) is extensively cultivated and used as animal feed (Eun and Beauchemin 2007) and human food owing to its high nutritional value, particularly its richness in protein and functional compounds (Hadidi *et al*. 2023). β-Carotene is susceptible to oxidative degradation when alfalfa is harvested, with prolonged exposure to oxygen, heat, light, and strong acids (Dunne *et al*. 2009; Kalač 2012; Liu *et al*. 2016). Immediately anaerobic fermentation of lactic acid bacteria (LAB) is a traditional and effective method to store fresh alfalfa, which can prolong the preservation time of alfalfa (Zong *et al*. 2023). The existing literature indicated that good anaerobic fermentation led to a decrease in β-carotene loss (Kalač 2012; Oloo *et al*. 2014; Wu *et al*. 2021; Zong *et al*. 2021). However, some studies showed that the β-carotene content increased (from 2.08 to 64.65%) in certain forage silages compared with the raw materials, such as wilted alfalfa (Zhang *et al*. 2020), yarrow (*Achillea millefolium* L.) (Goliński *et al*. 2012), Italian ryegrass (Lv *et al*. 2017), grass-clover mixture, and maize whole crop (Mogensen *et al*. 2012). Similarly, in the food industry, anaerobic lactic acid fermentation led to a substantial 2.6–5 and 1.6–4.8-fold rise in the β-carotene content in African nightshade pickles and relish products, respectively (Sangija *et al*. 2022). The fermentation with LAB increased the content of β-carotene from 0.89 mg per 100 g (in Cunero var.) and from 0.28 mg per 100 g (in Ronaldo var.) to 1.14 mg per 100 g (Bartkiene *et al*. 2013). Unfortunately, the increase mechanism of β-carotene during fermentation remains unclear. Recently, we found that alfalfa silage containing hexanoic acid (HA, 7.78% of relative content in 95% acetonitrile aqueous extracts) had an insignificant β-carotene loss, the specific information was shown in Appendices A–C. Therefore, HA might have the increase effect on β-carotene during alfalfa fermentation.

The carotenoid biosynthetic pathways are mevalonate (MVA) in bacteria and 2-C-methyl-Derythritol-4-phosphate (MEP) pathways in plants have been reported (Van Cuon 2017). The important C5 isoprenoid precursor (Liang *et al*. 2018) and detailed synthesis process were exhibited in our previous study (Zong *et al*. 2023), which contained various enzymes. Among them, geranylgeranyl diphosphate (GGPPS) is a key enzyme at the precursor of the β-carotene biosynthesis module (involving in the flow of modules, from C10-20 isoprenoid synthesis to β-carotene synthesis). Additionally, the synthesis of β-carotene includes phytoene synthase (PSY), phytoene desaturase (PDS), and lycopene β-cyclase (LYC) (Liang *et al*. 2017). By monitoring the dynamic of these enzymes and bacterial community succession, we might gain insights into the increase mechanism of β-carotene in ensiled alfalfa with added HA. Based on current research, we hypothesized that HA supplementations would increase β-carotene content possibly through multiple factors, including β-carotene-producing bacteria and enzymes associated with β-carotene synthesis.

To validate this assumption, alfalfa was ensiled with varying doses of HA supplementations and stored in an oxygen-free and dark conditions from May to August, 2021. The fermentation process lasted for 10, 40, and 80 d, allowing us to examine the changes in β-carotene levels, activities of β-carotene-related enzymes, bacterial communities, nutrient compositions, and fermentation characteristics. Furthermore, we employed operon *crtNM* identification (which identifies carotenoid-producing LAB), real-time fluorescent quantitative PCR (specifically for the carotenoid-related *crtE* gene in *Lactobacillus*), and single-molecule real-time (SMRT) sequencing technologies to track and confirm the presence of β-carotene-producing bacteria throughout the fermentation process. The primary factors for upraising the β-carotene content regulated by HA supplementations would be evaluated using a multiple linear regression model (MLR).

2. Materials and methods

2.1. Experimental design and treatments

A completely randomized design was performed with 4×3 factorial arrangements (4 treatments×3 fermentation days (10, 40, and 80 d)) with 6 replicates. The selection of fermentation days was based on the statement by Buxton *et al*. (2003). Based on the report of Ohyama *et al*. (1977), 0.05% HA was beneficial for the perseveration of silage, three gradients of HA supplementation were set. Therefore, four treatments included: without HA supplementation as the control (CON), or with HA supplementation at 0.05% (HA1), 0.1% (HA2), and 0.2% (HA3). The HA was applied based on the weight of fresh alfalfa, while the CON received an equal amount of distilled water.

2.2. Alfalfa planting site and climate conditions

Alfalfa was cultivated in the experimental field, which was divided into three equal sections at Baima teaching and research base of Nanjing Agricultural University (Humid subtropical climate with an average annual rainfall of 1106 mm and an annual average temperature of 15.4°C, 32°86´N, 120°02´E, 12.5 masl; Nanjing, China).

2.3. Sampling and anaerobic fermentation of alfalfa

Alfalfa was harvested at the full bloom stage on 5/18/2021, with a plant height of 60–70 cm. Immediately after harvest, the alfalfa was transported to the laboratory and cut into 1–2 cm lengths using a forage cutter (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China). The biochemical characteristics of fresh alfalfa were listed in Appendix D. Fresh alfalfa had moderate crude protein (CP, 190 g kg^{-1} dry matter (DM)) and buffering capacity (BC, 256 milligram equivalent (mEq) kg^{-1} DM). However, it had low contents of DM (228 g kg^{-1} fresh matter (FM) and water-soluble carbohydrate (WSC, 47.6 g kg^{-1} DM). The fiber fractions were 383 g kg^{-1} DM of neutral detergent fiber (NDF), and 233 g kg^{-1} DM of acid detergent fiber (ADF). The numbers of LAB, aerobic bacteria, and yeasts were 3.85 lg

colony-forming units (CFU)$^{-1}$g FM, 7.23 lgCFU g^{-1} FM, and 6.89 lgCFU g^{-1} FM, respectively. About 300 g mass of chopped alfalfa was filled in Nylon vacuum plastic bags (28 cm×35 cm). It was vacuumed to ensure an anaerobic environment and was stored in a dark cabinet at room temperature (20–38°C) for 10, 40, and 80 d (from May to August, 2021).

2.4. Microbial and chemical analysis

To determine the microbial populations epiphytic on alfalfa surface and in ensiled alfalfa, exact 10 g of fresh alfalfa were immersed in 90 mL of 0.85% sterile normal saline to obtain bacterial solution and serial dilutions (10^{-1} through 10^{-6}) were made in sterile saline solution. The counts of LAB, aerobic bacteria, and yeasts were determined by the method of plate count described in our previous study (Liu *et al*. 2016).

The fresh and ensiled alfalfa were used for chemical analyses. Approximately 20 g of fresh alfalfa were put in conical flasks (capacity: 250 mL) and immersed in 180 mL of distilled water at 4°C for 24 h. After filtering, BC was measured using the hydrochloric acid-sodium hydroxide method (Playne and McDonald 1966). To evaluate the fermentation quality of alfalfa, another 25 g of ensiled alfalfa were immersed in 75 mL of distilled water at 4°C for 24 h. The resulting extract was filtered to determine the pH value, organic acids, and ammonia-nitrogen (NH_3-N). The pH value was determined using a glass electrode pH meter (HI221, Hanna Ltd., Rome, Italy). The concentrations of lactic, acetic, propionic, and butyric acids were determined using a high-performance liquid chromatography system (1260 HPLC, Agilent Technologies, Inc., Waldbronn, Germany) equipped with a refractive index detector (column: Carbomix® H-NP5, Sepax Technologies, Inc., Newark, DE, USA; eluent: 2.5 mmol L^{-1} H_2SO_4, 0.5 Ml min^{-1}; temperature: 55°C) (Wen *et al*. 2017). The NH_3-N concentration was determined using the phenol-sodium hypochlorite colorimetric method (Broderick and Kang 1980).

For nutrient value evaluation, an additional 100 g of either fresh or ensiled alfalfa samples were dried at 105°C for 30 min and then at 65°C for 48 h in a forced-draft oven. The dried alfalfa samples were then ground to pass through a 1-mm screen using laboratory knife mills (FW100, Taisite Instrument Co., Ltd., Tianjin, China). The indexes of CP, WSC, NDF, and ADF were used to evaluate the nutrient value. The crude protein content was determined using a Kjeldahl nitrogen analyzer (Kjeltec 8200, Foss Analytics, Höganäs, Sweden), and the CP content was calculated by multiplying the total nitrogen content. The WSC content was determined using anthrone-sulfuric acid colorimetry (Thomas 1977). The contents of NDF and ADF were determined using an ANKOM 200i fiber analyzer (ANKOM Technologies, USA) based on the profile in the previous study (Van Soest *et al*. 1991).

