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Clean recovery and recycling of seasonal surplus forage grass by microbial driven anaerobic fermentation: a case study of napiergrass

Jie Zhao, Xue-Jing Yin, Jun-Feng Li, Si-Ran Wang, Zhi-Hao Dong and Tao Shao*

Abstract

In this study, the anaerobic fermentation technique was conducted to accomplish the clean recycling of surplus napiergrass. The surplus napiergrass harvested at two harvest dates (early vegetative stage, N_I; late vegetative stage, N_{II}) was treated as follows: (i) natural fermentation of N_I (NN_I); (ii) natural fermentation of N_{II} (NN_{II}) and stored for 1, 3, 7, 15, 30 and 60 days. After 60 days of anaerobic fermentation, NN_I had higher lactic acid concentration and ratio of lactic to acetic acid, but lower pH value and ammonia–nitrogen concentration than NN_{II}. *Lactobacillus* and *Enterobacter* were, respectively, dominant in both 7-day NN_I and NN_{II}, while *Lactobacillus* was the most abundant genus in 30-day NN_I and NN_{II}. Both harvest date and store time altered the bacterial co-occurrence networks of fresh and fermented napiergrass. The complexity of the bacterial networks decreased from N_{II}, N_I, NN_{II} to NN_I. The correlations were primarily positive in the bacterial networks of N_I, N_{II}, NN_{II}-7 and NN_{II}-30 with positive correlative proportion of 53.0%, 64.3%, 53.1% and 55.6%, but negative in those of NN_I-7 (47.4%) and NN_I-30 (46.2%) with positive correlative proportion of 47.4% and 46.2%, respectively. Overall, the fermentation quality and microbial community structure of napiergrass during anaerobic fermentation were highly influenced by harvest date and store time. Based on the principle of stable fermentation and high quality, anaerobic fermentation of N_I for at least 15 days is recommended. The in-depth understanding of microbial community dynamics and co-occurrence networks during anaerobic fermentation of napiergrass is important for revealing the fermentation mechanism and can contribute to resource recycling without increasing cost.

Keywords: Surplus forage grass, Harvest date, Anaerobic fermentation, Microbial community, Co-occurrence network, Recycling

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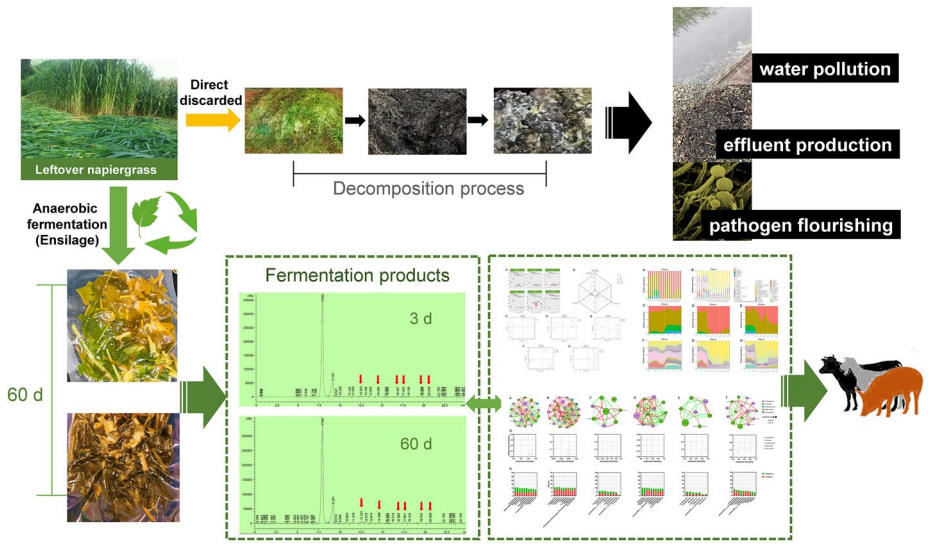
Graphical Abstract



Background

Feed production is closely related to the environment in terms of water consumption, land use and climate change, since it is an essential industry that required resources, such as water, land, and energy. Given the increasing global population growth and demand for the animal product, these relationships might be more tightly in the future. As a key link of the feed industry, forage grass production presents a distinct seasonal characteristic throughout the year [1]. Feedback from forage growers indicated that in the fast-growing season (May–October), the yield of forage grass increases wildly, accounting for more than 70% of the annual growth, especially in July and August, accounting for more than 40% of the annual growth. It frequently happens that the yield of forage grass exceeds the need for utilization [2]. It's common to leave surplus forage grass in the fields or discard directly without utilization. In resource waste assessment, the waste of forage grass is often not as prominent as the waste of food, and only a few studies have emphasized forage waste. Based on the concept of resource utilization, long-term preservation of surplus forage grass by anaerobic fermentation may increase its add-value and transform the original linear economy into a circular economy [3].

