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Using PICRUSt2 to explore the functional potential of bacterial community in alfalfa silage harvested at different growth stages

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Abstract

Background: This study evaluated the effects of growth stage and storage time on fermentation characteristics, bacterial communities and their functionality in alfalfa (*Medicago sativa* L.) silage. Alfalfa was harvested at initial flowering (10–20% bloom, AL1) and full flowering (> 80% bloom, AL2) stages, respectively. The harvested alfalfa was ensiled in 15 L plastic silos. Triplicate silos were sampled after 1, 3, 7, 15, 30 and 60 days of ensiling, respectively. Fermentation products were analyzed on each sampling day. The bacterial communities and their functional potential after 3 and 60 days were analyzed by high-throughput sequencing technique and PICRUSt2 method.

Results: AL2 had better fermentation quality than AL1 with lower pH, ammonia nitrogen and butyric acid concentrations and higher lactic acid concentrations on day 60. AL2 had higher abundances of *Weissella* and *Lactobacillus* after 3 days, and lower abundances of *Enterobacter* and Enterobacteriaceae on day 60 compared to AL1. In metabolic pathway analysis, ensiling promoted the carbohydrate and amino acid metabolism, and inhibited the signal transduction and membrane transport. In enzyme analysis, AL2 had lower abundances of nitrite reductase (NADH) and ornithine decarboxylase than AL1 on day 60. In phenotype analysis, AL2 had higher proportions of facultatively anaerobic and lower proportions of anaerobic, potentially pathogenic and gram negative than AL1 on day 60.

Conclusions: High throughput sequencing technique combined with PICRUSt2 can be successfully used to describe the changes of bacterial communities and their functionality in silage. This approach can improve our understanding of the silage microbiology to further regulate the fermentation products.

Keywords: Silage, Bacterial community, Functional potential, Alfalfa, Growth stage

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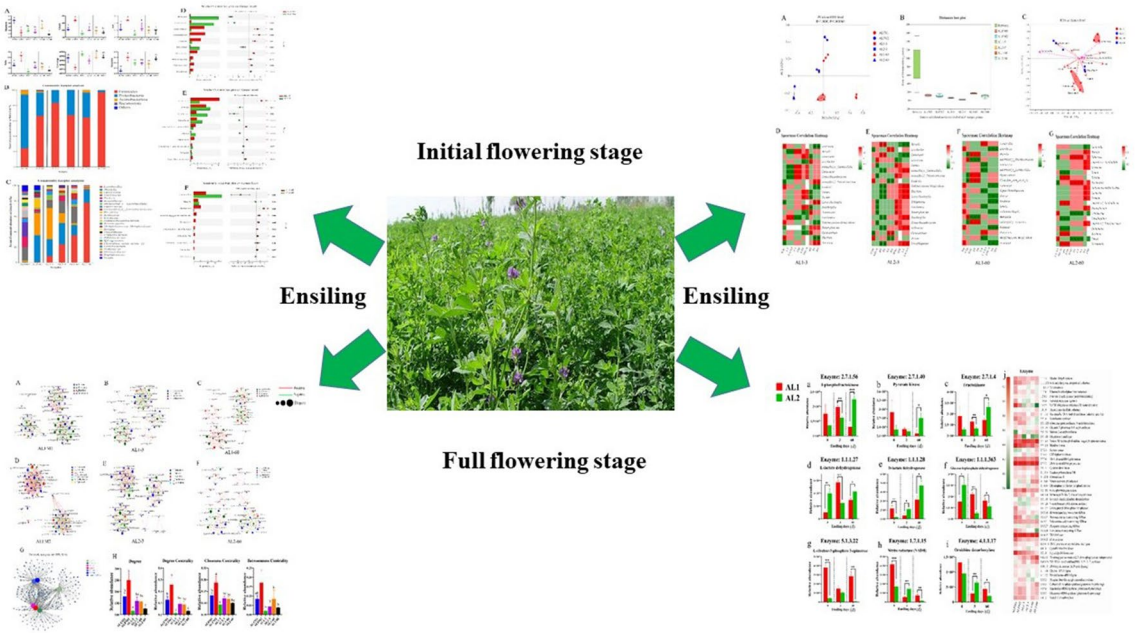
Graphical Abstract



Background

Ensiling is an effective method by which epiphytic lactic acid bacteria on forages utilize water soluble carbohydrates and produce organic acids (mainly lactic acid) in an anaerobic environment, thus lowering the pH and inhibiting the growth of undesirable microorganisms in silage to preserve the nutrition of forages at the optimum growth stage for use [1]. The ensiling process is strongly affected by material characteristics, such as buffering capacity, water soluble carbohydrate, dry matter and epiphytic microorganisms [2]. Hence, the composition of alfalfa (*Medicago sativa* L.) at harvest has a great influence on silage fermentation characteristics. Buxton et al. [3] reported that, with the advancing maturity of alfalfa, buffering capacity generally declines, and dry matter and water soluble carbohydrate concentrations increase, which are conducive to the improvement of fermentation quality. Whereas, the nutritive value of alfalfa silage is decreased due to the enhancement of acid detergent fiber, acid detergent lignin, and neutral detergent fiber concentrations as alfalfa matures. To date, numerous studies described the changes of chemical compositions in alfalfa silages at different growth stages. However, little information is available on investigating the effect of growth stage on bacterial communities and their functional potential in alfalfa silages.

Recent advances in microbial community analysis using high throughput sequencing technique have allowed new insights into the complexity of silage microbiology [4]. This technique could provide estimates of microbial abundances and diversity, whereas it could not directly show the functional potential of bacterial community. The shotgun sequencing technique could supply complete functional information of bacterial community, but its cost is prohibitive especially for large-scale investigations. Hence, several functional prediction tools, such as PICRUSt, Tax4Fun, FAPROTAX, BugBase and FUNGuild, have been developed to predict the functionality of microbial community from amplicon sequences and allow for initial exploration of hypotheses prior to shotgun sequencing. PICRUSt2 is such a prediction tool that is becoming increasingly popular in microbial ecology studies [5].

PICRUSt2 was first presented in 2020 and improved on the basis of PICRUSt1. PICRUSt2 is more accurate than PICRUSt1 and other competing methods overall, because PICRUSt2 contains an updated and larger database of gene families and reference genomes, and provides interoperability with any operational taxonomic unit (OTU)-picking or denoising algorithm [6]. Recently, Bai et al. [7] used PICRUSt2 to assess the predicted functionality of bacterial communities in maize silages. They found that

the functions of transcription, translation, and replication and repair of bacterial community were obviously up-regulated, and the amino acid metabolism was down-regulated in 60 day silages. However, there is a paucity of information regarding the functional potential of bacterial community in alfalfa silages analyzed by PICRUSt2.

Elucidating the community composition, variation, and functional potential of the bacteria community in silage will increase our understanding of the silage microbiology to further regulate the fermentation products. Therefore, this study aimed to evaluate the effects of growth stage and storage time on fermentation characteristics, bacterial communities and their predicted functional potential and phenotype in alfalfa silages. To the best of our knowledge, this is the first detailed investigation about functional potential and phenotype of bacterial community in alfalfa silages at different growth stages.

Materials and methods

Silage preparation and sampling

Alfalfa was planted in the experimental plots in Nanjing (31°36′ N, 119°10′ E, annual precipitation 1098 mm, average elevation 43.2 m, average temperate 16.1 °C), Jiangsu Province, China. First-cutting fresh alfalfa (AL) was harvested on 20 May and 8 June 2021 corresponding to the growth stages initial flowering (10–20% bloom, AL1) and full flowering (> 80% bloom, AL2). Alfalfa harvests were taken from a large experimental field plot that was further divided into two subplots (one for each growth stage of alfalfa). At each growth stage, the alfalfa from each plot was harvested by a small-scale forage harvester to a stubble height of 7 cm. Harvest of alfalfa always took place between 9 and 11 a.m. in sunny days. Without any additives and wilting, the harvested fresh alfalfa was directly cut to about 2 cm in length with a stationary chopper and mixed thoroughly for silage making. Then, about 11.4 kg of chopped alfalfa was placed tightly into the plastic silos (15 L capacity for one silo), hermetically sealed with screw tops and stored upside down in darkness at ambient temperature (22–27 °C). In total, 36 silos (6 ensiling days × 2 growth stages × 3 replicates = 36) were ready for the opening after 1, 3, 7, 15, 30 and 60 days of fermentation, respectively.

At sampling, fresh alfalfa or the silage mass was thoroughly mixed and collected after any wastage had been removed. After mixing, the sample mass was divided into three parts. One part of sample (20.0 g) was homogenized with 100 mL of distilled water, and filtrated with sterile gauze. The filtrate was stored at − 20 °C and used for measuring pH, buffering capacity, ammonia–nitrogen (NH_3–N), organic acid and ethanol contents. The second part of sample (150 g) was oven-dried at 65 °C for 48 h, and ground to pass a 1 mm screen and prepared for determining fiber, water soluble carbohydrate (WSC) and total nitrogen (TN) contents. The third part of sample (10.0 g) was mixed with 90 mL sterilized saline in a 200 mL conical flask and shaken for 10 min at 120 rpm in a constant temperature shaker, followed by filtration with sterile gauze and continuously dilutions (10^{-1}–10^{-7}) in sterilized saline in several 2 mL tubes. Then, 1 mL of liquid from each tube was sucked and used to count microbial populations. The residual filtered liquid (about 84 mL) in conical flask was collected and stored at − 80 °C and prepared for DNA extraction and sequencing analyses.

Chemical and microbial analyses

The pH was measured with a pH meter (Mettler Toledo, Zurich, Switzerland). The buffering capacity was measured based on method of Jasaitis et al. [8]. The concentrations of organic acid (lactic acid, acetic acid, propionic acid, butyric acid) and ethanol were analyzed by high performance liquid chromatography (HPLC) as described by Wang et al. [9]. In brief, the concentrations of organic acid (lactic acid, acetic acid, propionic acid, butyric acid) and ethanol contents were analyzed with the Agilent HPLC (High Performance Liquid Chromatography) 1260 (Agilent Technologies, Inc., Santa Clara, CA, United States; column, Carbomix H-NP5, Sepax Technologies, Inc., Delaware, United States; detector, refractive index detector, Agilent Technologies, Inc., Santa Clara, CA, United States; eluent, 2.5 mmol/L H_2SO_4, 0.5 mL/min; temperature, 55 °C). The concentration of NH_3–N was determined by the phenol–hypochlorite method [10].