2.5. β-Carotene and its related enzyme activities analysis

Consistent with the pre-processing method reported in our previous study (Zong *et al*. 2023), 3 g of liquid nitrogen frozen and grounded samples mixed with 7.5 mL 10% vitamin C solution, 7.5 mL methanol, 17.5 mL anhydrous ethanol, and 5 mL KOH solution (w/v=1:1). Subsequently, the mixture was then subjected to a 30-min bath at 80°C in darkness. After cooling to room temperature, the mixture was supplemented with 3 mL heptane and 1 mL distilled water, and subsequently centrifuged at 3,500 r min^{-1} for 5 min at room temperature. This step was repeated. A precise 1 mL of the supernatant was utilized for the analysis of β-carotene concentration. β-carotene concentration was determined using Agilent HPLC 1260 (Agilent Technologies, Inc., Germany; column: Intersil 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^{-1}, methanol-acetonitrile 9:1 (v/v); temperature: 45°C).

Another 10 g of fresh or ensiled alfalfa was frozen in liquid nitrogen and grounded. Exact 0.20 g samples were mixed with 1 mL phosphate-buffered saline (pH=7.4), thoroughly. The mixture was centrifuged at 4°C and 3,000 r min^{-1} for 20 min. According to the introduction manuals, the activities of plant endogenous oxidase (lipoxygenase and peroxidase) were determined by Lipoxygenase and Peroxidase ELISA Kits (Feiya Biotechnology Co., Ltd., Nanjing, China), and the activities of key synthases referred to GGPPS, PSY, PDS, and LYC were determined by GGPPS, PSY, PDS, and LYC ELISA Kits (Jiangsu Meibiao Biotechnology Co., Ltd., Yancheng, China), respectively.

2.6. Bacterial community analysis

DNA extraction, PCR amplification, and SMRT sequencing Following the manufacturer's instructions, microbial community genomic DNA was extracted from samples using a TIANamp Bacteria DNA Isolation Kit (DP302-02, Tiangen, Beijing, China). The extracted DNA was checked using 1% agarose gel and its concentration and purity were measured using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). To clarify the bacterial community, the bacterial 16S rRNA genes were amplified using the universal bacterial primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′). Primers were tagged with PacBio barcode sequences to distinguish each sample. Amplification reactions (20-μL volume) consisted of forward primer (5 μmol L^{-1}) 0.8 μL, reverse primer (5 μmol L^{-1}) 0.8 μL, 5× FastPfu buffer 4 μL, 2.5 mmol L^{-1} dNTPs 2 μL, FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng and DNase-free water. The PCR procedure was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C (ABI GeneAmp® 9700 PCR thermocycler, CA, USA). PCR products were purified using the AMPure® PB beads (Pacific Biosciences, CA, USA) and quantified with *Quantus*™ fluorometer (Promega, WI, USA). Purified PCR products were pooled in equimolar and the DNA library was constructed using the SMRTbell® Express Template Prep Kit 2.0 (Pacific Biosciences, CA, USA) according to PacBio's instructions. Purified SMRTbell libraries were sequenced on the Pacbio Sequel

II System (Pacific Biosciences, CA, USA) by Majorbio Bio-Pharm Technology Co., Ltd., (Shanghai, China). Circular consensus sequence (CCS) reads were generated from the raw PacBio sequencing data using the SMRTLink 8.0 (minfullpass=3, minPredictedAccuacy=0.99). CCS reads were barcode-identified and length-filtered. For bacterial communities, sequences outside of a range of <1,400 bp and >1,600 bp were removed. Optimized CCS were used for further downstream analyses. Operational taxonomic units (OTUs) with a 99% similarity cutoff were clustered using USEARCH (version 7.0), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (version 2.13) against the 16S rRNA database (Silva v138) using a confidence threshold of 75%. All sequences were submitted to the NCBI Sequence Read Archive database (PRJNA884150).

Sequencing analysis Alpha diversity analysis (Shannon and Chao 1 index) and beta diversity analysis were consistent with our previous studies (Zong *et al*. 2023). Bar charts of genus and species levels were constructed by R software (version 3.3.1) for further delving into the core and unique information of fresh and ensiled alfalfa. A heatmap of the mantel test (linkET package) was used to illuminate the factors affecting β-carotene according to Pearson's correlation matrices. To visualize the relationship between the bacterial community, enzyme activities, fermentation indices, and β-carotene, Pearson's correlation heatmaps (pheatmap package) and Canonical correspondence analysis (CCA, vegan package) were accomplished. Utilizing the KEGG database, microbial functions were clarified by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2, https://github.com/picrust/picrust2; Version 2.2.0). The co-occurrence network (WGCNA package) was statistically calculated and shown the correlations of predominant species.

2.7. PCR the operon *crtNM* and *crtE* gene

PCR was used to detect the presence of the *crtNM* operon responsible for carotenoid-producing LAB on fresh and ensiled alfalfa, using the crtN-for and crtM-rev primers and the same PCR conditions as described by Turpin *et al*. (2016). PCR procedure was performed according to the method reported by Garrido-Fernández *et al*. (2010).

The carotenoids-related gene only found in *Lactobacillus* was *the crtE* gene (coding the GGPPS) (Turpin *et al*. 2016), and its concentration was measured in an *L*. *plantarum* strain (GenBank accession no. 2417264) using quantitative PCR. To clarify the *crtE* gene concentration relative to total microbial DNA during alfalfa fermentation, quantitative PCR was carried out on a *MiniOpticon*™ System (Bio-Rad Laboratories, Inc.). The *crtE*f (5′-ACGCCATGCGTTATTCGGTA-3′) and *crtE*r (5′-GGCAACGTCTTCCCCAAACT-3′) primers were used for *crtE*, and known concentrations of *L*. *plantarum* DNA carrying the *crtE* gene were used for serial dilution. The amplification reactions were carried out in a 20-μL volume containing 2 μL of DNA solution, 23 μL of a PCR mixture containing 12.5 μL of KAPA SYBR FAST Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), and 1.0 μL of each 8 μmol L^{-1} primer, under the following PCR conditions: one cycle at 95°C for 30 s, 35 cycles at 95°C for 30 s, at 60°C for 10 s, and at 72°C for 15 s, using a *MiniOpticon*™ System (Bio-Rad Laboratories, Inc.).

2.8. Statistical analysis of data

Statistical analysis was conducted using the IBM Statistical Package for the Social Sciences (SPSS 22.0 for Windows). Each Nylon vacuum plastic bags treated as an experimental unit. The effects of hexanoic acid supplementations, fermentation days, and their interaction on fermentation characteristics, nutrient compositions, β-carotene, lipoxygenase, peroxidase, and β-carotene synthesis-related enzymes during alfalfa fermentation were analyzed using a two-way analysis of variance (ANOVA, General Linear Models). The model for the analysis of variance was $Y_{ij}=\mu+T_i+D_j+(T\times D)_{ij}+e_{ij}$; where Y_{ij} represented the dependent variable; μ denoted the overall mean; T_i represented the effects of hexanoic acid supplementation; D_j represented the effects of fermentation days; $(T\times D)_{ij}$ represented the interaction effect of hexanoic acid supplementation and fermentation days; and e_{ij} represented the residual error term.

The multiple comparisons for samples were conducted only if the effect of the interaction was significant using Tukey's test (P<0.05). Otherwise, multiple comparisons for the means from different fermentation days or supplementations of hexanoic acid were conducted when the effect of fermentation days or hexanoic acid supplementations was significant using Tukey's test (P<0.05).

MLR is a statistical method that utilizes multiple variables to create a reliable model. To ensure model accuracy, the variables must be independent and free from multicollinearity. By employing stepwise regression, the explanatory variables retained in the model are deemed both important and not severely multicollinear.