Anaerobic fermentation of forages (ensilage) is based on the principle that lactic acid bacteria (LAB) ferment available sugars in plants to produce lactic acid-based organic acids, and rapidly reduce pH value so as to effectively inhibit the activities of harmful microorganisms to achieve the purpose of long-term preservation [4]. However, it is worth noting that the fermentation quality varies greatly in the anaerobic fermentation of forage at different harvest dates. Oliveira et al. [5] and Toruk et al. [6] have shown that the harvest date of forage is the main factor determining the nutritional value and fermentation quality. In the case of guinea grass, van Niekerk et al. [7] reported that the anaerobic fermentation of guinea grass prepared at early vegetative and boot stages was lactate-type, while the anaerobic fermentation of guinea grass prepared at the full bloom stage was acetate-type. Similarly, there is also a need to determine the appropriate harvest date of surplus napiergrass for anaerobic fermentation. However, to the authors' knowledge, no studies regarding the effects of different harvest dates on the fermentation quality of surplus forage grass were reported.

Along with the study of anaerobic fermentation, culture-based methods are no longer sufficient to clearly present the microbial community succession from fresh forages to fermentative products. Recent advances in culture-independent analyses, such as high-throughput sequencing technology, have enabled researchers to explore the microbial population shifts involved in the growing and anaerobic fermentation process, and to mine more crucial biological information [8]. Therefore, in this study, surplus napiergrass (*Pennisetum purpureum* Schumach.) was used to evaluate the effects of harvest date and store time on its fermentation quality, microbial community

and co-occurrence networks during anaerobic fermentation. The obtained results may, therefore, provide a basic reference for contributing to resource recycling without increasing cost and transferring a single economy to a circular economy.

Methods

Surplus forage grass collection

Napiergrass was surplus and left in the field of Baima National Agricultural-tech Zone (Jiangsu, China) without harvest. A 30 m^2 field was separated into three equal blocks (for replicates) and the obtained each block was further divided into two equal plots (for two harvest dates). After 12 weeks of planting, half of the napiergrass was harvested on August 15, 2018, and after 18 weeks of planting, the remaining napiergrass was harvested on September 26, 2018, to obtain two batches of napiergrass (N_I, the early vegetative stage; N_{II}, the late vegetative). The harvest time was in the morning with clear weather, and the stubble height was about 15 cm. Each batch of fresh napiergrass was immediately cut into about 2 cm lengths by a forage cutter, mixed thoroughly and split into two parts for fresh sample analysis and anaerobic fermentation preparation, respectively.

Anaerobic fermentation preparation

A total of 36 bags (2 harvest dates × 6 store time × 3 replicates per treatment) were prepared and the treatments were set as follows: (i) natural fermentation of N_I (NN_I) and (ii) natural fermentation of N_{II} (NN_{II}). Specifically, approximately 0.45 kg of thorough-mixing material was packed into a UV-sterilized polythene bag (size: 300 × 400 mm), sealed by an automatic vacuum sealer and stored under surrounding temperature (25–30 ℃) for 1, 3, 7, 15, 30 and 60 days of anaerobic fermentation.

Bio-chemical composition analyses

Before analyses, the fresh or fermented sample was blended thoroughly. About 300 g sample was dried at 65 °C for 48 h in an air-forced oven to determine dry matter (DM) content. The oven-dried sample was then milled to pass through a 1-mm sieve. The water-soluble carbohydrates (WSC) content of fresh and fermented samples was analyzed with anthrone-sulfuric acid [9]. The buffering capacity (BC) of the fresh sample was quantified by titration [10]. The neutral and acid detergent fiber (NDF and ADF) content of the fresh sample was quantified by the method of Van Soest et al. [11]. The total nitrogen (TN) content of the fresh sample was quantified by a Kjeltec 8200 Kjeldahl N analyzer (Foss Analytical AB, Höganäs, Sweden). The crude protein (CP) content of fresh and fermented samples was obtained through multiplying TN by 6.25.