The dry matter (DM) of fresh and ensiled alfalfa was measured by drying the samples in a forced-air oven until reaching a constant weight. The water soluble carbohydrate concentrations were determined with the methods of Owens et al. [11]. Total nitrogen contents were measured based on the method 978.04 of AOAC [12]. Crude protein (CP) contents were analyzed by multiplying total nitrogen value by 6.25. The acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were analyzed according to the description of Soest et al. [13].

Enumeration of Enterobacteriaceae, lactic acid bacteria (LAB), yeasts, and aerobic bacteria was determined by the method of Wang et al. [14]. Briefly, for enumeration of the microorganisms, 10 g sample (fresh material or silage) was shaken well with 90 mL of sterilized saline solution at 120 rpm for 2 h. Then, 1 mL solution was used for tenfold serial dilution for microorganism counting, and then the remaining solution was filtered through 4 layers of medical gauze and stored in the − 80 °C refrigerator for DNA extraction. The colonies of lactic acid bacteria (LAB) were counted on MRS agar medium after incubation in an anaerobic incubator

(N_2:H_2:CO_2 = 85:5:10, YQX-II; CIMO Medical Instrument Manufacturing Co., Ltd., Shanghai, China) at 37 °C for 3 days. Aerobic bacteria were cultured and counted on nutrient agar medium (Nissuiseiyaku Ltd., Tokyo, Japan). Yeasts were counted on potato dextrose agar (Nissui-seiyaku Ltd., Tokyo, Japan) and acidified with sterilized tartaric acid solution to pH 3.5. These agar plates were incubated at 37 °C for 3 days. Enterobacteriaceae was counted on the Violet Red Bile Glucose Agar medium after 24 h of incubation at 37 °C under aerobic conditions. The microbial data were obtained as colony-forming units (cfu) and were transformed to a logarithmic scale on a fresh weight basis.

High throughput sequencing analyses

The DNA extraction kit (MP Biomedicals, Santa Ana, CA, USA) was used to extract the total genomic DNAs of bacteria in samples. The quantity and quality of extracted DNA were checked by 1% agarose gel electrophoresis and spectrophotometry (optical density at 260/280 nm ratio). The PCR amplification, purification and quantification were conducted based on the reports of Cao et al. [15]. The amplicons were paired-end sequenced on the Illumina MiSeq PE300 platform.

The alpha diversity analysis (Chao1, Simpson, Sobs, Coverage, Shannon and Ace indexes) was analyzed by QIIME software (Version 1.9.1). After comparing the Silva 138 database, the bacterial compositions in fresh alfalfa and silage samples were determined on Phylum and Genus levels. The principal coordinate analysis (PCoA), analysis of similarities (ANOSIM), redundancy analysis (RDA), Spearman's correlation heatmap were graphically constructed by R software (Version 4.2.0). The co-occurrence network plots were performed by NetworkX (Version 2.8) according to Spearman's correlation matrices based on the description of Qiu et al. [16]. The predicted functional characteristics of bacterial community were performed from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database via Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2; Version 2.2.0), which predicts the function of bacterial community according to the proportion of marker gene sequences in samples [6]. The sample heatmap analysis on the abundance of Pathway level 3, associated enzymes and modules were graphically constructed by R program (Version 4.2.0). Phenotypic prediction of bacterial community in samples was performed using BugBase (https://bugbase.cs.umn.edu/index.html). The abundances of Pathway level 1 and 2, associated enzymes, modules, and phenotype in bacterial community were presented using Graphpad Prism (Version 9). The raw sequencing data have been deposited in NCBI's Sequence Read Archive (SRA) under the accession number PRJNA837105.

Statistical analysis

Data on fermentation characteristics and chemical compositions were subjected to two-way analysis of variance (ANOVA) and analyzed by SPSS software according to a 2×6 factorial design with two growth stages and six storage timepoints:

$$Y_{ij} = \mu + G_i + T_j + (G \times T)_{ij} + e_{ij}$$

where Y_{ij} represents the dependent variable; μ is overall mean; G_i is the effect of growth stage of alfalfa; T_j is the effect of storage time; $(G \times T)_{ij}$ is the effect of interaction between growth stage of alfalfa and storage time; and e_{ij} is the residual error term. Statistical difference was determined by Tukey's multiple comparison.

Differences of chemical compositions and microbial populations of fresh alfalfa at two growth stages, and fermentation characteristics between the full flowering and initial flowering stages were analyzed using the non-parametric Kruskal–Wallis test. Data on proportions of bacterial compositions on Genus level (the 10 most abundant) and predicted functional characteristics (including enzyme and module) were analyzed by student's t test. The network topological properties of Degree, Degree Centrality, Closeness Centrality and Betweenness Centrality were analyzed by one-way analysis of variance (ANOVA). The relative abundance of phenotype in bacterial community was analyzed by Kruskal–Wallis H test. Values of $P < 0.05$ were declared significant.

Results

Chemical compositions and microbial populations in fresh alfalfa at initial and full flowering stages

The chemical compositions and microbial populations of fresh alfalfa (AL) at initial (ALFM1) and full flowering (ALFM2) stages are described in Table 1. The contents of dry matter, water soluble carbohydrate, neutral detergent fiber and acid detergent fiber, and populations of lactic acid bacteria, yeasts, Enterobacteriaceae and aerobic bacteria increased ($P < 0.05$), and buffering capacity declined ($P < 0.05$) with advancing maturity of fresh alfalfa.

Fermentation characteristics of alfalfa silages at initial and full flowering stages

Fermentation characteristics of alfalfa silages at initial flowering (AL1) and full flowering (AL2) stages are presented in Table 2. The pH, acetic acid (AA), lactic acid

Table 1 Chemical compositions (g/kg of DM, unless otherwise stated) and microbial populations (fresh matter basis) of fresh alfalfa at two growth stages

Items	Growth stages		SEM	*P* value
	Initial flowering	Full flowering		
Chemical compositions (g/kg DM)				
pH	5.81	5.59	0.054	0.165
Dry matter (g/kg FW)	219^{b}	238^{a}	2.121	< 0.001
Water soluble carbohydrates	47.1^{b}	56.4^{a}	1.425	< 0.001
Buffering capacity (mEq/kg DM)	208^{a}	131^{b}	1.136	< 0.001
Crude protein	187	190	2.579	0.086
Neutral detergent fiber	314^{b}	337^{a}	2.336	0.046
Acid detergent fiber	284^{b}	296^{a}	2.843	0.032
Microbial populations (Log_{10} cfu/g FW)				
Lactic acid bacteria	3.76^{b}	4.33^{a}	0.143	0.026
Aerobic bacteria	6.76^{b}	7.39^{a}	0.108	0.038
Yeasts	3.02^{b}	3.89^{a}	0.112	0.015
Enterobacteriaceae	6.18^{b}	6.96^{a}	0.130	0.036

Means with different letters in the same row ($^{a-b}$) differ ($P < 0.05$)

DM dry matter, *FW* fresh weight, *mEq* milligram equivalent, *cfu* colony-forming units, *SEM* standard error of means

Table 2 Fermentation characteristics (g/kg of DM, unless otherwise stated) in alfalfa silages at two growth stages

Items	Growth stages	Storage time (day)						SEM	*P* value		
		1	3	7	15	30	60		G	T	G × T
pH	Initial flowering	6.06^{Aa}	5.95^{Aab}	5.82^{Abc}	5.62^{Ac}	5.28^{Ad}	5.75^{Abc}	0.004	< 0.001	< 0.001	< 0.001
	Full flowering	5.26^{Ba}	5.28^{Ba}	5.07^{Bb}	4.81^{Bc}	4.79^{Bc}	4.70^{Bd}				
Lactic acid	Initial flowering	18.2^{Bd}	27.1^{Bbc}	28.1^{Bbc}	32.7^{Bab}	34.5^{Ba}	23.7^{Bcd}	2.864	< 0.001	< 0.001	< 0.001
	Full flowering	23.7^{Ad}	36.8^{Ac}	47.0^{Aab}	50.1^{Aab}	46.4^{Ab}	52.6^{Aa}				
Acetic acid	Initial flowering	15.3^{Ac}	22.0^{Ab}	21.7^{Ab}	23.8^{b}	25.1^{b}	31.4^{Aa}	2.931	< 0.001	< 0.001	0.643
	Full flowering	11.1^{Bd}	15.6^{Bcd}	17.8^{Bbcd}	20.6^{abc}	21.4^{ab}	26.1^{Ba}				
Lactic acid/acetic acid	Initial flowering	1.19^{Ba}	1.24^{Ba}	1.29^{Ba}	1.38^{Ba}	1.37^{Ba}	0.75^{Bb}	0.093	< 0.001	< 0.001	0.004
	Full flowering	2.14^{A}	2.35^{A}	2.66^{A}	2.44^{A}	2.17^{A}	2.02^{A}				
Propionic acid	Initial flowering	5.33^{c}	5.38^{c}	5.45^{Bc}	5.44^{Bc}	6.57^{Ab}	12.0^{Aa}	0.014	< 0.001	< 0.001	< 0.001
	Full flowering	5.56^{b}	5.59^{b}	6.41^{Aa}	6.53^{Aa}	6.19^{Bab}	6.30^{Bab}				
Butyric acid	Initial flowering	5.54^{c}	5.58^{Bc}	5.66^{Bc}	5.67^{Bc}	6.32^{Ab}	7.24^{Aa}	0.031	< 0.001	< 0.001	< 0.001
	Full flowering	5.76^{c}	6.14^{Aab}	6.30^{Aa}	6.42^{Aa}	5.63^{Bc}	5.90^{Bbc}				
Ethanol	Initial flowering	15.8^{c}	18.5^{Abc}	18.7^{Ab}	20.5^{Aab}	21.1^{Aab}	21.8^{Aa}	0.734	< 0.001	< 0.001	< 0.001
	Full flowering	15.0^{b}	16.1^{Bab}	17.0^{Ba}	17.5^{Ba}	15.1^{Bb}	15.9^{Bab}				
Dry matter (g/kg FW)	Initial flowering	219^{Ba}	217^{Bab}	217^{Babc}	216^{Babc}	215^{Bbc}	213^{Bc}	2.902	< 0.001	< 0.001	< 0.001
	Full flowering	238^{Aa}	235^{Aa}	230^{Ab}	226^{Ac}	223^{Acd}	219^{Ad}				
Ammonia nitrogen (g/kg TN)	Initial flowering	47.7^{Ae}	92.2^{Ad}	100^{Ad}	123^{Ac}	157^{Ab}	183^{Aa}	3.078	< 0.001	< 0.001	< 0.001
	Full flowering	20.1^{Bf}	49.5^{Be}	71.8^{Bd}	105^{Bc}	122^{Bb}	143^{Ba}				
Water soluble carbohydrates	Initial flowering	43.6^{Ba}	33.0^{Bb}	24.5^{Bc}	16.5^{Bd}	11.7^{Be}	6.24^{f}	1.602	< 0.001	< 0.001	< 0.001
	Full flowering	47.7^{Aa}	41.0^{Ab}	32.0^{Ac}	20.0^{Ad}	14.1^{Ae}	6.64^{f}				

G: effect of growth stages of alfalfa; T: effect of storage time; G × T: the interaction between growth stages and storage time. Means with different letters in the same row ($^{a-f}$) or column ($^{A-B}$) differ ($P < 0.05$)

DM dry matter, *FW* fresh weight, *TN* total nitrogen, *SEM* standard error of means

(LA), butyric acid (BA), propionic acid (PA), ethanol, ammonia nitrogen (NH_3–N), dry matter (DM) and water soluble carbohydrate (WSC) concentrations and ratios of lactic to acetic acid (LA/AA) were significantly ($P<0.001$) affected by the effect of growth stage and storage time. The interaction between growth stage and storage time significantly ($P<0.01$) affected pH, LA, PA, BA, ethanol, DM, NH_3–N, WSC and ratios of LA/AA.