3. Result

3.1. Effects of HA supplementations on β-carotene and related synthetases at different fermentation days

As shown in Table 1, β-carotene content, and the activities of LOX, POD, GGPPS, PSY, PDS, and LYC were influenced by HA supplementations, fermentation days, and their interaction (P<0.05). The β-carotene lost about 17% on fermentation for 40 d (from 38.5 to 32.0 mg kg^{-1} DM) and was slightly increased by about 2.86% (from 38.5 to 39.6 mg kg^{-1} DM) relative to the fresh material by the end of fermentation phase (80 d). Interestingly, the β-carotene content increased by more than 26% in each HA supplementation on fermentation for 40 d (P<0.05), and then further increased by over 85% on fermentation for 80 d relative to the fresh material (P<0.05). The β-carotene content (100 mg kg^{-1} DM) in HA2 was higher (P<0.05) than that in fresh alfalfa (38.5 mg kg^{-1} DM), with an increase of approaching 160%. HA3 supplementation had the highest (P<0.05) LOX activity on fermentation for 40 d, followed by HA2 on fermentation for 40 d. The CON had the highest (P<0.05) POD activity on fermentation for 80 d, which was lowered by all HA supplementations on fermentation for

Table 1 Effects of HA supplementations and fermentation days on β-carotene and β-carotene related enzymes during alfalfa fermentation

Fermentation days (d)[1]	HA supplementations	β-carotene (mg kg^{-1} DM)[2]	LOX	POD	GGPPS	PSY	PDS	LYC
			(U g^{-1} DM)[3]					
Fresh alfalfa		38.5 ghi	7.54 def	12.8 h	0.37 cde	4.49 de	2.64 d	5.35 f
10	CON	33.7 hi	8.77 de	22.0 cd	0.42 ab	5.14 cd	2.46 de	12.1 c
	HA1	40.6 g	7.66 def	25.0 c	0.34 ef	6.58 a	3.17 b	7.43 ef
	HA2	40.5 g	13.6 c	28.9 b	0.33 ef	3.92 e	2.50 d	9.83 cde
	HA3	36.6 ghi	7.98 def	18.5 def	0.30 f	4.02 e	2.49 d	14.8 b
40	CON	32.0 i	9.03 d	22.0 cd	0.37 cde	5.31 bcd	2.50 d	9.44 de
	HA1	62.7 e	7.37 ef	24.7 c	0.42 ab	6.32 ab	4.75 a	16.5 b
	HA2	78.0 c	17.2 b	19.8 de	0.47 a	6.75 a	2.62 d	23.8 a
	HA3	48.7 f	24.7 a	17.0 efg	0.43 b	5.01 cde	2.25 e	9.30 de
80	CON	39.6 gh	7.56 def	39.7 a	0.44 ab	5.99 abc	3.33 b	11.7 cd
	HA1	71.5 d	6.82 f	14.2 gh	0.40 bcd	4.87 cde	2.24 e	7.29 ef
	HA2	100 a	12.6 c	18.8 def	0.41 bc	5.69 abc	2.90 c	7.32 ef
	HA3	89.7 b	13.2 c	15.2 fgh	0.36 de	3.94 e	2.45 de	8.56 e
SEM		3.12	0.81	1.14	0.008	0.171	0.105	0.785
Fermentation days means								
10		37.8	9.49	23.6	0.35	4.91	2.66	11.1
40		55.4	14.6	20.9	0.42	5.85	3.03	14.8
80		75.2	10.0	22.0	0.40	5.12	2.73	8.70
SEM		1.50	0.89	1.59	0.009	0.299	0.199	1.220
HA supplementations means								
CON		35.1	8.45	27.9	0.41	5.48	2.76	11.1
HA1		58.3	7.28	21.3	0.39	5.92	3.39	10.4
HA2		72.9	14.5	22.5	0.40	5.45	2.67	13.7
HA3		58.3	15.3	16.9	0.36	4.32	2.40	10.9
SEM		1.73	1.03	1.84	0.011	0.345	0.230	1.41
Significance								
HA supplementations		<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001
Fermentation days		<0.001	<0.001	0.014	<0.001	0.003	<0.001	<0.001
Interaction		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] HA, hexanoic acid; CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; SEM, standard error of the means. Interaction, the interaction of hexanoic acid supplementations and fermentation days.
[2] DM, dry matter.
[3] LOX, lipoxygenase; POD, peroxidase; GGPPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; LYC, lycopene β-cyclase.
Different little letters in the same column indicated significant differences at P<0.05. Data from each treatment represent the means of six replicates (n=6); data from fermentation days means represent the means of twenty-four replicates (n=24); data from HA supplementations means represent the means of eighteen replicates (n=18).

80 d (P<0.05). HA supplementations had higher (P<0.05) GGPPS activity than the CON on fermentation for 40 d. HA1 supplementation had higher (P<0.05) PSY activity than the CON on fermentation for 10 d, while HA3 supplementation had lower (P<0.05) PSY activity than the CON on fermentation for 10 and 80 d. HA1 supplementation had the highest PDS activity on fermentation for 40 d (P<0.05). HA1 supplementation had higher (P<0.05) PDS activity than the CON and supplementations of HA2 and HA3 on fermentation for 10 and 40 d but had lower (P<0.05) PDS activity than the CON and HA2 on fermentation for 80 d. HA2 had the highest (P<0.05) LYC activity on fermentation for 40 d while the fresh alfalfa had the lowest (P<0.05) LYC activity on fermentation for 40 d.

3.2. Effects of HA supplementations on microbial composition and β-carotene-related bacterial community at different fermentation days

As shown in Table 2, the interaction between HA supplementation and fermentation days had significant impacts on the counts of LAB, aerobic bacteria, and yeasts (P<0.05). Additionally, fermentation days significantly affected the counts of mold (P<0.05). HA supplementations resulted in fewer LAB counts compared to the CON on fermentation 40 d (P<0.05). Among the HA supplementations, HA1 had the highest LAB counts on fermentation after 80 d (P<0.05). HA3 supplementation had the lowest counts of aerobic bacteria on fermentation after 80 d (P<0.05), while the CON had the highest counts of aerobic bacteria on fermentation after 10 d (P<0.05) but all HA supplementations decreased (P<0.05) the counts of aerobic bacteria. On fermentation after 10 d, HA1 supplementation had the highest counts of yeast (P<0.05), followed by the CON. The counts of mold decreased as the fermentation days increased, with an average across the HA supplementations (P=0.031).

The α- and β-diversity were shown in Appendix E. The rarefaction curves of samples tended to be stable. According to PCoA1 (PERMANOVA, P=0.001), HA caused diverse

Table 2 Effects of HA supplementations and fermentation days on the microorganism composition during alfalfa fermentation

Fermentation days (d)[1)]	HA supplementations	LAB	AB	Yeasts	Mold
		(lgCFU g^{-1} FW)[2)]			
10	CON	9.28 c	6.09 a	3.35 bc	3.09
	HA1	8.97 d	5.47 b	3.99 a	<2.60
	HA2	8.98 d	4.75 cd	2.75 de	<2.60
	HA3	9.04 cd	5.15 bc	2.95 cde	2.85
40	CON	8.60 e	4.00 ef	3.24 bcd	<2.60
	HA1	8.22 f	4.29 de	3.24 bcd	<2.60
	HA2	8.06 f	3.75 fgh	3.08 cde	<2.60
	HA3	8.12 f	3.72 fgh	2.68 e	<2.60
80	CON	9.81 ab	3.47 gh	2.60 e	<2.60
	HA1	10.0 a	4.13 ef	3.59 ab	<2.60
	HA2	8.93 d	3.88 efg	2.99 cde	<2.60
	HA3	9.59 b	3.36 h	2.68 e	<2.60
SEM		0.092	0.125	0.069	0.034
Fermentation days means					
10		9.07	5.37	3.26	2.79 a
40		8.25	3.94	3.06	2.60 b
80		9.59	3.71	2.96	2.60 b
SEM		0.069	0.116	0.109	0.077
HA supplementations means					
CON		9.23	4.52	3.06	<2.76
HA1		9.06	4.63	3.61	<2.60
HA2		8.66	4.13	2.94	<2.60
HA3		8.92	4.08	2.77	<2.68
SEM		0.080	0.134	0.126	0.089
Significance					
HA supplementations		<0.001	<0.001	<0.001	0.219
Fermentation days		<0.001	<0.001	0.032	0.031
Interaction		<0.001	0.001	0.006	0.190

[1)] CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; SEM, standard error of the means. Interaction, the interaction of hexanoic acid supplementations and fermentation days.
[2)] LAB, lactic acid bacteria; AB, aerobic bacteria.
Different little letters in the same column indicated significant differences at P<0.05. Data from each treatment represent the means of six replicates (n=6); data from fermentation days means represent the means of 24 replicates (n=24); data from HA supplementations means represent the means of eighteen replicates (n=18).

bacterial communities. Compared with the fresh alfalfa, fermentation reduced the Shannon and Chao 1 index (P<0.05). HA2 supplementation had lower (P<0.05) Shannon indices than the CON on fermentation for 10 d.