After extraction of the fresh or fermented sample with deionized water (1:3 ratio) at 4 °C for 24 h, the above extracts were filtered with 4 layers of sterile cheesecloth and filter paper. The pH of fresh or fermented samples was immediately recorded by a glass electrode pH meter. The ammonia–nitrogen (NH_3–N) concentration of the fermented sample was quantified by the phenol–hypochlorite procedure [12]. The organic acid concentrations of fermented sample including lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid (BA) were quantified by the 1260 Infinity HPLC system (Agilent Technology Co., Ltd., Waldbronn, Germany) [13].

After homogenization of fresh or fermented sample with sterile saline solution (1:9 ratio) at 120 rpm, 37 °C for 2 h, 1 mL homogenized solution was serial-diluted for the enumeration of LAB, aerobic bacteria, yeasts, molds and enterobacteria [14]. The microbial number was recorded in colony-forming units (CFU), transformed to logarithmic form and expressed on a fresh material (FM) basis. After filtrating with 2 layers of sterile cheesecloth, the obtained filtrate was collected for subsequent bacterial DNA extraction.

High-throughput sequencing analysis

Bacterial DNA extraction, PCR amplification and 16 s rRNA paired-end sequencing were conducted as reported in our previous study [15]. Briefly, after quality filtering and chimeric sequences removal, the qualified reads were obtained and further clustered into operational taxonomic units (OTUs). The OTUs were analyzed at phylum and genus levels based on the SILVA database. Bacterial alpha diversities (Shannon, Chao1, Ace, Sobs, Simpson and Coverage indexes) and beta diversity (Bray–Curtis distance metric) were calculated by the QIIME software. Through R software (ver. 4.1.3), the vegan package was run to construct principal coordinates analysis (PCoA) plots for beta diversity analysis, the ggplot2 package was to construct stream graphs showing the bacterial community successions, and the pheatmap package was to construct heatmaps visualizing the Spearman's correlation relationships between fermentation products and bacterial communities.

Co-occurrence network analysis

The co-occurrence pattern was constructed by calculating multiple abundance correlations based on a genus-level matrix using Networkx (ver. 2.6.3). Only genera of relative abundance > 0.05% were considered. If Spearman correlation coefficient (ρ) > 0.50 and $p < 0.05$, co-occurrence is considered to be robust. The co-occurrence networks were visualized using Gephi (ver. 0.9.2). Nodes represent individual bacterial genera, and edges represent

the pairwise correlation between nodes in the bacterial network. The calculated topological characteristics of bacterial networks include positive (co-occurrence) and negative (mutually exclusive) correlation numbers, network diameter, average shortest path length, average clustering coefficient, average connectivity (degree), closeness centrality, betweenness centrality, modularity, etc.

Statistical analysis

The effects of harvest date, store time and their interactions on chemical composition, fermentation quality and microbial population were investigated using the GLM of SAS (ver. 9.2; SAS Institute Inc., NC, USA) following the model as follows:

$$Y_{ij} = \mu + G_i + D_j + (G \times D)_{ij} + e_{ijk}$$

where Y_{ij} refers to the dependent variable; μ refers to the overall mean; G_i refers to the effect of harvest date ($i = 2$, N_I vs. N_{II}); D_j refers to the effect of store time ($j = 6$, 1, 3, 7, 15, 30 and 60); $(G \times D)_{ij}$ refers to the interaction effects of harvest date and store time; and e_{ijk} refers to the residual error. Comparisons between two harvest date were performed through *t* test when the fixed effect of harvest date was significant. The differences were considered statistically significant at $p < 0.05$.

Results

Characteristics of surplus napiergrass before anaerobic fermentation

The harvest date significantly ($p < 0.05$) affected all measured chemical and microbial parameters except the pH value and mold count of napiergrass (Table 1). The WSC and CP content, the BC and the LAB and enterobacteria number decreased ($p < 0.05$), while the DM, NDF and ADF contents and the aerobic bacteria and yeasts numbers increased ($p < 0.05$) as harvest date was delayed.