The pH in AL1 group were slowly decreased from days 1 to 30 while increased ($P<0.05$) on day 60. In contrast, the pH in AL2 group decreased rapidly during ensiling. The LA concentrations in AL1 group were slowly accumulated from days 1 to 30 while declined ($P<0.05$) on day 60. The LA contents in AL2 group were rapidly produced during ensiling. AL2 group always had higher ($P<0.05$) LA concentrations and lower ($P<0.05$) pH than AL1 group on the same opening day. Higher ($P<0.05$) concentrations of PA, BA and ethanol were observed in AL1 group compared to AL2 group on day 60. The NH_3–N contents were gradually accumulated in AL1 and AL2 groups during ensiling, and AL1 had higher ($P<0.05$) NH_3–N contents than AL2 group on the same ensiling day.

Bacterial community diversity, compositions and successions in fresh alfalfa and silages at initial and full flowering stages

The alpha diversity of bacterial community in fresh alfalfa and silages at two growth stages are shown in Fig. 1A. ALFM1 group had higher ($P<0.05$) Shannon and Coverage values, and lower ($P<0.05$) Chao1, Ace, Sobs and Simpson values than ALFM2 group. On day 60, AL1 group had higher ($P<0.05$) Shannon values, and lower ($P<0.05$) Simpson values than AL2 group.

The bacterial communities on Phylum level in fresh alfalfa and silages at two growth stages are described in Fig. 1B. On day 3, the abundance of Proteobacteria was increased from 16.5% to 32.3%, and the abundance of Firmicutes was decreased from 83.4% to 67.4% with growth stage. On day 60, the abundance of Proteobacteria was decreased from 32.6% to 2.82%, and the abundance of Firmicutes was increased from 64.2% to 97.0% with growth stage.

The bacterial communities on Genus level in fresh alfalfa and silages at two growth stages are presented in Fig. 1C. After 3 days, the abundances of *Weissella* were increased from 21.7% to 27.7% and the abundance of *Lactobacillus* was increased from 8.45% to 23.6% with growth stage. On day 60, the abundance of *Enterobacter* was decreased from 20.2% to 0.70% and the abundance of Enterobacteriaceae was decreased from 7.83% to 0.08% with growth stage.

As shown in Fig. 1D, E, F, the bacterial compositions on Genus level in fresh alfalfa and silages at two growth stages were compared. The abundances of *Weissella* and *Lactobacillus* in AL1 group were significantly ($P<0.05$) lower than that in AL2 group after 3 days. The abundances of *Enterobacter* and *Enterobacteriaceae* were significantly ($P<0.05$) higher than that in AL2 group after 60 days.

The correlation between fermentation products and bacterial community in alfalfa silages at initial and full flowering stages

As presented in Fig. 2A, the variance of bacterial communities in fresh alfalfa and silages at two growth stages was described by PCoA analysis ($R=1.000$, $P=0.001$). The Component 2 and 1 explained 32% and 34.93%, respectively. The bacterial communities in ALFM1 were separated from that in ALFM2, while they were divided into different quadrants.

As shown in Fig. 2B, analysis of similarities (ANOSIM) analysis exhibited greater differences in bacterial communities on Genus level among groups than within group.

Results of redundancy analysis (RDA) are presented in Fig. 2C. *Lactobacillus* are positively related to lactic acid and ratios of LA/AA. *Lactococcus* are positively correlated with WSC and pH.

Spearman correlations between bacterial compositions and fermentation characteristics (the 20 most abundant) in alfalfa silages at two growth stages (AL1, AL2) on day 3 (AL1–3. AL2–3) and 60 (AL1–60, AL2–60) are described in Fig. 2D, E, F, G. In AL1-3 group, a positive relationship ($P<0.001$) was found between acetic acid and *Weissella*. In AL1-60 group, Enterobacteriaceae was positively ($P<0.001$) related to ethanol.

Bacterial co-occurrence, co-occurrence network complexity, and stability in fresh alfalfa and silages at initial and full flowering stages

As shown in Fig. 3A–F, the networks of bacterial community in fresh alfalfa and silages at two growth stages after 3 and 60 days of ensiling exhibited different co-occurrence patterns. Longer storage time decreased the bacterial network complexity in alfalfa silages, indicated by simpler bacterial community structure in AL1-60 and AL2-60 groups.

As shown in Fig. 3G, H, the network topological properties of Degree, Degree Centrality, Closeness Centrality and Betweenness Centrality were used to evaluate the bacterial network complexity. The Degree of a node represents the number of nodes directly connected to this node in the network. Higher abundances

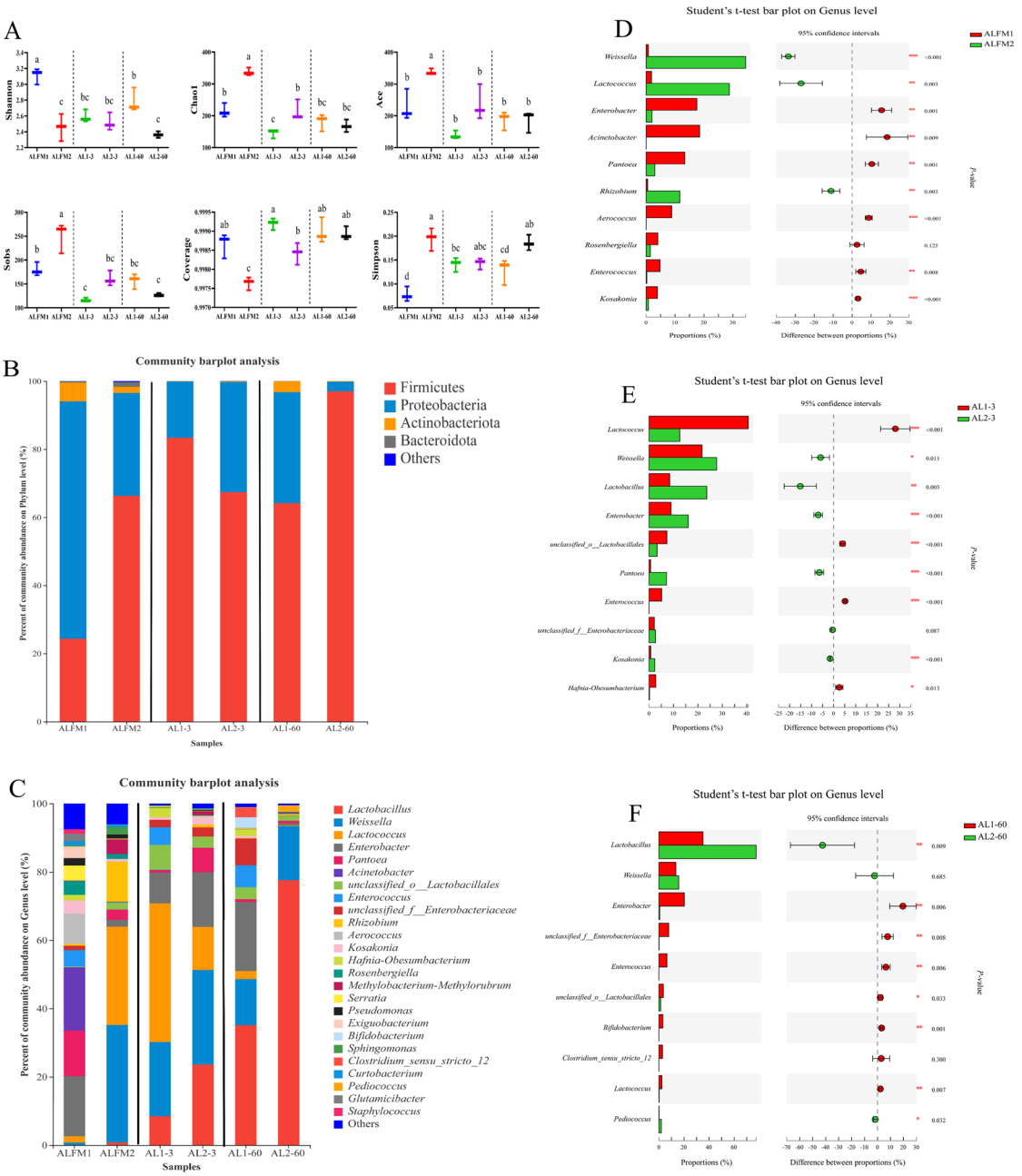


Fig. 1 Bacterial community diversity, compositions and successions in fresh alfalfa and silages at two growth stages. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60

of Degree and Degree Centrality indicate higher importance of the node in the entire network. The higher abundance of Closeness Centrality is, the closer the node is to the center of the network. The Betweenness Centrality was used to evaluate the node's role in maintaining tight connectivity throughout the network. The results exhibited that ensiling time possessed a more remarkable influence on bacterial communities than growth stage. Irrespective of which growth stage the alfalfa was, ensiling obviously decreased the complexity of the bacterial community network. AL2 had similar bacterial community stability (Degree, Degree Centrality, Closeness Centrality, Betweenness Centrality) with AL1 after 3 and 60 days.