The bacterial communities on fresh alfalfa were primarily composed of *Pantoea* (29.50%), *Alorhizobium* (13.46%), *Methylobacterium* (9.74%), *Pseudomonas* (5.88%), and *Sphingomonas* (6.29%) (Fig. 1-A). Compared with the fresh alfalfa, β-carotene-related *Pantoea* disappeared in ensiled alfalfa. After fermentation, the above bacteria were replaced by LAB (Fig. 1-A and B). There was bacterial succession during alfalfa fermentation, mainly on *Lactobacillus kullabergensis*, *L. apis*, and *L. rennini* appearing on fermentation for 40 and 80 d relative to 10 and 0 d (Fig. 1-C). Additionally, the bacterial succession and abundances of LAB were altered by HA supplementations. Briefly, on fermentation for 80 d, *L. senioris* had higher (P<0.05) abundance in each HA supplementation and was barely found in the CON. HA1 supplementation had a higher (P<0.05) abundance of *L. apis* than HA2, HA3, and the CON on fermentation for 80 d. HA3 supplementations had the highest abundance of *L. kullabergensis* (P<0.05) and all HA supplementations lowered (P<0.05) the abundance of *L. rennini* on fermentation for 80 d. HA2 and HA3 supplementations had a higher (P<0.05) abundance of unclassified *Lactobacillus* than the CON on fermentation for 10 d (P<0.05).

The correlation heatmap generated from the Mantel's test was used to identify the key factors driving β-carotene synthesis during fermentation (Fig. 2). β-Carotene production was affected by bacterial diversity, HA supplementation, and fermentation duration, with varying degrees of impact (Fig. 2-A). Higher (P<0.05) bacterial diversity was in the CON compared with HA2 and HA3 on fermentation for 10 d (Fig. 2-B) but no difference (P>0.05) of bacterial diversity occurred between HA supplementations and the CON on fermentation for 40 and 80 d. β-Carotene had a positive correlation with *L. kullabergensis* (P<0.001) and *L. senioris* (P<0.01) (Fig. 2-C). The db-RDA visualized the potential correlations between β-carotene, bacterial species, enzymes, HA supplementation, and fermentation days (Fig. 2-D and E). Among the bacterial species, *Lactobacillus* had the strongest positive influence on β-carotene production (P<0.05).

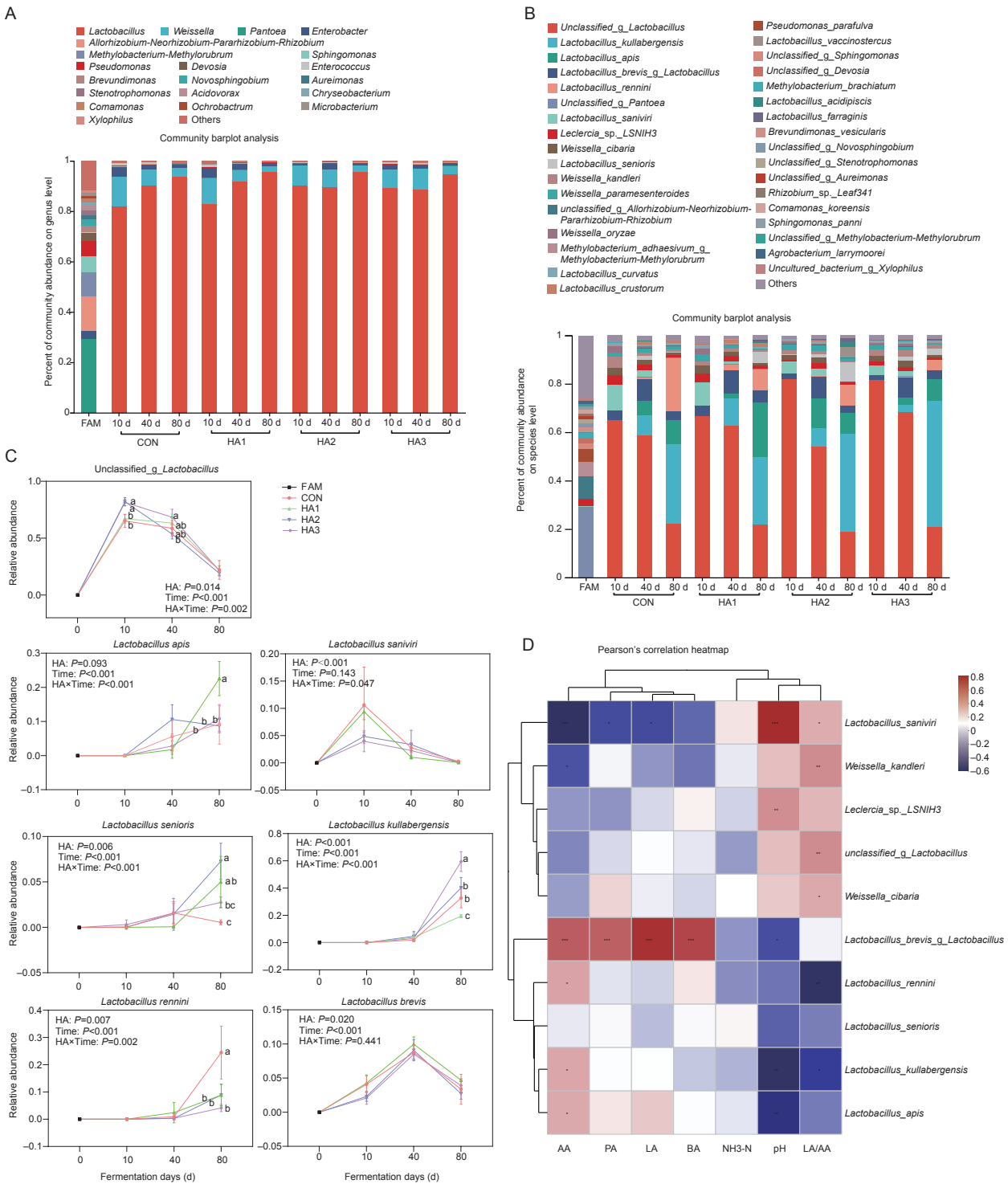


Fig. 1 Composition of the bacterial community on genus (A) and species (B) level in fresh alfalfa material and ensiled alfalfa; dynamic changes of 7 top *Lactobacillus* species (C), Pearson's correlation heatmap (D). FAM, fresh alfalfa material; CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; LA, lactic acid; AA, acetic acid; LA/AA, the ratio of lactic acid to acetic acid; PA, propionic acid; BA, butyric acid; HA, hexanoic acid; data from each treatment represent the means of three replicates and the error bars represent standard deviation of the mean (n=3); means with different letters in the same days differed at P<0.05.

3.3. Effects of HA supplementation on metabolic modules related to β-carotene biosynthesis at different fermentation days

As shown in Fig. 3, four modules (M00095 for C5 isoprenoid biosynthesis, mevalonate pathway; M00097 for β-Carotene biosynthesis, GGAP≥β-carotene; M00364 for C10–C20 isoprenoid biosynthesis, bacteria; and M00367 for C10–C20 isoprenoid biosynthesis, non-plant eukaryotes) in