Fermentation quality of surplus napiergrass after anaerobic fermentation

Harvest date or store time had significant ($p < 0.05$) effects on the pH, the LA, AA and BA concentrations and the ratio of lactic to acetic acid (LA/AA), while their interaction significantly ($p < 0.05$) affected the LA concentration and LA/AA (Table 2). The pH of NN_I and NN_{II} sharply ($p < 0.05$) declined during the first 7 days of anaerobic fermentation reaching the lowest value (3.66 and 4.00) on day 15 and day 30 of anaerobic fermentation, respectively, then increased slightly. During the whole anaerobic fermentation, NN_I always had a lower pH value than NN_{II} ($p < 0.05$). The LA concentration presented the opposite trend to the variation of pH value, with the highest concentration of 52.0 and 39.2 g/kg DM on day 30 of anaerobic fermentation in NN_I and NN_{II}, respectively. Regardless of harvest date, the AA concentration increased with the store time prolonged. Along with the anaerobic fermentation process, the LA/AA value showed an upward and then downward tendency, with a maximum of 9.58 in NN_I on day 15 of anaerobic fermentation and a maximum of 4.89 in NN_{II} on day 30 of anaerobic fermentation. The BA of NN_I and NN_{II} was always less than 2 g/kg DM.

Table 1 Chemical composition and epiphytic microbial populations of fresh napiergrass at two harvest dates

Harvest date	Early	Late	SEM[E]	*P* value
Items[C] and treatments[D]	N_I	N_{II}		
pH	5.91	5.88	0.038	0.740
DM (g/kg FM)	242[B]	282[A]	9.518	0.003
WSC (g/kg DM)	64.5[B]	40.1[A]	5.578	0.001
BC (mEq/kg DM)	61.2[A]	51.8[B]	2.302	0.011
NDF (g/kg DM)	656[B]	691[A]	8.422	0.007
ADF (g/kg DM)	376[B]	418[A]	10.87	0.027
CP (g/kg DM)	46.8[A]	31.9[B]	3.427	0.001
LAB (Log_{10} CFU/g FM)	5.11[B]	3.92[A]	0.301	0.019
Aerobic bacteria (Log_{10} CFU/g FM)	6.22[B]	9.58[A]	0.758	< 0.001
Yeasts (Log_{10} CFU/g FM)	3.77[B]	4.41[A]	0.161	0.018
Molds (Log_{10} CFU/g FM)	3.53	4.06	0.181	0.157
Enterobacteria (Log_{10} CFU/g FM)	8.31[A]	6.31[B]	0.451	< 0.001

[A–B] Means with different uppercase in the same row differ at $p < 0.05$

[C] *DM* dry matter, *FM* fresh material, *WSC* water-soluble carbohydrates, *BC* buffering capacity, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *CP* crude protein, *LAB* lactic acid bacteria, *CFU* colony-forming units

[D] N_I napiergrass harvested at the early vegetative stage, N_{II} napiergrass harvested at the late vegetative stage

[E] *SEM* standard error of means

The DM content was affected by harvest date, the WSC content was affected by store time, and the NH_3–N concentration was affected by their interaction ($p < 0.001$). As anaerobic fermentation proceeded, the DM content remained relatively stable, but the WSC content decreased and the NH_3–N concentration increased ($p < 0.05$). NN_{II} always had higher DM content and NH_3–N concentration than NN_I ($p < 0.05$).

Harvest date and store time significantly ($p < 0.001$) affected LAB, aerobic bacteria, yeasts, molds and enterobacteria numbers, while their interaction significantly ($p < 0.05$) affected LAB and aerobic bacteria numbers (Table 3). The LAB number of NN_I and NN_{II} showed an upward and then downward tendency, but the aerobic bacteria, yeast, molds and enterobacteria numbers constantly decreased to a low or undetected level. The LAB

Table 2 Effects of harvest dates and store time on fermentation quality of fermented napiergrass