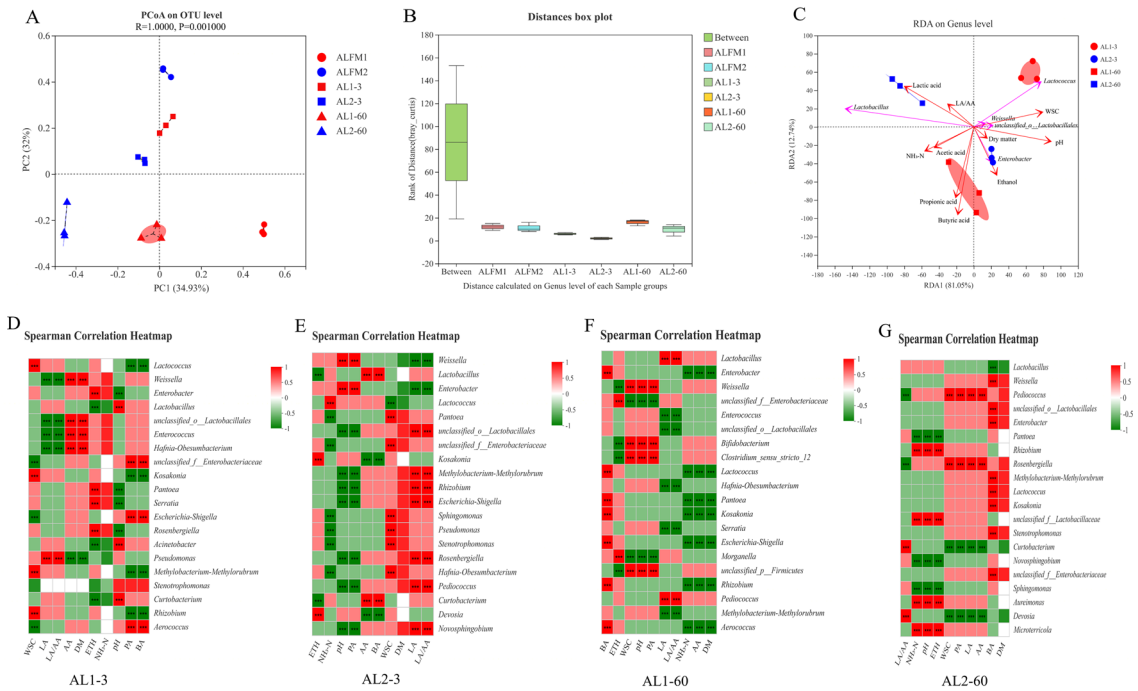


Fig. 2 Correlation between fermentation products and bacterial community in fresh alfalfa and silages at two growth stages. ETH, ethanol; NH_3–N, ammonia nitrogen; *PA* propionic acid, *AA* acetic acid, *BA* butyric acid, *WSC* water soluble carbohydrate, *DM* dry matter, *LA* lactic acid, *LA/AA* ratio of lactic acid to acetic acid. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$

Predicted pathways of bacterial communities in fresh alfalfa and silages at initial and full flowering stages

Figure 4 shows the differences of predicted metabolic pathway of bacterial communities on different levels. On the first pathway level (Fig. 4A), AL1 had higher ($P < 0.05$) proportions of cellular processes, genetic information processing, environmental information processing, organismal systems, and metabolism than AL2 on day 0. AL1 had higher ($P < 0.01$) proportions of metabolism and genetic information processing than AL2 on day 3. A higher ($P < 0.01$) abundance of cellular processes was observed in AL1 than AL2 on day 60.

On the second pathway level (Fig. 4B–F), higher proportions of amino acid and carbohydrate metabolism were found in Metabolism pathway. In genetic information processing pathway, relatively higher abundances of folding, sorting and degradation, translation, and replication and repair were observed in AL2-60 group. In cellular processes and organismal systems pathways, ensiling obviously decreased the abundances of cell motility, cellular community-prokaryotes, and environmental adaptation.

On the third pathway level (Fig. 4G), the heat-map presents the functional potential of bacterial community in fresh alfalfa and silages at two growth stages during ensiling. The metabolic pathways were always up-regulated in all samples, whereas the biosynthesis of amino acids, ABC transporters, carbon metabolism, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, and two-component system were gradually down-regulated during ensiling regardless of growth stage.

Predicted enzymes of bacterial community in fresh alfalfa and silages at initial and full flowering stages

The activity of some predicted enzymes of bacterial community in fresh alfalfa and silages at two growth stages are described in Fig. 5. As shown in Fig. 5a–i, AL1 had higher ($P < 0.01$) proportions of 1-phosphofructokinase, glucose-6-phosphate dehydrogenase, L-lactate dehydrogenase and fructokinase, and lower ($P < 0.05$) proportions of nitrite reductase (NADH), D-lactate dehydrogenase and ornithine decarboxylase compared to AL2 on day 3. On day 60, AL1 had higher ($P < 0.05$) proportions of L-ribulose-5-phosphate 3-epimerase,

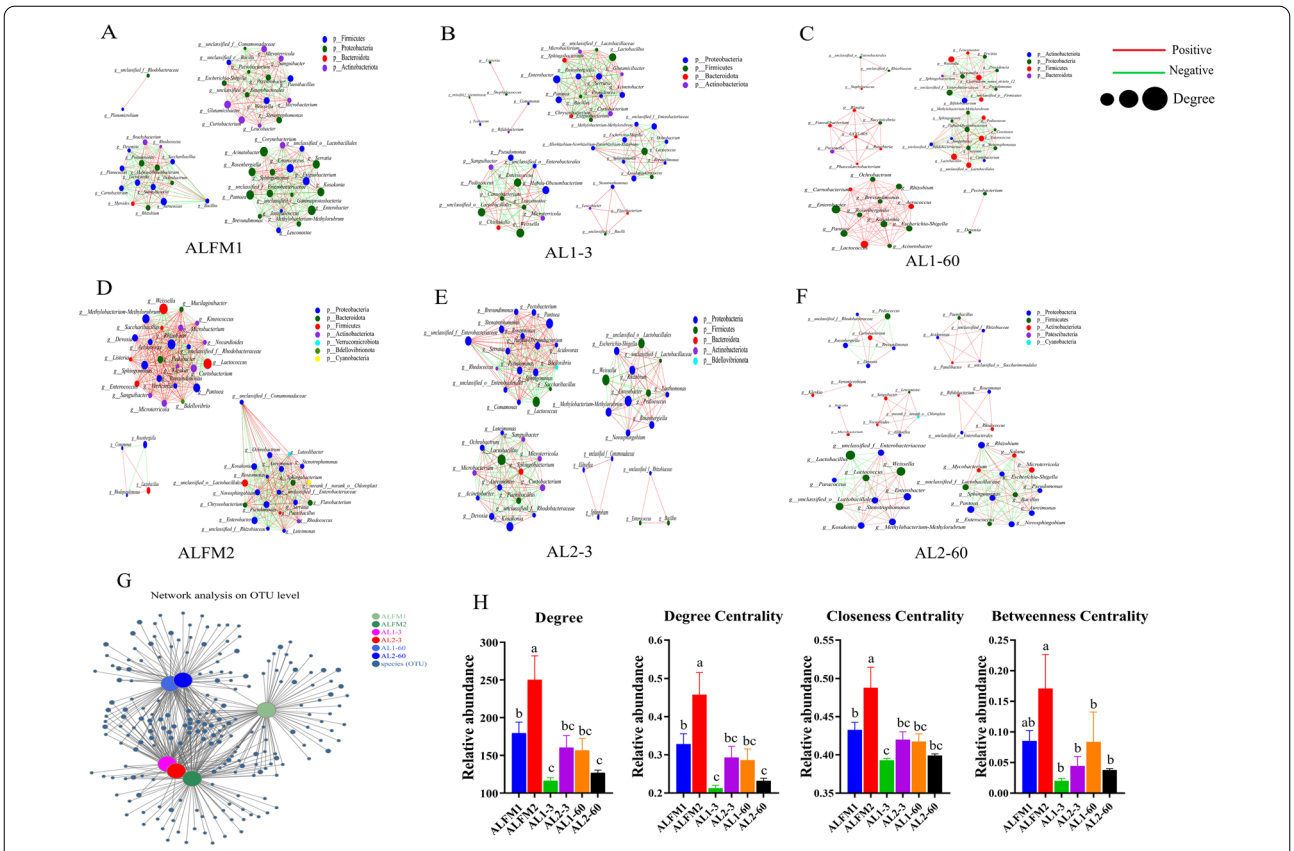


Fig. 3 Bacterial co-occurrence, co-occurrence network complexity, and stability in fresh alfalfa and silages at two growth stages (Spearman correlation, the most abundant 50 species, *P* value < 0.05, correlation > 0.5). ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60

glucose-6-phosphate dehydrogenase, nitrite reductase (NADH) and ornithine decarboxylase, and lower ($P < 0.05$) proportions of fructokinase, pyruvate kinase, 1-phosphofructokinase, L- and D-lactate dehydrogenase compared to AL2.

As shown in Fig. 5j, the enzyme abundance heat-map exhibits the enzyme activity of bacterial communities in fresh alfalfa and silages at two growth stages during ensiling. The protein–N(pi)–phosphohistidine–sugar phosphotransferase, histidine kinase, DNA-directed DNA polymerase and DNA helicase were always up-regulated in all samples, whereas the NADH: ubiquinone reductase, Glutathione transferase, Iron–chelate-transporting ATPase and peptidylprolyl isomerase were gradually down-regulated during ensiling regardless of growth stage.

Predicted modules of bacterial community in fresh alfalfa and silages at initial and full flowering stages

The predicted modules of bacterial communities in fresh alfalfa and silages at two growth stages are shown in Fig. 6. As shown in Fig. 6a–c, AL1 group had higher ($P < 0.01$) abundances of pentose phosphate pathway (pentose phosphate cycle) and glycolysis (Embden–Meyerhof pathway), glucose = > pyruvate than AL2 group on day 3. On day 60, AL1 had higher ($P < 0.05$) proportions of citrate cycle (TCA cycle, Krebs cycle) than AL2.

As described in Fig. 6d, the module abundance heat-map exhibits the module activity of bacterial community in fresh alfalfa and silages at two growth stages during ensiling. The module of NADH: quinone oxidoreductase, prokaryotes was obviously down-regulated in AL2-60 group compared to AL1-60 group.