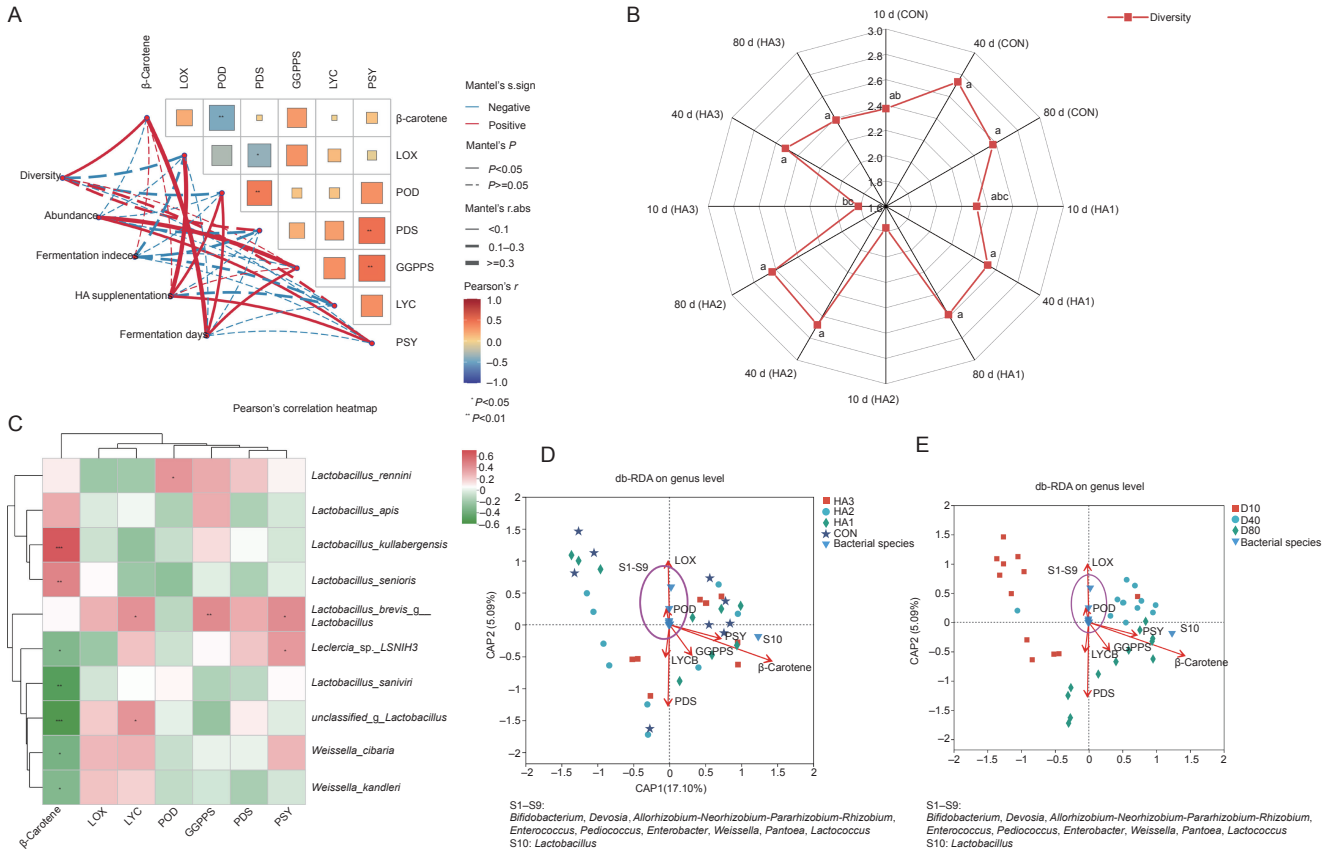


Fig. 2 Integrated analysis of bacterial diversity, fermentation characteristics, and β-carotene-related factors in alfalfa silage treated with hexanoic acid. A, the relationships among the bacterial diversity, abundance, fermentation indices, HA supplementations, and fermentation days in ensiled alfalfa. B, effect of HA supplementations on the bacterial diversity. C, Pearson's correlation heatmap of bacterial species (top 10 in relative abundance) with β-carotene, lipoxygenase, peroxidase, and β-carotene synthesis related enzymes. D and E, db-RDA analysis on HA supplementations or fermentation days. CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; HA, hexanoic acid; LOX, lipoxygenase; POD, peroxidase; GGPPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; LYC, lycopene β-cyclase. Data from each treatment represent the means of three replicates (n=3); means with different letters differed at P<0.05.

microorganism metabolism pathways on KEGG categories were involved in β-carotene synthesis. The interaction of HA supplementations and fermentation days significantly influenced M00095, M00364, and M00367 (P<0.05), but did not influence M00097 (P>0.05). There was an increase in M00095 (P<0.05) during fermentation in HA supplementations relative to the fluctuation in the CON. In HA supplementations and the CON, sharp decreases (P<0.05) in value were shown in M00364 during 10 d of fermentation, followed by an increasing tendency (0.05<P<0.01) from fermentation 10 to 80 d. Fluctuation values were presented in M00367 for HA1, HA2, and the CON during fermentation for 80 d. In contrast, an increase in value (P<0.05) was shown in M00367 for HA3 supplementation.

3.4. Some Lactobacillus involved in metabolic modules

As demonstrated in Fig. 4-A, species *L. apis* (r=0.569), *L. senioris* (r=0.703), *L. kullabergensis* (r=0.680), and *L. rennini* (r=0.652) were positively correlated with M00095 (P<0.05). Species *L. senioris* (r=0.643), *L. kullabergensis* (r=0.624), *L. rennini* (r=0.616), and *L. brevis* (r=0.560) were positively correlated with M00367 (P<0.05). Additionally, a co-occurrence network analysis was conducted among bacteria on the species level (Fig. 4-B), revealing that *L. senioris* has significant (P<0.05) correlations with *L. rennini*, *L. kullabergensis*, and *L. crustorum*.

3.5. Factors of promoting β-carotene synthesis in the condition of HA supplementations

The values of the Variance Inflation Factor (VIF) and Durbin-Watson regulate the multicollinearity and independence of variables, respectively. A higher VIF indicates more serious multicollinearity between variables. It has been observed that multicollinearity exists when VIF is greater than 10 (Salmerón *et al*. 2020). In multiple linear regression (MLR), a Durbin-Watson value close to 2 indicates a good model (Zhang *et al*. 2008). In our study, all variables met the requirement (Durbin-Watson value was 2.61 and VIF was below 10) (Table 3). The CP, POD, PDS, LYC, *L. apis*, *L. saniviri*, *L. senioris*, and *L. kullabergensis* showed a positive correlation with β-carotene concentration based on the positive beta value.

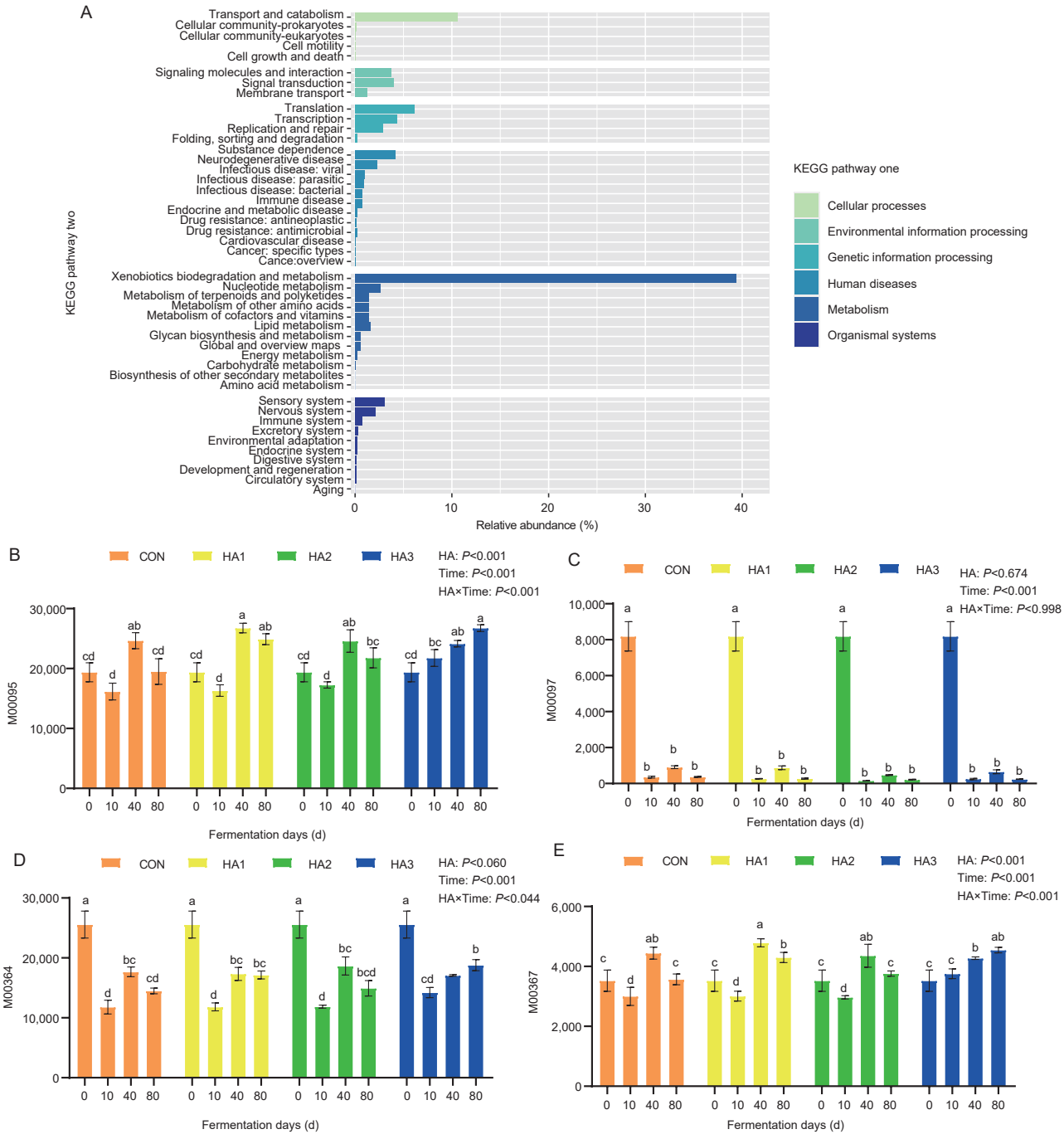


Fig. 3 Four modules of β-carotene synthesis in microorganism metabolism pathways on KEGG categories. CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d. Data from each treatment represent the means of three replicates and the error bars represent standard deviation of the mean (n=3); means with different letters in the same days differed at P<0.05.