Items[C]	Treatments[D]	Store time (*d*)						SEM[E]	*P* value[F]		
		1	3	7	15	30	60		G	D	G × D
pH	NN_I	5.31^B	4.63	4.13	3.66^B	3.76	3.99^B	0.142	< 0.001	< 0.001	0.082
	NN_{II}	5.88^A	4.86	4.39	4.19^B	4.00	4.20^A	0.159			
DM (g/kg FM)	NN_I	236^B	232^B	231^B	231^B	230^B	226^B	1.801	< 0.001	0.066	0.760
	NN_{II}	267^A	261^A	255^A	254^A	253^A	242^A	2.705			
LA (g/kg DM)	NN_I	5.51^A	21.2^A	34.5^A	51.1^A	52.0^B	43.2^A	4.153	< 0.001	< 0.001	0.017
	NN_{II}	1.99^B	10.1^B	17.6^B	32.8^B	39.2^A	25.6^B	3.142			
AA (g/kg DM)	NN_I	2.78^B	3.60^B	4.83^B	5.39^B	8.19	14.4	1.018	0.047	< 0.001	0.186
	NN_{II}	3.67^A	5.99^A	7.43^A	7.67^A	8.11	12.9	0.708			
LA/AA	NN_I	1.99^A	5.88^A	7.19^A	9.58^A	6.39^A	3.17^A	0.631	< 0.001	< 0.001	< 0.001
	NN_{II}	0.54^B	1.69^B	2.39^B	4.33^B	4.89^B	2.00^B	0.376			
PA (g/kg DM)	NN_I	ND	ND	ND	ND	ND	ND	–	–	–	–
	NN_{II}	ND	ND	ND	ND	ND	ND	–			
BA (g/kg DM)	NN_I	0.34B	1.18	1.38	1.23	1.18	1.45	0.079	0.002	0.005	0.986
	NN_{II}	0.66A	1.49	1.63	1.59	1.69	1.84	0.096			
WSC (g/kg DM)	NN_I	45.2	24.1	17.9	12.1	10.1	7.41	3.214	0.286	< 0.001	0.288
	NN_{II}	40.6	27.8	23.6	11.2	11.7	10.3	2.756			
NH_3–N (g/kg TN)	NN_I	36.6^B	42.1^B	53.9^B	64.1^B	92.3^B	98.9^B	6.177	< 0.001	< 0.001	< 0.001
	NN_{II}	47.6^A	68.2^A	119^A	145^A	150^A	187^A	11.87			

[A–B] Means with different uppercase in the same row differ at $p < 0.05$

[C] *DM* dry matter, *FM* fresh material, *LA* lactic acid, *AA* acetic acid, *LA/AA* the ratio of lactic to acetic acid, *PA* propionic acid, *BA* butyric acid, *WSC* water soluble carbohydrates, NH_3*–N* ammonia–nitrogen, *TN* total nitrogen

[D] NN_I natural fermentation of napiergrass harvested at the early vegetative stage, NN_{II} natural fermentation of napiergrass harvested at the late vegetative stage

[E] *SEM* standard error of means

[F] *G* the effect of harvest date, *D* the effect of store time; *G* × *D* the interaction between harvest date and store time

Table 3 Effects of harvest dates and store time on the microbial number of fermented napiergrass

Items[C]	Treatments[D]	Store time (*d*)						SEM[E]	*P* value[F]		
		1	3	7	15	30	60		G	D	G × D
LAB (log_{10} CFU/g FM)	NN_I	6.03^A	7.85^A	7.91^A	8.66^A	7.58^A	6.47^A	0.225	< 0.001	< 0.001	0.011
	NN_{II}	4.38^B	5.51^B	6.02^B	7.39^B	6.06^B	5.30^B	0.227			
Aerobic bacteria (log_{10} CFU/g FM)	NN_I	5.63^B	4.81^B	2.61^B	$< 2.00^B$	< 2.00	< 2.00	0.459	< 0.001	< 0.001	0.013
	NN_{II}	8.09^A	7.39^A	5.98^A	3.15^A	< 2.00	< 2.00	0.695			
Yeasts (log_{10} CFU/g FM)	NN_I	3.52^B	3.46^B	2.41^B	$< 2.00^B$	< 2.00	< 2.00	0.299	< 0.001	< 0.001	0.905
	NN_{II}	4.44^A	4.39^A	3.68^A	2.93^A	2.34	2.41	0.223			
Molds (log_{10} CFU/g FM)	NN_I	3.03^B	2.42	$< 2.00^B$	ND	ND	ND	0.307	< 0.001	< 0.001	0.601
	NN_{II}	3.88^A	3.07	2.66^A	< 2.00	< 2.00	< 2.00	0.305			
Enterobacteria (log_{10} CFU/g FM)	NN_I	8.16^A	6.89^A	4.44^B	3.82^B	2.91	3.08	0.447	< 0.001	< 0.001	0.216
	NN_{II}	6.11^B	5.76^B	5.63^A	4.86^A	3.35	2.99	0.347			

[A–B] Means with different uppercase in the same row differ at $p < 0.05$

[C] *LAB* lactic acid bacteria, *CFU* colony-forming units, *FM* fresh material

[D] NN_I natural fermentation of napiergrass harvested at the early vegetative stage, NN_{II} natural fermentation of napiergrass harvested at the late vegetative stage