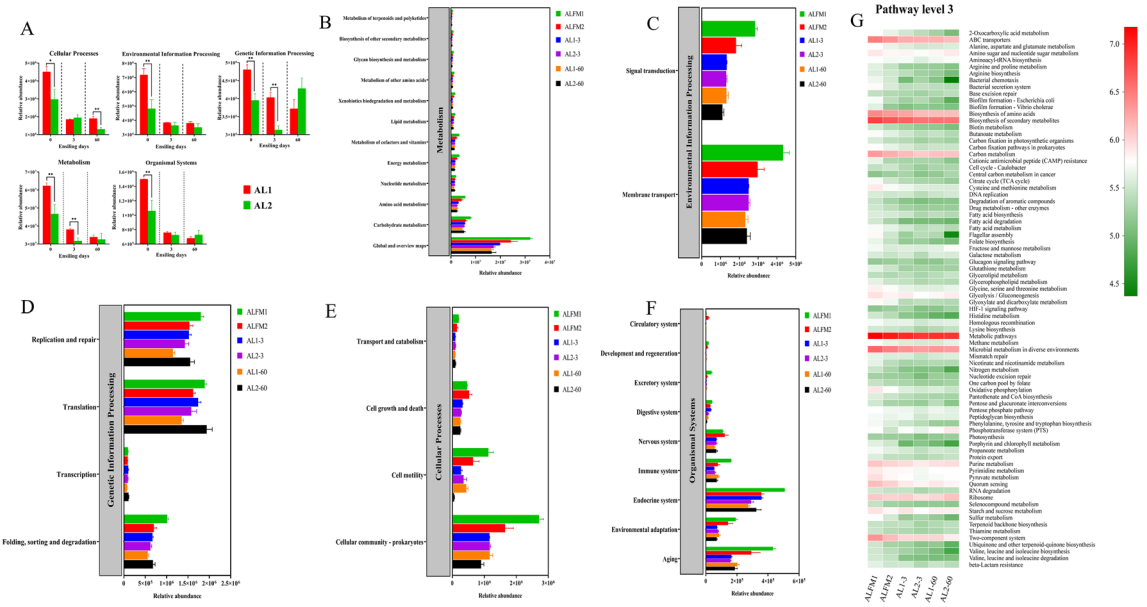


Fig. 4 Predicted pathways of bacterial community in fresh alfalfa and silages at two growth stages. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$

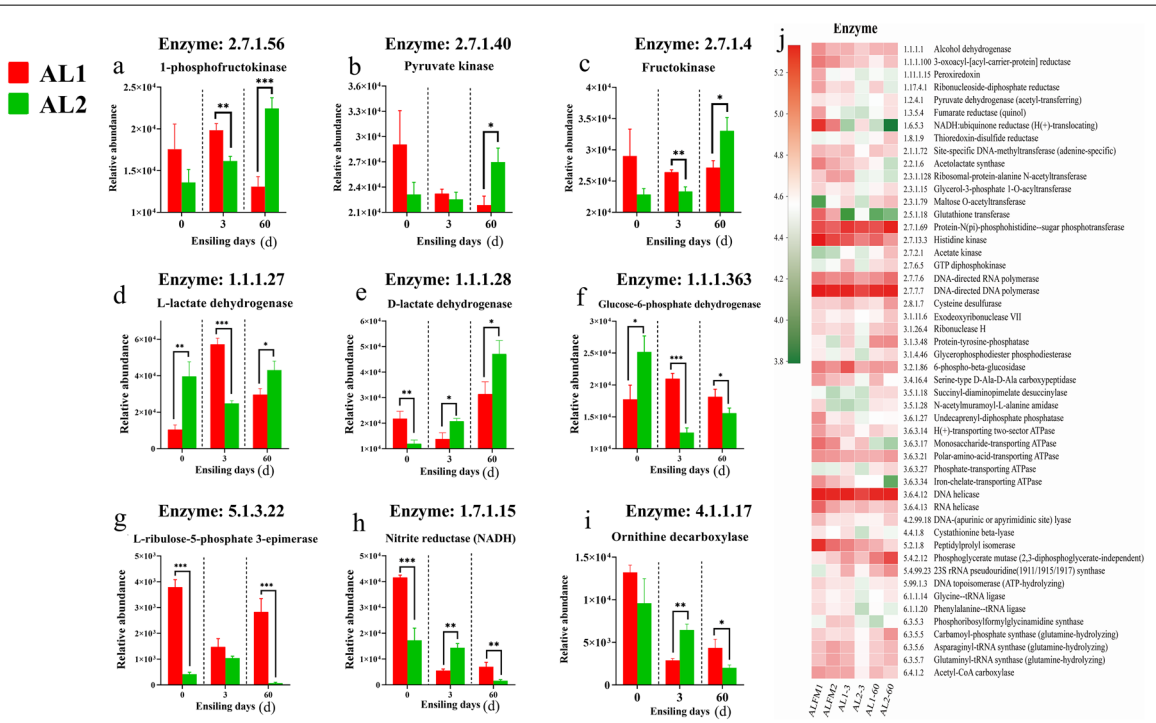


Fig. 5 Predicted enzymes of bacterial community in fresh alfalfa and silages at two growth stages. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$

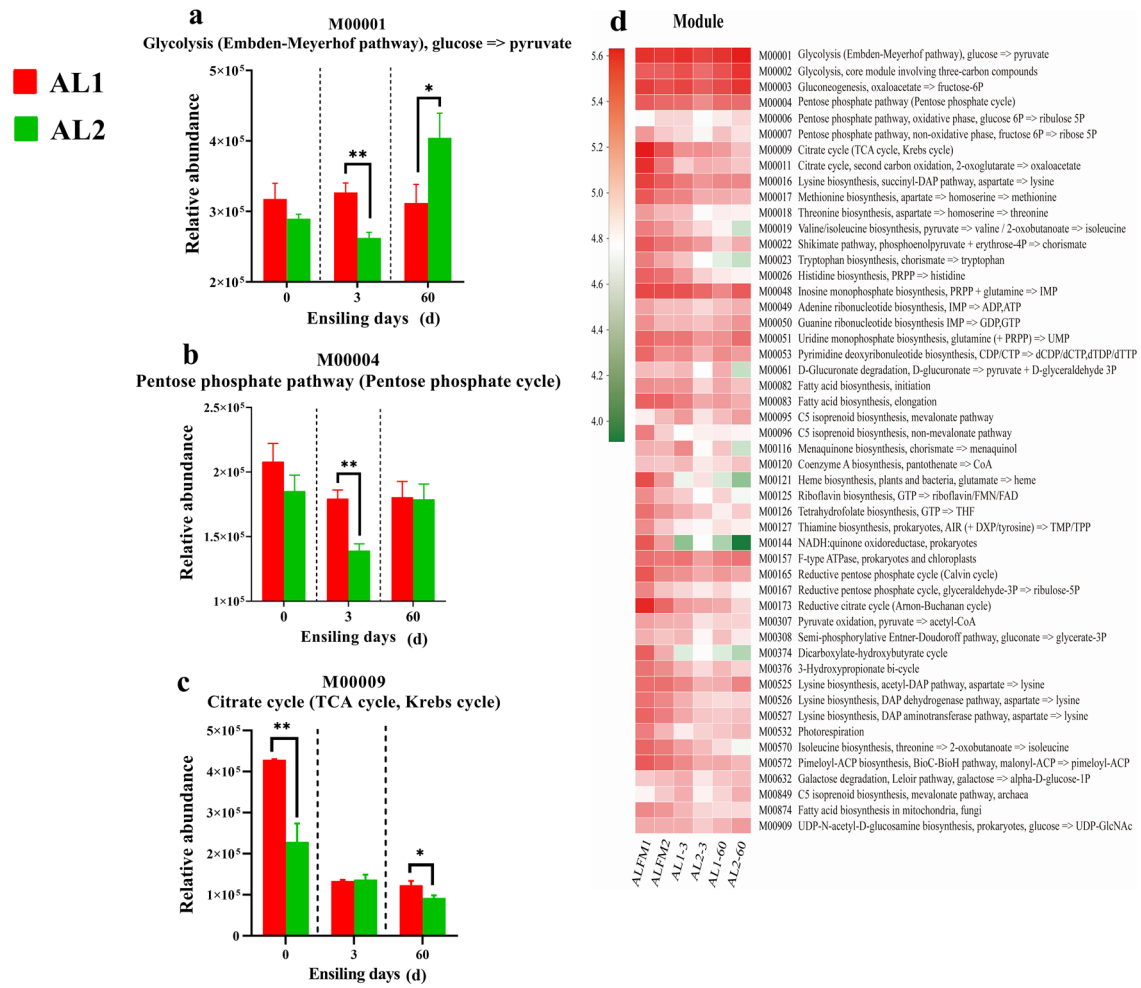


Fig. 6 Predicted modules of bacterial community in fresh alfalfa and silages at two growth stages. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$

Predicted phenotype of bacterial community in fresh alfalfa and silages at initial and full flowering stages

The predicted phenotypes of bacterial communities in fresh alfalfa and silages at two growth stages are presented in Fig. 7. AL1 group had relatively lower proportions of facultatively anaerobic and aerobic than AL2 group on days 3 and 60. Higher proportions of potentially pathogenic and gram negative were observed in AL1-60 group than AL2-60 group. AL1 group had relatively lower proportions of gram positive than AL2 group on day 60.

Discussion

Chemical compositions and microbial populations in fresh alfalfa at initial and full flowering stages

It is well-known that the chemical compositions of forage are strongly influenced by the growth stage. This study described that the dry matter, water soluble carbohydrate, neutral detergent fiber, and acid detergent fiber contents increased as alfalfa matured. This may be because the ratio of leaf to stem declines with plant elongation and cell wall contents in stems increase with the maturity of alfalfa [2]. In addition, the buffering capacity in alfalfa decreased with advancing maturity. McDonald and Henderson [17] reported that the buffering capacity of plants is mainly ascribed to the potassium, calcium and magnesium salts of organic acids. It is possible that the decline in buffering capacity may be linked with a reduction in the organic acid fraction with the maturity of alfalfa. Similarly, Greenhill [18] found that the least mature harvest of ryegrass, clover and Lucerne was more highly buffered than the advanced maturity, which could be due to the decrease in organic acids with maturity.