On the other hand, *L. rennini* and *L. brevis* exhibited a negative correlation with β-carotene concentration. Furthermore, the positive effects of CP, POD, PDS, LYC, *L. apis*, *L. saniviri*, *L. senioris*, and *L. kullabergensis* on β-carotene concentration were greater than those of *L. rennini* and *L. brevis* due to the negative constant in the regression equation. Therefore, the major factors influencing β-carotene synthesis under HA supplementation conditions were identified.

3.6. Existence of β-carotene-producing bacteria *via* examining the *crtNM* and *crtE* gene

The *crtNM* operon was responsible for LAB-producing carotenoid compounds (including α- and β-carotene etc.), which were found in both the CON and all HA supplementations due to the clear band (Fig. 5). This indicated that *crtNM* operon was successfully amplified, and suggested the presence of LAB capable of producing β-carotene in ensiled alfalfa. In contrast, no band was observed for the

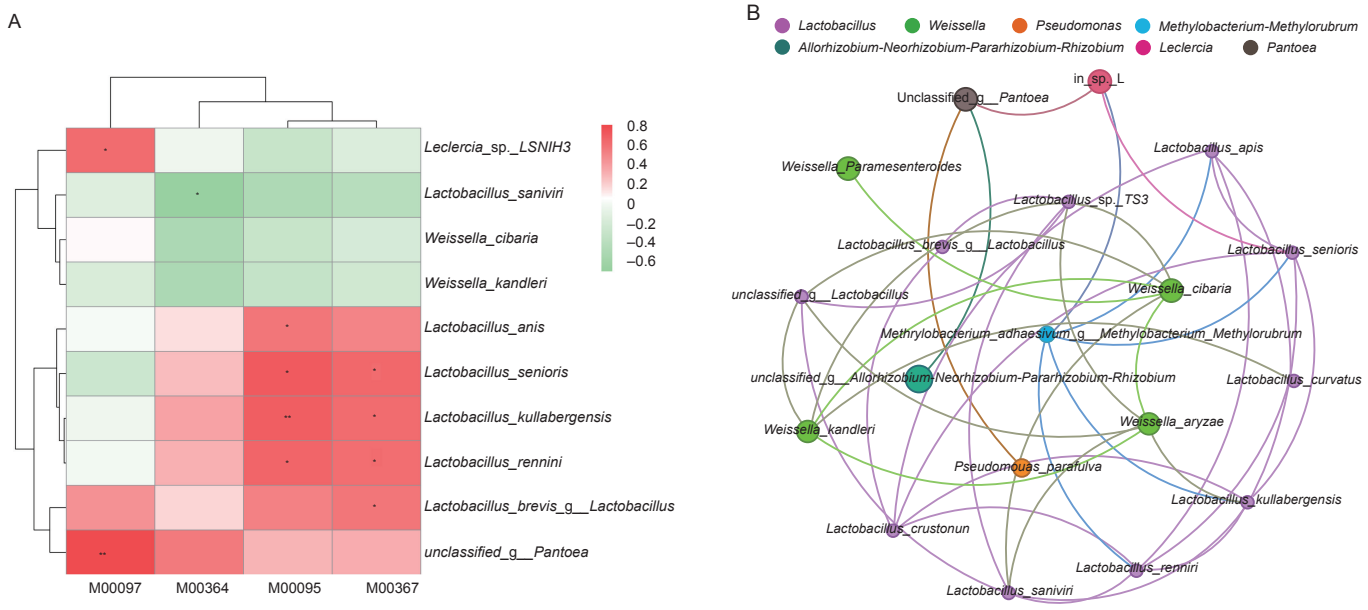


Fig. 4 Pearson's correlation analysis of the relative abundance of the top 10 bacterial species and metabolic modules (A) and co-occurrence network of bacteria (B). M00095 for C5 isoprenoid biosynthesis, mevalonate pathway; M00097 for β-carotene biosynthesis, GGAP≥β-carotene; M00364 for C10–C20 isoprenoid biosynthesis, bacteria; and M00367 for C10–C20 isoprenoid biosynthesis, non-plant eukaryotes.

Table 3 Regression coefficients of β-carotene

Model (R^2=0.981)[1)]	Unstandardized coefficients		Standardized coefficients β	T	Significance	Collinearity statistics	
	B	Standard error				Tolerance	VIF
Constant	–117	28.4		–4.13	<0.001		
CP	0.336	0.129	0.106	2.60	0.013	0.314	3.19
POD	1.74	0.168	0.390	10.3	<0.001	0.362	2.76
PDS	19.9	1.46	0.589	13.6	<0.001	0.277	3.61
LYC	1.00	0.16	0.209	6.24	<0.001	0.462	2.17
Lactobacillus apis	1.79	0.183	0.518	9.80	<0.001	0.185	5.40
L. saniviri	0.828	0.292	0.125	2.84	0.007	0.269	3.72
L. senioris	3.88	0.422	0.389	9.21	<0.001	0.290	3.45
L. kullabergensis	0.854	0.058	0.651	14.7	<0.001	0.264	3.78
L. rennini	–2.07	0.159	–0.569	–13.0	<0.001	0.271	3.69
L. brevis	–2.46	0.389	–0.295	–6.31	<0.001	0.237	4.22

[1)] POD, peroxidase; PDS, phytoene desaturase; LYC, lycopene β-cyclase. $Y_{\beta\text{-carotene}}=-117+0.336X_{CP}+1.74X_{POD}+19.9X_{PDS}+1.00X_{LYC}+1.79C_{L.\ apis}+0.828X_{L.\ saniviri}+3.88X_{L.\ senioris}+0.854X_{L.\ kullabergensis}-2.07X_{L.\ rennini}-2.46X_{L.\ brevis}$.

fresh alfalfa, indicating the absence or scarcity of LAB with the ability to produce β-carotene in the fresh alfalfa. Similarly, the *crtE* gene in *Lactobacillus*, coding the GGPPS for the production of C_{40} carotenoids, did not exist in the fresh alfalfa according to its relative abundance (Fig. 6). However, after fermentation, the *crtE* relative abundances were shown in the CON and all HA supplementations. Furthermore, the interaction between HA supplementations and fermentation days had an impact on the relative abundance of the bacterial *crtE* gene (P<0.05). Generally, compared with the CON, HA supplementations decreased (P<0.05) the *crtE* gene relative abundance on fermentation for 40 and 80 d. The highest bacterial *crtE* gene relative abundance was found in the CON (P<0.05) on fermentation for 40 d.

3.7. Effects of HA supplementation on fermentation and nutritive quality at different fermentation days

The interaction of HA supplementation and fermentation days affected the contents of lactic acid, acetic acid, propionic acid, butyric acid, NH_3-N, and the ratio of lactate to acetate (LA/AA) (P<0.05) (Table 4). The pH was significantly affected by HA supplementation and fermentation days (P<0.05). As the supplementations of HA were increased, the pH was declined (P<0.05). After fermentation for 40 d, the HA1 and HA2 supplementations had the highest lactic acid content (P<0.05), and the HA1 supplementation had the highest

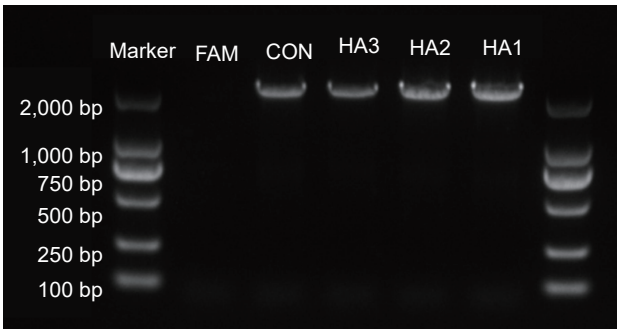


Fig. 5 The *crtNM* operon for identifying LAB synthesizing β-carotene during alfalfa fermentation with hexanoic acid supplementation. FAM, fresh alfalfa material; CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis.