[E] *SEM* standard error of means

[F] *G* the effect of harvest date, *D* the effect of store time, *G* × *D* the interaction between harvest date and store time

number of NN_I and NN_{II} increased rapidly during the first 3 days of anaerobic fermentation and detected the maximum (8.66 and 7.39 log_{10} CFU/g FM) on day 15 of anaerobic fermentation. Wherein, NN_I had more LAB than NN_{II} ($p < 0.05$). Although the number of aerobic bacteria, yeasts and enterobacteria in NN_I was significantly ($p < 0.05$) lower than that in NN_{1I} within the first 15 days of anaerobic fermentation, there was no difference in the number of these microorganisms between NN_I and NN_{II} at the end of anaerobic fermentation (60 d).

Bacterial community of surplus napiergrass before and after anaerobic fermentation

The alpha diversities of the microbial community in fresh and fermented napiergrass are presented in Fig. 1A. The Shannon, $Chao_1$ and Sobs indexes were highest in fresh N_{II}, followed by fresh N_I and finally fermented samples. Among all samples, NN_I always had numerically ($p > 0.05$) or statistically ($p < 0.05$) lower Shannon, Chao1, Ace and Sobs indexes than NN_{II}. Compared with fresh samples (N_I and N_{II}), the Shannon, Chao1, Ace and Sobs indexes