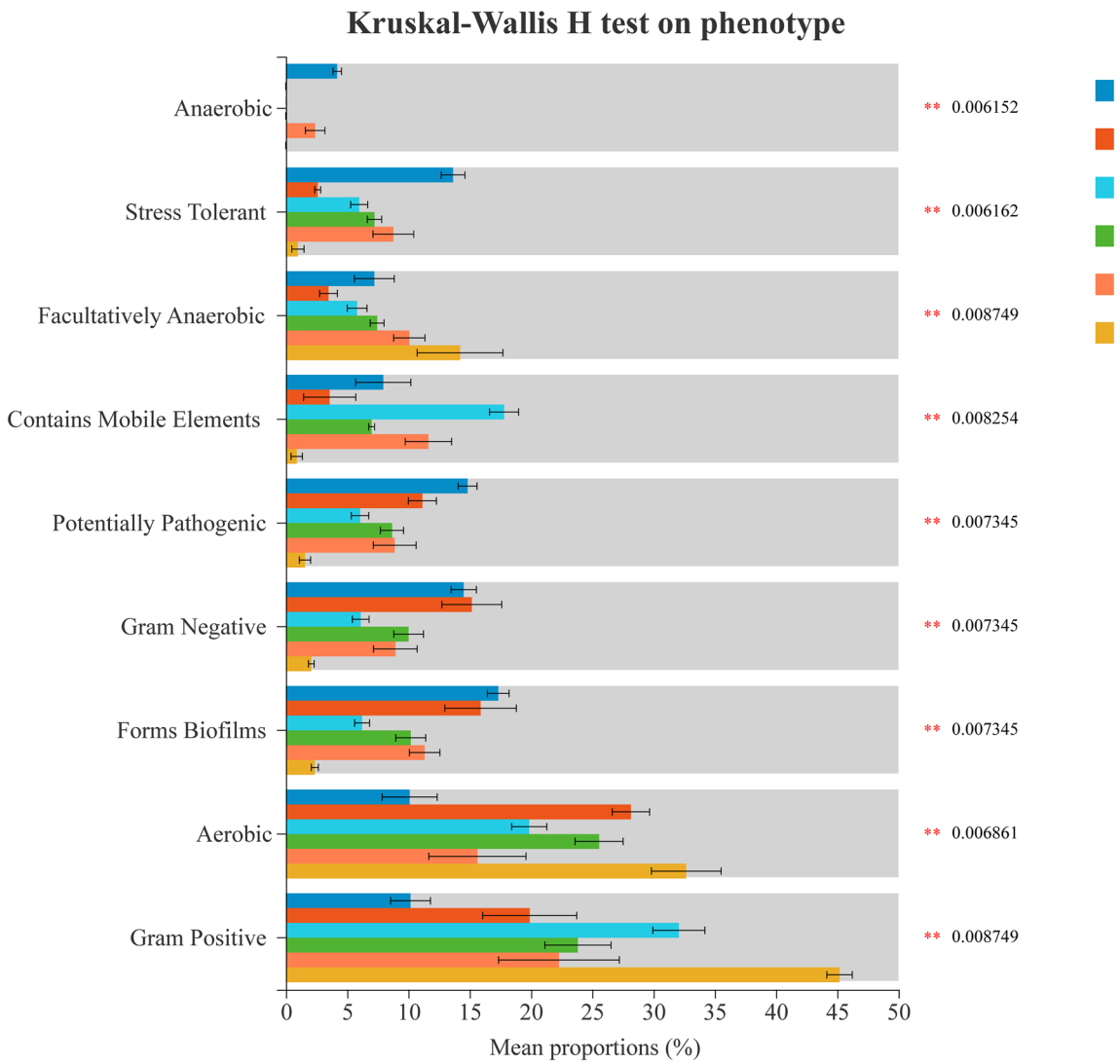


Fig. 7 Predicted phenotype of bacterial community in fresh alfalfa and silages at two growth stages. ALFM1, fresh alfalfa harvested at initial flowering stage; ALFM2, fresh alfalfa harvested at full flowering stage; AL1-3, alfalfa silage at initial flowering stage on day 3; AL2-3, alfalfa silage at full flowering stage on day 3; AL1-60, alfalfa silage at initial flowering stage on day 60; AL2-60, alfalfa silage at full flowering stage on day 60. **, $0.001 < P < 0.01$

The growth stage of plant can obviously influence the microbial diversity and populations [19]. In this study, the epiphytic lactic acid bacteria, yeast, Enterobacteriaceae and aerobic bacteria populations increased with the maturity of alfalfa. There are two possible explanations for this phenomenon. One is related to the accumulated water soluble carbohydrate contents as alfalfa matured. Lindow and Brandl [20] found that the availability of carbon-containing nutrients on leaves is critical in determining the epiphytic colonization, and bacterial community on forages was first limited by carbon availability and secondarily by nitrogen availability. The other reason is probably due to the increased matured tissues. Thompson et al. [21] reported that some nutrients released from matured tissues are important for microbial growth and microbial community diversity, which may be the reason for the differences in epiphytic microbiota at different growth stages.

Fermentation characteristics in alfalfa silages at initial and full flowering stages

The fermentation quality of silage is highly dependent on the growth stage of forage at harvesting [22]. Present study exhibited that AL2 group had better fermentation quality than AL1 group with lower pH, NH_3–N, ethanol and butyric acid concentrations, and higher lactic acid concentrations. This could be mainly attributed to

the lower buffering capacity and moisture contents and higher water soluble carbohydrate contents in ALFM2 group compared with ALFM1 group. Guo et al. [2] concluded that suitable dry matter contents, sufficient water soluble carbohydrate contents and lactic acid bacteria populations, low buffering capacity and undesirable microbial populations are critical for successful ensiling.

Lactic acid is a metabolite produced by lactic acid bacteria during fermentation. The higher lactic acid contents in AL2 group than AL1 group were probably correlated with the higher water soluble carbohydrate contents and lactic acid bacteria populations in ALFM2 group, stimulating the lactic acid fermentation. Furthermore, the rate of pH decline is mainly determined by the organic acids produced during ensiling and buffering capacity of raw material. Hence, the lower pH in AL2 group than AL1 group during fermentation could be due to the rapid accumulation of lactic acid and lower buffering capacity in ALFM2 group, making it easier to change in pH.

In conserved silages, butyric acid and ethanol are both undesirable. Butyric acid was mainly produced by undesirable microorganisms which break down amino acid resulting in nutrient loss. The lower butyric acid contents in AL2 group may be correlated with the rapid drop of pH in AL2 group, inhibiting the propagation and metabolism of harmful microorganisms. Kung et al. [23] reported that the ethanol contents (> 30–40 g/kg DM) may be associated with the metabolism of yeast. Herein, the ethanol concentrations in AL1 and AL2 groups were both less than 25 g/kg DM, indicating the ethanol contents in alfalfa silages were mainly produced by other microbes, such as hetero-fermentative lactic acid bacteria.

The NH_3–N content is a critical indicator for assessing the silage fermentation quality, reflecting the crude protein degradation through proteolysis by clostridium fermentation and plant protease activity [24]. In the current study, the relatively higher NH_3–N contents (> 140 g/kg TN) in AL1 and AL2 groups on day 60 could be ascribed to the insufficient acidic conditions at the initial fermentation stage. However, the AL2 group with an NH_3–N content of 143 g/kg TN; less than 150 g/kg TN is reported by Mahanna and Chase [25] to be acceptable for well-preserved legume silage.

Bacterial community diversity, compositions and successions in fresh alfalfa and silages at initial and full flowering stages

Alpha diversity, including Shannon, Chao1, Ace, Sobs, Coverage and Simpson indices, describes the coverage, diversity, richness and evenness of entire bacterial community in samples. Herein, higher Shannon values and lower Simpson values in ALFM1 group than ALFM2 group indicated that higher bacterial diversity existed in ALFM1 group. In addition to the inherent changes in alfalfa with growth stage, Wang et al. [14] reported that the environmental factors, such as temperature, humidity and solar radiation, can also influence the epiphytic bacterial community of forage. After 60 days, higher Shannon values and lower Simpson values in AL1 group indirectly proved that AL2 group had better fermentation quality than AL1 group. Muraro et al. [26] concluded that a successful fermentation with a decline in diversity indexes, from raw material to final silage by establishing a parallel with changes occurring within a silo, where the complex bacterial community of fresh material is gradually replaced by lactic acid bacteria strains in silage when anaerobic condition is reached and pH decreases.

Noteworthy, the decreased abundance of Firmicutes and increased abundance of Proteobacteria with growth stage after 3 days seemed to imply that anaerobic condition was more difficult to realize in matured alfalfa silage. Conversely, lower pH and higher lactic acid concentrations were found in AL2 group compared to AL1 group on day 3. This is probably correlated with the species, interaction, activity and metabolic characteristics of epiphytic lactic acid bacteria strains in alfalfa at different growth stages. After 60 days, the higher abundances of Firmicutes and lower abundances of Proteobacteria in AL2 group than AL1 group may be because AL2 group had a more acidic environment than AL1 group, benefiting the growth and reproduction of Firmicutes and suppressing the multiplication of Proteobacteria [27].

Higher proportions of *Weissella* and *Lactobacillus* in AL2 group than AL1 group on day 3 could partly explain the result that AL2 group had lower pH and higher lactic acid concentrations after 3 days. *Weissella* are recognized as the initial colonizer microbes during fermentation and they could convert water soluble carbohydrates to lactic and acetic acids [28]. Regarding *Lactobacillus*, they are often applied to occupy the predominant position at the beginning of fermentation, and guarantee good silage quality due to their capacity of producing lactic acid via consuming glucose [1].

After 60 days, higher abundances of *Enterobacter* and Enterobacteriaceae in AL1 group than AL2 group may result in higher NH_3–N contents in AL1 group. Enterobacteriaceae and *Enterobacter* were both reported for their proteolytic activity, which can lead to inefficient lactic acid fermentation, higher NH_3–N contents and pH [29, 30]. Herein, *Enterobacter* was the subdominant bacteria in AL1 group on day 60, likely responsible for the large amounts of NH_3–N produced during fermentation [1]. Santos et al. [31] described most *Enterobacter* frequently found in silage are nonpathogenic. Whereas, their development is unacceptable, because they are in competition with LAB for water soluble carbohydrates

during fermentation. Silva et al. [32] also reported *Enterobacter* are detrimental to silage quality, because they can produce NH_3–N and slow down the drop in pH during fermentation.

Correlation between fermentation products and bacterial community in alfalfa silages at initial and full flowering stages

The ALFM1 group was separated from ALFM2 group in PCoA plot, indicating that growth stage had great effects on the epiphytic bacterial community of forages. ANOSIM analysis presented greater discrepancies in bacterial communities between groups than within group. The positive correlation between *Lactobacillus* and lactic acid in RDA plot demonstrated that *Lactobacillus* play an important role in accumulating lactic acid and decreasing pH throughout the fermentation process [1].