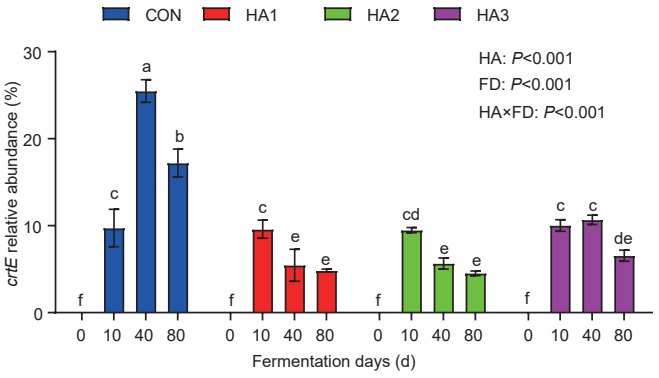


Fig. 6 The *crtE* gene abundance relative to total microbial DNA in alfalfa supplemented with hexanoic acid. CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; data from each treatment represent the means of six replicates and the error bars represent standard deviation of the mean (n=6); means with different letters differed at P<0.05. HA, hexanoic acid supplementation; FD, fermentation days; HA×FD, the interaction of hexanoic acid supplementation and fermentation days.

acetic acid content (P<0.05). In contrast, the CON silage had the lowest acetic acid content after ensiling for 10 d (P<0.05). HA3 supplementation had the highest LA/AA (P<0.05) and propionic acid content (P<0.05). Additionally, the butyric acid content decreased to zero with HA supplementation as fermentation time increased, and only HA2 and HA3 supplementations had lower (P<0.05) NH_3-N content than the control after 40 d of fermentation.

The interaction of HA supplementation and fermentation days affected (P<0.05) the contents of WSC and CP. The highest WSC content (P<0.05) was found in HA1 on fermentation for 10 d. The HA2 and HA3 had higher (P<0.05) CP content than HA1 and the CON on fermentation for 40 and 80 d (Table 5）.

4. Discussion

In this study, the data verified our hypothesis. All HA supplementations increased the β-carotene content of alfalfa silages with high quality relative to the fresh alfalfa. This might be attributed by that HA increased the abundance of *L. kullabergensis* and facilitated the emergence of β-carotene-related *L. senioris*. Furthermore, MLR predicted that the increased β-carotene content of alfalfa silage by HA supplementation might be *via* enhancing the positive actions of multiple factors, including *L. kullabergensis*, *L. apis*, *L. saniviri*, *L. senioris*, POD, PDS, and LYCB, to overpass negative effects of *L. rennini* and *L. brevis*. Our study explored the possible mechanisms that β-carotene content could be increased by HA possibly *via* the β-carotene-related LAB flora and enzymes during alfalfa ensiling. This will provide potential theoretical foundation and additive technical support for the research and production of functional and high-quality silage.

In fresh alfalfa, β-carotene of 38.5 mg kg^{-1} DM was detected, which was low and contradicted our previous study on alfalfa (Zong *et al*. 2021). This difference was attributed to the poor quality of this alfalfa, which had been planted for 5 years and undergone degeneration in the current year (Cai *et al*. 2020). Interestingly, β-carotene content decreased after 10 and 40 d of fermentation, consistent with our previous study (Zong *et al*. 2023) that observed increased LOX activity and the absence of β-carotene-producing bacteria, such as *Pantoea* genera, in the acidic fermentation environment. However, after 80 d of fermentation, β-carotene content in the fermented alfalfa did not decrease, but instead slightly increased by 2.82% compared to the fresh alfalfa. This result is consistent with the previous studies on fermented alfalfa (Zhang *et al*. 2020), yarrow (*Achillea millefolium* L.) (Goliński *et al*. 2012), Italian ryegrass (Lv *et al*. 2017), grass-clover mixture, and maize whole crop (Mogensen *et al*. 2012). We believed that the increase in β-carotene during the fermentation of those biomass materials was not coincidental, suggesting the presence of certain substances that play a key role in enhancing β-carotene content during fermentation. Unfortunately, limited information was available until the discovery of HA in fermented alfalfa with low β-carotene loss. HA supplementation was demonstrated to increase β-carotene content by up to 159% compared with the fresh alfalfa in this study. One indirect literature was that 2-diethylaminoethanol esters of HA could lead to the accumulation of large amounts of β-carotene (Poling *et al*. 1977).

The addition of HA increased β-carotene content without decreasing the activities of lipoxygenase and peroxidase, which was consistent with our previous study (Zong *et al*. 2023). The Mantel's test indicated that β-carotene content was not influenced by β-carotene synthesis enzymes, significantly (Fig. 2-A). However, β-carotene content was dependent on the bacterial diversity and fermentation days. HA primarily influenced bacterial diversity, as evidenced by *L. senioris* in all HA supplementations rather than in the CON (Fig. 1-C). Acidity plays a crucial role in microbial population succession (Tan *et al*. 2020), and HA supplementation created a different acidic environment relative to natural fermentation in the CON. This led to the activation of anaerobic β-carotene-producing bacteria, as supported by the positive relationship between *L. senioris* and β-carotene.

Table 4 Effects of hexanoic acid and fermentation days on fermentation indices of alfalfa

Fermentation days (d)[1)]	HA supplementations	pH	LA (g kg^{-1} DM)[2)]	AA (g kg^{-1} DM)[2)]	LA/AA[3)]	PA (g kg^{-1} DM)[4)]	BA (g kg^{-1} DM)[4)]	NH_3-N (g kg^{-1} N)
10	CON	5.11	48.2 e	37.6 e	1.29 ab	3.38 e	1.86 b	89.3 de
	HA1	5.06	57.7 d	47.0 d	1.23 abc	3.25 e	2.05 b	80.6 e
	HA2	4.95	51.1 de	42.8 de	1.20 bcd	3.23 e	1.98 b	81.0 e
	HA3	4.90	57.5 d	46.8 d	1.23 abc	3.24 e	0.00 b	81.9 e
40	CON	4.80	67.7 c	62.5 bc	1.09 de	4.21 d	8.71 a	122 a
	HA1	4.76	84.3 a	71.3 a	1.19 bcd	4.88 c	0.00 b	111 ab
	HA2	4.70	83.2 a	64.6 b	1.29 ab	5.96 b	0.00 b	93.4 de
	HA3	4.69	76.5 b	56.9 c	1.35 a	7.44 a	0.00 b	102 bcd
80	CON	4.79	65.3 c	65.3 ab	1.00 e	4.53 cd	0.00 b	109 abc
	HA1	4.66	65.9 c	60.9 bc	1.08 de	4.42 cd	0.00 b	94.9 cde
	HA2	4.63	65.8 c	55.7 c	1.18 bcd	4.61 cd	0.00 b	88.3 de
	HA3	4.59	67.0 c	59.3 bc	1.14 cde	4.62 cd	0.00 b	102 bcd
SEM		0.026	1.68	1.51	0.017	0.178	0.348	1.99
Fermentation days means								
10		5.00 a	53.6	43.5	1.24	3.27	1.47	83.2
40		4.74 b	77.9	63.8	1.23	5.62	2.18	107
80		4.67 c	66.0	60.3	1.10	4.54	0.00	98.6
SEM		0.030	1.66	1.52	0.030	0.126	0.045	2.30
HA supplementations means								
CON		4.90 a	60.4	55.1	1.13	4.04	3.52	107
HA1		4.83 b	69.3	59.7	1.17	4.18	0.68	95.4
HA2		4.76 bc	66.7	54.4	1.22	4.60	0.66	87.6
HA3		4.72 c	67.0	54.3	1.24	5.10	0.00	95.5
SEM		0.035	1.92	1.76	0.035	0.145	0.052	2.65
Significance								
HA supplementations		<0.001	<0.001	0.011	0.010	<0.001	<0.001	<0.001
Fermentation days		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction		0.797	0.009	<0.001	0.008	<0.001	<0.001	<0.001

[1)] CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; HA, hexanoic acid; SEM, standard error of the means.
[2)] LA, lactic acid; AA, acetic acid.
[3)] LA/AA, the ratio of lactic acid to acetic acid.
[4)] PA, propionic acid; BA, butyric acid.
Different little letters in the same column indicated significant differences at P<0.05. Data from each treatment represent the means of six replicates (n=6); data from fermentation days means represent the means of 24 replicates (n=24); data from HA supplementations means represent the means of eighteen replicates (n=18).