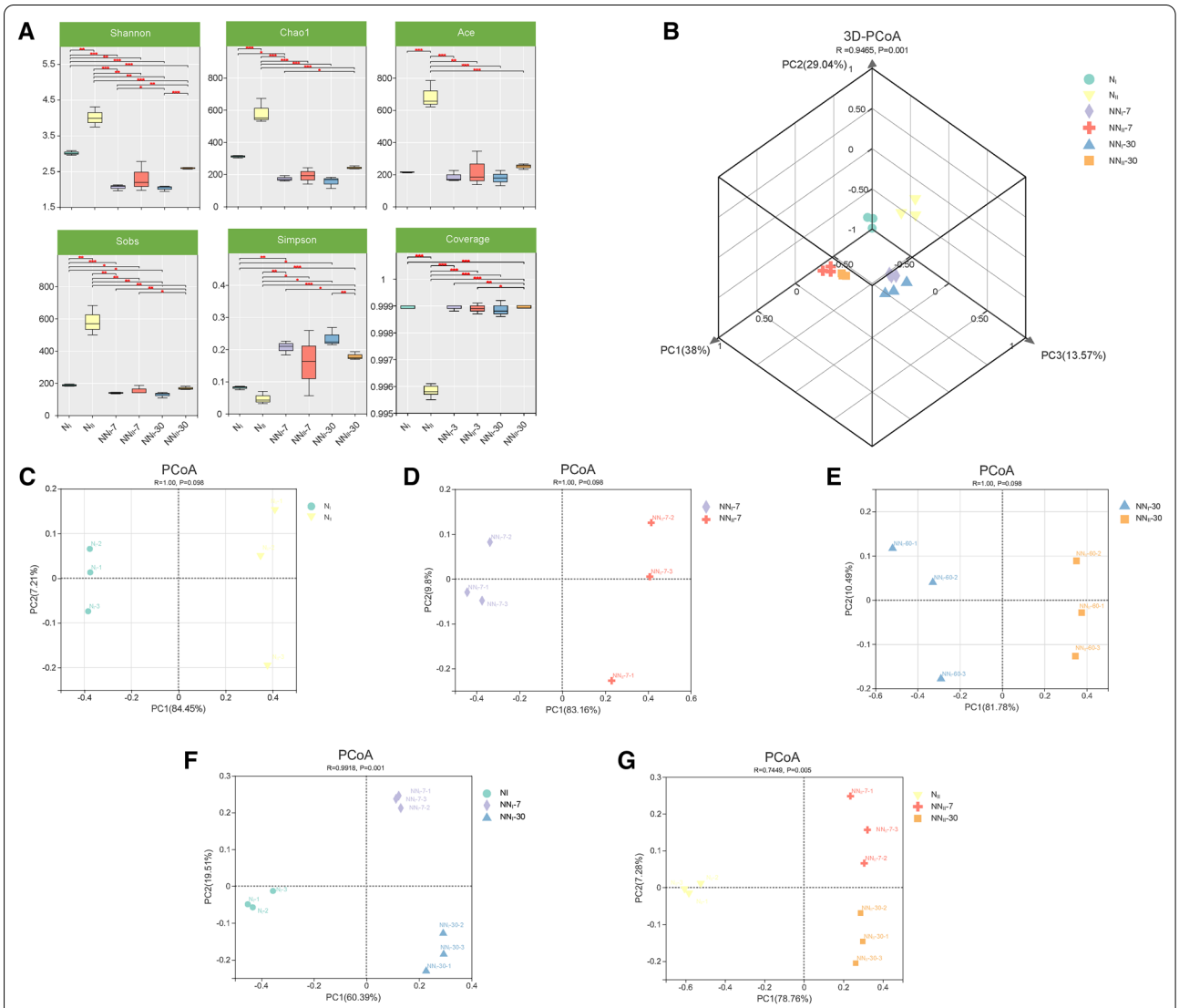


Fig. 1 Bacterial community diversities of fresh and fermented napiergrass harvested at different harvest dates. **A** Alpha diversities (Shannon, Chao1, Ace, Sobs, Simpson and Coverage indexes) of bacterial community; **B** beta diversities of bacterial community, calculated by three-dimensional principal coordinates analysis (3D-PCoA) based on the Bray–Curtis distance metric; **C** PCoA plot of N_I and N_{II}; **D** PCoA plot of NN_I-7 and NN_{II}-7; **E** PCoA plot of NN_I-30 and NN_{II}-30; **F** PCoA plot of N_I, NN_I-7 and NN_I-30; **G** PCoA plot of N_{II}, NN_{II}-7 and NN_{II}-30. *N_I* napiergrass harvested at the early vegetative stage, *N_{II}* napiergrass harvested at the late vegetative stage, *NN_I* natural fermentation of napiergrass harvested at the early vegetative stage, *NN_{II}* natural fermentation of napiergrass harvested at late vegetative stage

of fermented samples (NN_I and NN_{II}) decreased after 7 days of anaerobic fermentation, and further decreased in NN_I but increased in NN_{II} after 30 days of anaerobic fermentation. The lowest Shannon, Chao1, Ace and Sobs indexes were observed in NN_I on day 30 of anaerobic fermentation. The average Coverage index of all sequenced samples was greater than 0.99. The beta diversities of the microbial community in fresh and fermented napiergrass were assessed by PCoA. A well separation was found between the symbols of fresh and fermented sample in the 3D-PCoA plot except for NN_{II}-7 and NN_{II}-30 (Fig. 1B). Among them, N_I and N_{II}, NN_I-7 and NN_{II}-7 as well as NN_I-30 and NN_{II}-30 were well-separated in Fig. 1C–E. In addition, N_I, NN_I-7 and NN_I-30 as well as N_{II} and NN_{II} (NN_{II}-7 and NN_{II}-30) were well-separated in Fig. 1F and G.

As shown in Fig. 2A, Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota were detected in both N_I and N_{II} and Deinococcota was an additional phylum in N_{II}. Proteobacteria was the abundant phyla in N_I and N_{II}, with a relative abundance of 64.3% and 69.2%, respectively. With the growth of napiergrass, the relative abundances of Actinobacteriota and Bacteroidota increased from 5.55% and 0.21% to 20.0% and 6.86%, but Proteobacteria and Firmicutes decreased from 74.3% and 19.9% to 59.2% and 10.8%, respectively. After 7 days of anaerobic fermentation, the relative abundance of Firmicutes in NN_I and NN_{II} increased in