In Spearman Correlation Heatmap plots, the positive correlation between the abundance of *Weissella* and acetic acid contents in AL1-3 group was in accordance with the fact that most *Weissella* species are obligate heterofermentative bacteria, generating acetic and lactic acids via converting WSC [28]. The positive correlation between the abundance of Enterobacteriaceae and ethanol contents in AL1-60 group was consistent with the results of Sun et al. [33], who reported that Enterobacteriaceae could thrive in weak acidic and anaerobic conditions, fermenting water soluble carbohydrates and lactic acid to ethanol, succinic acids, acetic acid, or 2,3-butanediol.

Bacterial co-occurrence, co-occurrence network complexity, and stability in fresh alfalfa and silages at initial and full flowering stages

The separate bacterial network analysis was applied to assess the effects of growth stage and storage time on bacterial network complexity and stability of alfalfa silages at two growth stages. The results proved ensiling time possessed a more remarkable influence on bacterial community compositions than growth stage. Herein, longer storage time decreased the bacterial network complexity in alfalfa silages, indicated by simpler bacterial community structure in 60 day silages. This may be related to the acidic environment and inadequate fermentable substrates in 60 day silage, restricting the growth and reproduction of most microorganisms.

AL2 group had similar bacterial community stability (Degree, Degree Centrality, Closeness Centrality, Betweenness Centrality) with AL1 group on days 3 and 60. The higher bacterial community stability in AL1 group might be attributed to the inferior fermentation quality in AL1 group with lower amounts of lactic acid and higher pH, resulting in large amounts of undesirable bacteria. Regarding AL2 group, its higher bacterial community stability was probably due to the rapid drop of pH and quick production of lactic acid in AL2 group, resulting from the dominant role of beneficial lactic acid bacteria.

Predicted pathways of bacterial communities in fresh alfalfa and silages at initial and full flowering stages

Currently, an increasing number of highly accurate reference microbial genomes (> 50000) supply a tremendous resource of taxonomy-anchored functional information [5]. Bioinformatic tools can use this resource to predict functional characteristics of microbial communities based on 16S rRNA gene profiles. Herein, PICRUSt2, which expands the capabilities of the original PICRUSt1 method to predict functional potential of a community based on marker gene sequencing profiles [6], was used to explore the effects of growth stage and storage time on functional profiles of bacterial communities in fresh alfalfa and silages at two growth stages.

On the Pathway level 1, higher proportions of environmental information processing, cellular processes, metabolism, organismal systems, and genetic information processing in AL1 group than AL2 group prior to ensiling might be linked with the higher diversity of epiphytic bacterial community in AL1 group. After ensiling, the higher proportions of metabolism and genetic information processing on day 3, and Cellular Processes on day 60 in AL1 group than AL2 group may reflect the flourishment of undesirable bacteria due to the less lactic acid production and higher pH environment.

On the Pathway level 2, higher proportions of amino acid and carbohydrate metabolism in silage samples confirmed the bacterial communities in silage possessed strong capacity to metabolize carbohydrates and amino acids during ensiling. The lower abundances of signal transduction and membrane transport in silages may be due to the production of organic acids and acidification during fermentation, inhibiting the signal transduction and membrane transport of bacterial community. The higher abundances of folding, sorting and degradation, translation, and replication and repair in AL2-60 group than other silage samples suggested that the beneficial LAB of bacterial community in AL2 still rapidly proliferated after 60 days, consistent with the higher lactic acid production in AL2 on day 60. Kilstrup et al. [34] showed nucleotides can be used by microbes to replicate and synthesize DNA and RNA, and supply energy for cellular process. The decreased abundances of cell motility, cellular community-prokaryotes, and environmental adaptation after ensiling were probably because the fermentation products (mainly organic acids) and acidic

environment in silage were adverse to the bacterial cell motility, cellular community and their adaptive capacity.

On the Pathway level 3, the up-regulated metabolic pathways in all samples reflected that the bacterial community compositions of fresh alfalfa and silages at two growth stages always remained high metabolic activity. The gradually down-regulated pathways of ABC transporters, biosynthesis of secondary metabolites, biosynthesis of amino acids, carbon metabolism, two-component system, and microbial metabolism in diverse environments during ensiling indicated that the membrane transport, biosynthesis and metabolism of bacterial community in silages might be strongly influenced by the anaerobic environment, bacterial interaction and fermentation products (e.g., lactic acid, acetic acid and ethanol).

Predicted enzymes of bacterial community in fresh alfalfa and silages at initial and full flowering stages

The enzyme activity of different individual microorganisms may differ, leading to different functional contributions to certain reaction end-products [35]. It is common knowledge that 1-phosphofructokinase, fructokinase and pyruvate kinase are mainly responsible for the Embden–Meyerhof pathway (EMP). Compared to AL2 group, the higher abundances of 1-phosphofructokinase and fructokinase in AL1 group on day 3 may be more related to the higher metabolic activity of undesirable bacteria due to the slower acidification at initial fermentation stage in AL1 group. The higher abundances of fructokinase, pyruvate kinase and 1-phosphofructokinase on day 60 in AL2 group than AL1 group were primarily related to the higher metabolic activity of beneficial LAB in AL2 group.

In anaerobic glycolysis, lactate dehydrogenase is the terminative enzyme in the sequence of reactions that promote the breakdown of glucose to lactate [36]. Compared to AL1 group, the higher abundances of D-lactate dehydrogenase and lower abundances of L-lactate dehydrogenase in AL2 group on day 3 suggested D-lactate dehydrogenase was more critical than L-lactate dehydrogenase in stimulating lactic acid fermentation during the early stages. It is in accordance with the findings of Wang et al. [9], who reported D-lactate dehydrogenase was important in the production of LA at the beginning of ensiling. In the later research, more attention should be paid to investigating the role of D-lactate dehydrogenase in regulating the LA production during the initial stages of fermentation. After 60 days, higher proportions of D- and L-lactate dehydrogenase in AL2 than AL1 agreed with the large accumulation of LA in AL2 at the late stages of fermentation.

The ribulose-5-phosphate and glucose-6-phosphate dehydrogenase played important roles in pentose phosphate pathway [37]. The higher proportions of L-ribulose-5-phosphate 3-epimerase and glucose-6-phosphate dehydrogenase in AL1 group than AL2 group on day 60 implied that the bacterial communities in well-preserved silage were less involved in pentose phosphate pathway. More research needs to be conducted to explore the role of pentose phosphate pathway during ensiling.

The nitrite reductase (*nirB*) and ornithine decarboxylase (*odcA*) in bacterial community were mainly responsible for the ammonia–nitrogen (NH_3–N) and biogenic amine formation in alfalfa silages [30]. In the current study, the higher NH_3–N contents and lower abundances of Nitrite reductase (NADH) and Ornithine decarboxylase in AL1 group on day 3 seemed to be contradictory. However, it can be explained by the reports of Heinritz et al. [38], who concluded that the formation of ammonia during ensiling was related to plant enzyme and bacterial activity. During the initial stages of fermentation, higher NH_3–N contents in AL1 group could be mainly ascribed to the plant proteolytic enzyme due to the less lactic acid production and slower acidification in AL1 group. After 60 days, the higher NH_3–N concentrations in AL1 group may be more correlated with the bacterial activity because of the higher abundances of Nitrite reductase (NADH) and Ornithine decarboxylase in AL1 group.

The up-regulated protein–N(pi)–phosphohistidine–sugar phosphotransferase, histidine kinase, DNA-directed DNA polymerase and DNA helicase of bacterial community in silage samples indicated that these bacterial enzymes were positively involved in various biochemistry reactions during ensiling. The down-regulated NADH: ubiquinone reductase, glutathione transferase, iron–chelate-transporting ATPase and peptidylprolyl isomerase of bacterial community in silage samples suggested that these enzymes were inhibited by ensiling due to the acidic environment and fermentation products. However, the specific function of abovementioned enzymes requires further study.

Predicted modules of bacterial community in fresh alfalfa and silages at initial and full flowering stages

Genome-scale metabolic models including functional network modules, can provide important insights into cell function. These modules are defined as groups of reactions with correlated fluxes, and the identification of modules can enable researchers to know the internal "wiring" of genetic networks [39, 40]. On day 3, the higher proportions of glycolysis (Embden–Meyerhof pathway),

glucose = > pyruvate, and Pentose phosphate pathway (pentose phosphate cycle) in AL1 group than AL2 group were in agreement with the variations of abovementioned key enzymes involved in Embden–Meyerhof pathway and pentose phosphate pathway, demonstrating the consistency of predicted enzymes and modules in bacterial communities. On day 60, lower proportions of citrate cycle (TCA cycle, Krebs cycle) in AL2 group than AL1 group confirmed that the bacterial community in well-preserved silage was less involved in TCA cycle.

Notably, the module of NADH: quinone oxidoreductase, prokaryotes was obviously down-regulated in well-fermented AL2 group on day 60, consistent with the changes of abovementioned NADH: ubiquinone reductase. Brandt [41] described that the NADH: quinone oxidoreductase can catalyze electron transfer from NADH to quinone, and it is by far the largest enzyme of respiratory chain. Hence, it was speculated that the downregulation of NADH: quinone oxidoreductase may reflect the inhibition of undesirable bacteria in well-fermented silage. However, the role of NADH: quinone oxidoreductase in bacterial community during ensiling needs to be further investigated.

Predicted phenotype of bacterial community in fresh alfalfa and silages at initial and full flowering stages

Nowadays, shotgun metagenomics and marker gene amplicon sequencing (16S) can be used to directly measure or predict the functional profiles of microbiota, but current methods do not readily estimate the functional capability of individual microorganisms. Hence, an algorithm named BugBase was used to predict biologically interpretable phenotypes, such as oxygen tolerance, Gram staining and pathogenic potential, within complex microbiome based on whole-genome shotgun or marker gene sequencing data [42]. To our knowledge, this is the first investigation to predict the phenotype of bacterial community in the field of silage.

Herein, the higher proportions of facultatively anaerobic and lower proportions of anaerobic in AL2 than AL1 group on day 60 implied that most lactic acid bacteria strains in AL2-60 group belonged to facultatively anaerobic bacteria rather than obligate anaerobic bacteria. The higher proportions of potentially pathogenic and gram negative in AL1 group than AL2 group on day 60 could be explained by the less lactic acid accumulation and higher pH environment in AL1-60 group, promoting the growth of some pathogenic and/or gram negative bacteria. The higher proportions of gram positive in AL2 group than AL1 group on day 60 was probably because the bacterial community in AL2-60 group was mainly occupied by the lactic acid bacteria, which belonged to gram positive bacteria [43].