A previous study reported *L. senioris* with a beige color isolated from faeces of a 100-year-old elderly female person (Oki *et al*. 2012). Some LAB with yellow color were identified to have the ability to produce carotenoids (Garrido-Fernández *et al*. 2010; Turpin *et al*. 2016; Xu *et al*. 2020). Additionally, fermentation is an acidic bio-process that can destroy plant substrates and clusters of cells, resulting in the release and bioaccessibility of carotenoids (Sangija *et al*. 2022). The results of our study suggest that HA supplementation enhanced the acidic progress, as evidenced by lower pH and higher contents of lactic acid and acetic acid relative to the CON on fermentation for 10 d. Mapelli-Brahm *et al*. (2020) summarized that LAB strains with enzyme activity and lactic acid fermentation benefited carotenoid extraction. This has benefited carotenoid extraction in various plants, including marigold, chili, alfalfa, tomato, and carrots (Sowbhagya and Chitra 2010). Fermentation days contributed to the emergence of anaerobic β-carotene-producing bacteria, such as β-carotene positive related *L. kullabergensis* and *L. apis*, on fermentation for 40 and 80 d. In this study, the crtNM operon identification indicated the potential functional LAB of synthesizing β-carotene was present in ensiled alfalfa (Fig. 5). *L. kullabergensis* and *L. senioris* positively correlated with β-carotene (Fig. 2-C) and their abundances could be evaluated by HA supplementations (Fig. 1-C). The analysis of db-RDA on genus level according to the HA supplementation or fermentation days revealed that *Lactobacillus* genera exhibited a positive relationship with β-carotene (Fig. 2-D and E). Therefore, HA favored to facilitate the increase abundance and emergence of β-carotene-producing bacteria during alfalfa fermentation.

Based on Pacbio's sequencing technology, the PICRUSt tools have been used to predict the microbial function according to the KEGG function profiles of microbial communities (Bai *et al*. 2021; Du *et al*. 2021). The metabolism of cofactors and vitamins, and the metabolism of terpenoids and polyketides were the essential role of microbial function. In this study, four modules (M00095, M00097, M00364, and M00367) related to β-carotene biosynthesis were used to gain insight into the role of HA in increasing the β-carotene. However, only M00095 and M00367 reflected the β-carotene increase regulated by HA supplementation. This was inconsistent with the results reported in our previous study (Zong *et al*. 2023). The reason for the variation was that HA supplementation possibly changed the biosynthesis pathway, supported by the decrease in the *crtE* (responsible for coding GGPPS in

Table 5 Effects of HA supplementations and fermentation days on nutrition composition during alfalfa fermentation

Fermentation days (d)[1)]	HA supplementations	DM (g kg^{-1} FW)[2)]	WSC	CP	NDF	ADF
			(g kg^{-1} DM)[3)]			
10	CON	203	17.5 c	225 ab	315	250
	HA1	198	25.8 a	227 ab	364	237
	HA2	199	16.9 c	225 ab	342	231
	HA3	198	22.4 b	228 a	345	226
40	CON	199	14.3 d	214 cd	348	255
	HA1	196	14.3 d	211 d	343	209
	HA2	201	15.9 cd	224 ab	303	221
	HA3	200	15.6 cd	221 ab	315	230
80	CON	194	6.84 e	211 d	336	219
	HA1	199	7.64 e	212 d	363	255
	HA2	197	7.62 e	221 ab	330	226
	HA3	197	8.90 e	220 bc	331	234
SEM		0.6	0.85	1.0	3.8	3.5
Fermentation days means						
10		200	20.6	226	341	236
40		199	15.1	217	327	229
80		197	7.75	216	340	234
SEM		1.5	0.54	1.6	7.8	8.1
HA supplementations means						
CON		199	12.9	217	333 b	241
HA1		198	15.9	216	356 a	233
HA2		199	13.5	223	325 b	226
HA3		198	15.6	223	330 b	230
SEM		1.7	0.62	1.8	9.0	9.3
Significances						
HA supplementations		0.883	<0.001	<0.001	0.007	0.418
Fermentation days		0.100	<0.001	<0.001	0.150	0.661
Interaction		0.130	<0.001	0.044	0.063	0.054

[1)] CON, HA1, HA2, HA3, the doses of hexanoic acid supplementations were 0, 0.05, 0.1, and 0.2% of the weight of fresh alfalfa basis; 10, 40 and 80, alfalfa was ensiled for 10, 40 and 80 d; SEM, standard error of the means.
[2)] DM, dry matter; FW, fresh weight.
[3)] WSC, water-soluble carbohydrates; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber.
Different little letters in the same column indicated significant differences at $P<0.05$. Data from each treatment represent the means of six replicates ($n=6$); data from fermentation days means represent the means of 24 replicates ($n=24$); data from HA supplementations means represent the means of eighteen replicates ($n=18$).

Lactobacillus) gene relative abundance. Although M00367 corresponds to the C10–C20 isoprenoid biosynthesis of non-plant eukaryotes, the actual result was yeasts and molds restrained by HA supplementation. One interesting finding was that some LAB, *L. kullabergensis* and *L. senioris*, were positively correlated with M00095 and M00367. Predicted Modules might not provide insight into how HA changes the β-carotene biosynthesis pathway. Using MLR based on stepwise analysis could eliminate the multidisciplinary of indexes to clarify the factors influencing β-carotene. In this study, MLR was employed to discover the factors of β-carotene in the condition of HA supplementation. According to the MLR, the *L. kullabergensis*, *L. apis*, *L. saniviri*, *L. senioris*, POD, PDS, and LYC positively regulated β-carotene. In comparison, *L. rennini* and *L. brevis* negatively adjusted β-carotene. However, the positive regulation of strains and enzymes had a stronger effect on increasing β-carotene than the negative adjustments of *L. rennini* and *L. brevis*. M00095 (responsible for C5 isoprenoid biosynthesis in mevalonate pathway) and M00367 (charging C10-C20 isoprenoid biosynthesis in non-plant eukaryotes) related to the β-carotene increase were adjusted by HA supplementation. These results reflected that β-carotene biosynthesis up-regulated by HA supplementation included multiple factors. Interestingly, the CP, three β-carotene related enzymes, and some LAB species were involved in the regression equation. This might indicate that the roles of other nutrient compositions, LOX, GGPPS, PSY, and other bacteria were excluded due to their multicollinearity on β-carotene. There were potential cooperations of POD, PDS, and LYC with *L. kullabergensis*, *L. apis*, *L. saniviri*, and *L. senioris* on β-carotene increase in the condition of HA supplementation. The future work is to deeply explore how above the β-carotene-related LAB flora interact with POD, PDS, and LYC.

HA supplementation enhanced the acidification of alfalfa in the first phase (10 d), supported by lower pH and higher contents of LA and AA. This was attributed to the acidity and bacteriostatic ability of HA (Caccalano *et al*. 2021; Wang W *et al*. 2021). Subsequently, HA supplementation inhibited aerobic bacteria, yeasts, and molds on fermentation for 10 d. As the fermentation progressed to 40 and 80 d, the acetic fermentation became more vigorous, resulting in LA/AA approaching 1 in all HA supplementations and the

CON group. The acetic fermentation caused by the LAB succession, demonstrated by the emergence of acetic acid positively related *L. kullabergensis*, *L. apis*, *and L. rennini* on fermentation for 40 and 80 d relative to 10 and 0 d (Fig. 2-B and D). HA supplementation did not further enhance the LA fermentation, but it restrained the formation of NH_3-N. Generally, high concentration of NH_3-N (>150 g kg^{-1} N) (Kung *et al*. 2018) indicated poor fermentation since undesired microbes, such as aerobic bacteria and clostridia, could multiply and degrade protein into NH_3-N. Consequently, this leads to poor nutritive quality. However, in this study, HA supplementation showed a tendency to decrease undesired aerobic bacteria, yeast, and molds, as well as reduced butyric acid levels, but increased the contents of WSC and CP. This indicated that HA supplementations improved fermentation and nutritive value compared to the CON group.

5. Conclusion

HA supplementation at 0.1% mostly increased the β-carotene content of alfalfa silage compared with fresh alfalfa material in an oxygen-free and dark conditions. The presence of β-carotene-producing LAB was indicated in ensiled alfalfa through the identification of *crtNM* operon. *Lactobacillus* was proposed as the source responsible for the β-carotene increase. HA supplementation specifically facilitated the increase abundance of *L. kullabergensis* and the emergence of *L. senioris*. β-Carotene content of ensiled alfalfa might be adjusted by HA supplementation *via* enhancing the combined action of multiple factors, including *L. kullabergensis*, *L. apis*, *L. saniviri*, *L. senioris*, POD, PDS, and LYCB, to overpass negative effects of *L. rennini* and *L. brevis*. This study provided useful information to regulate the β-carotene content of alfalfa silage. Further studies will be needed to confirm the above possible mechanisms using gene silencing and over-expression techniques as well as microbe removal or addition techniques.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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