Conclusions

Compared to alfalfa silage harvested at initial flowering stage, the alfalfa silage harvested at full flowering stage had better fermentation quality with lower pH, ammonia nitrogen and butyric acid contents and higher lactic acid contents after 60 days of ensiling. Moreover, the high throughput sequencing technique combined with PICRUSt2 method can be successfully used to describe the variations of bacterial communities and their predicted functional potential in silage. This approach can improve our understanding of the silage microbiology to further regulate the fermentation products.

Abbreviations

FW: Fresh weight; ETH: Ethanol; NH_3–N: Ammonia–nitrogen; PA: Propionic acid; AA: Acetic acid; BA: Butyric acid; WSC: Water soluble carbohydrate; DM: Dry matter; LA: Lactic acid; LA/AA: Ratio of lactic acid to acetic acid; ALFM1: Fresh alfalfa harvested at initial flowering stage; ALFM2: Fresh alfalfa harvested at full flowering stage; AL1-3: Alfalfa silage at initial flowering stage on day 3; AL2-3: Alfalfa silage at full flowering stage on day 3; AL1-60: Alfalfa silage at initial flowering stage on day 60; AL2-60: Alfalfa silage at full flowering stage on day 60.

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Author contributions

Designed experiments, TS; carried out experiments, SW, YW, HL, XL and JZ; analyzed experimental results, SW, TS, ZD and JL; wrote and edited the manuscript, SW, NAK and MN All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. McDonald P, Henderson AR, Heron S. The biochemistry of silage. Abersytwyth UK: Chalcombe publications; 1991.

2. Guo G, Shen C, Liu Q, Zhang SL, Wang C, Chen L, Xu QF, Wang YX, Huo WJ. Fermentation quality and in vitro digestibility of first and second cut alfalfa (*Medicago sativa* L.) silages harvested at three stages of maturity. Anim Feed Sci Technol. 2019;257:114274.
3. Buxton DR, Muck RE, Harrison JH, Buxton DR, O'Kiely P. Preharvest plant factors affecting ensiling. Agron Monogr. 2003;42:c5.
4. McAllister TA, Dunière L, Drouin P, Xu S, Wang Y, Munns K, Zaheer R. Silage review: using molecular approaches to define the microbial ecology of silage. J Dairy Sci. 2018;101(5):4060–74.
5. Toole DR, Zhao J, Martens-Habbena W, Strauss SL. Bacterial functional prediction tools detect but underestimate metabolic diversity compared to shotgun metagenomics in southwest Florida soils. Appl Soil Ecol. 2021;168:104129.
6. Douglas GM, Maffei VJ, Zaneveld J, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI. PICRUSt2: an improved and customizable approach for metagenome inference. BioRxiv. 2020;672295:1–16.
7. Bai J, Ding Z, Su R, Wang M, Cheng M, Xie D, Guo X. Storage temperature is more effective than lactic acid bacteria inoculations in manipulating fermentation and bacterial community diversity, co-occurrence and functionality of the whole-plant corn silage. Microbiol Spectr. 2022;10(2):e00101-e122.
8. Zhang H, Cai Y. Lactic acid bacteria. Berlin Heidelberg: Springer; 2014.
9. Wang S, Shao T, Li J, Zhao J, Dong Z. Fermentation profiles, bacterial community compositions, and their predicted functional characteristics of grass silage in response to epiphytic microbiota on legume forages. Front Microbiol. 2022;13:830888.
10. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. J Dairy Sci. 1980;63:64–75.
11. Owens V, Albrecht K, Muck R, Duke S. Protein degradation and fermentation characteristics of red clover and alfalfa silage harvested with varying levels of total nonstructural carbohydrates. Crop Sci. 1999;39(6):1873–80.
12. AOAC. Official methods of analysis. 19th ed. Gaithersburg MD: Association of Official Analytical Chemistry International; 2012.
13. Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 1991;74:3583–97.
14. Wang S, Zhao J, Dong Z, Li J, Shao T. Sequencing and microbiota transplantation to determine the role of microbiota on the fermentation type of oat silage. Bioresour Technol. 2020;309:123371.
15. Cao C, Bao W, Li W, Zhao F, Kwok LY, Zhang W, Zhang HP. Changes in physico-chemical characteristics and viable bacterial communities during fermentation of alfalfa silages inoculated with *Lactobacillus plantarum*. World J Microbiol Biotechnol. 2021;37(7):1–14.
16. Qiu L, Zhang Q, Zhu H, Reich PB, Banerjee S, van der Heijden MGA, Sadowsky MJ, Ishii S, Jia X, Shao M, Liu B, Jiao H, Li H, Wei X. Erosion reduces soil microbial diversity, network complexity and multifunctionality. ISME J. 2021;15:2474–89.
17. McDonald Henderson PAR. Buffering capacity of herbage samples as a factor in ensilage. J Sci Food Agr. 1962;13(7):395–400.
18. Greenhill WL. The buffering capacity of pasture plants with special reference to ensilage. Aust J Agr Res. 1964;15(4):511–9.
19. Macarisin D, Patel J, Bauchan G, Giron JA, Ravishankar S. Effect of spinach cultivar and bacterial adherence factors on survival of *Escherichia coli* O157:H7 on spinach leaves. J Food Prot. 2013;76:1829–37.
20. Lindow SE, Brandl MT. Microbiology of the phyllosphere. Appl Environ Microb. 2003;69(4):1875–83.
21. Thompson IP, Bailey MJ, Fenlon JS, Fermor TR, Lilley AK, Lynch JM, Mccormack PJ, Mcquilken MP, Purdy KJ, Rainey PB, Whipps JM. Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta Vulgaris*). Plant Soil. 1993;150:177–91.
22. Filya I. Nutritive value of whole crop wheat silage harvested at three stages of maturity. Anim Feed Sci Technol. 2003;103(1–4):85–95.
23. Kung L Jr, Shaver RD, Grant RJ, Schmidt RJ. Silage review: interpretation of chemical, microbial, and organoleptic components of silages. J Dairy Sci. 2018;101(5):4020–33.
24. Ogunade IM, Jiang Y, Pech Cervantes AA, Kim DH, Oliveira AS, Vyas D, Weinberg ZG, Jeong KC, Adesogan AT. Bacterial diversity and composition of alfalfa silage as analyzed by illumina miseq sequencing: effects of *Escherichia coli* O157:H7 and silage additives. J Dairy Sci. 2018;101:2048–59.
25. Mahanna B, Chase LE. Practical application and solution to silage problems. Silage Sci Technol. 2003;42:31–93.
26. Muraro GB, de Almeida C-E, de Oliveira Pasetti MH, Santos MC, Nussio LG. Bacterial dynamics of sugarcane silage in the tropics. Environ Microbiol. 2021;23(10):5979–91.
27. Keshri J, Chen Y, Pinto R, Kroupitski Y, Weinberg ZG, Sela SS. Microbiome dynamics during ensiling of corn with and without *Lactobacillus plantarum* inoculant. Appl Microbiol Biotechnol. 2018;102:4025–37.
28. Graf K, Ulrich A, Idler C, Klocke M. Bacterial community dynamics during ensiling of perennial ryegrass at two compaction levels monitored by terminal restriction fragment length polymorphism. J Appl Microbiol. 2016;120:1479–91.
29. Du G, Zhang G, Shi J, Zhang J, Ma Z, Liu X, Yuan C, Li X, Zhang B. Keystone taxa *Lactiplantibacillus* and *Lacticaseibacillus* directly improve the ensiling performance and microflora profile in co-ensiling cabbage byproduct and rice straw. Microorganisms. 2021;9:1099.
30. Li R, Zheng M, Zheng M, Cai R, Cui X, Wang Y, Xu C. Metagenomic analysis reveals the linkages between bacteria and the functional enzymes responsible for potential ammonia and biogenic amine production in alfalfa silage. J Appl Microbiol. 2022;132(4):2594–604.
31. Santos AO, Ávila CLS, Pinto JC, Carvalho BF, Dias DR, Schwan RF. Fermentative profile and bacterial diversity of corn silages inoculated with new tropical lactic acid bacteria. J Appl Microbiol. 2015;120:266–79.
32. Silva VP, Pereira OG, Leandro ES, Da Silva TC, Ribeiro KG, Mantovani HC, Santos SA. Effects of lactic acid bacteria with bacteriocinogenic potential on the fermentation profile and chemical composition of alfalfa silage in tropical conditions. J Dairy Sci. 2016;99:1895–902.
33. Sun L, Jiang Y, Ling Q, Na N, Xu H, Vyas D, Adesogan AT, Xue Y. Effects of adding pre-fermented fluid prepared from red clover or Lucerne on fermentation quality and *in vitro* digestibility of red clover and Lucerne silages. Agriculture. 2021;11:454.
34. Kilstrup M, Hammer K, Jensen PR, Martinussen J. Nucleotide metabolism and its control in lactic acid bacteria. FEMS Microbiol Rev. 2005;29:555–90.
35. Hu M, Dong J, Tan G, Li X, Zheng Z, Li M. Metagenomic insights into the bacteria responsible for producing biogenic amines in sufu. Food Microbiol. 2021;98(2):1–8.
36. Everse J, Kaplan NO. Lactate dehydrogenases: structure and function. Adv Enzymol Relat Areas Mol Biol. 1973;37:61–133.
37. Arese P, Gallo V. Pentose phosphate pathway. In: Hommel M, Kremsner P, editors. Encyclopedia of Malaria. New York NY: Springer; 2013.
38. Heinritz SN, Martens SD, Avila P, Hoedtke S. The effect of inoculant and sucrose addition on the silage quality of tropical forage legumes with varying ensilability. Anim Feed Sci Technol. 2012;174(3–4):201–10.
39. Shakhnovich BE, Reddy TE, Galinsky K, Mellor J, Delisi C. Comparisons of predicted genetic modules identification of co-expressed genes through module gene flow. Genome Inform. 2004;15(1):221–8.
40. Bundy JG, Papp B, Harmston R, Browne RA, Clayson EM, Burton N, Brindle KM. Evaluation of predicted network modules in yeast metabolism using NMR-based metabolite profiling. Genome Res. 2007;17(4):510–9.
41. Brandt U. Energy converting NADH: quinone oxidoreductase (complex I). Annu Rev Biochem. 2006;75(1):69–92.
42. Ward T, Larson J, Meulemans J, Hillmann B, Lynch J, Sidiropoulos D, Knights D. BugBase predicts organism-level microbiome phenotypes. BioRxiv. 2017;62:1479.
43. Jasaitis DK, Wohlt JE, Evans JL. Influence of feed ion content on buffering capacity of ruminant feedstuffs *in vitro*. J Dairy Sci. 1987;70:1391–403.

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