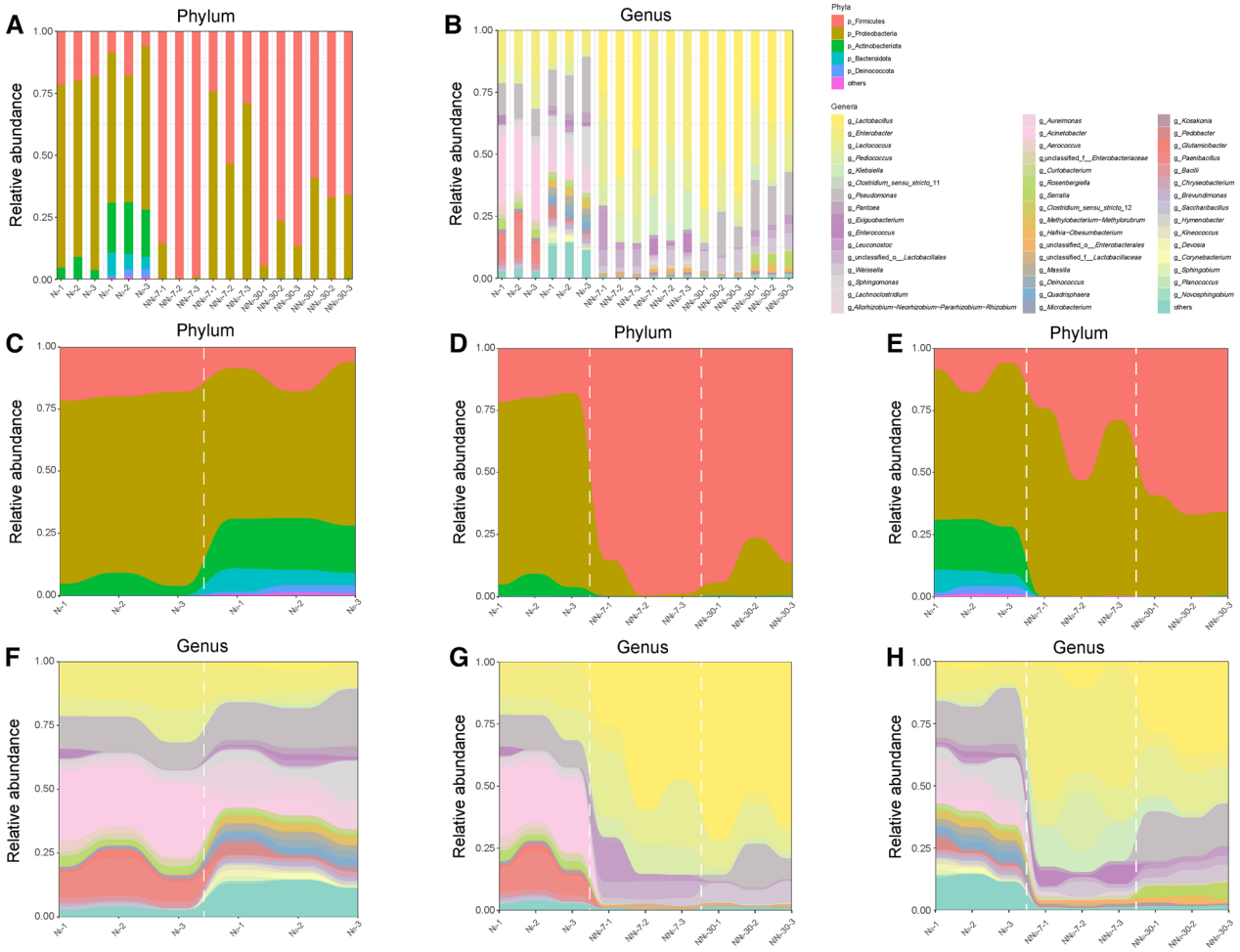


Fig. 2 Bacterial community composition and succession of fresh and fermented napiergrass harvested at different harvest dates. **A** Relative abundance of bacterial community at the phylum level; **B** relative abundance of bacterial community at the genus level; **C**, **F** bacterial community successions during the growth of fresh napiergrass are aggregated and colored on a stream-graph by phylum and genus, respectively; **D**, **G** bacterial community successions during anaerobic fermentation of N_I are aggregated and colored on a stream-graph by phylum and genus, respectively; **E**, **H** bacterial community successions during anaerobic fermentation of N_{II} are aggregated and colored on a stream-graph by phylum and genus, respectively. N_I napiergrass harvested at the early vegetative stage, N_{II} napiergrass harvested at the late vegetative stage, NN_I natural fermentation of napiergrass harvested at the early vegetative stage, NN_{II} natural fermentation of napiergrass harvested at late vegetative stage

varying degrees, accompanied by a decrease in the relative abundance of Proteobacteria, Actinobacteriota and Bacteroidota. After 30 days of anaerobic fermentation, Firmicutes (> 60%) dominated in the microbiota of all samples, especially in that of NN_I.

The number of genera with a relative abundance greater than 1% in N_I and N_{II} was 10 and 24, respectively (Fig. 2B). The most abundant genus in N_I was *Acinetobacter* (26.5%), followed by *Enterobacter* (15.0%) and *Pseudomonas* (12.4%), while *Pseudomonas* (17.6%), *Enterobacter* (9.14%), *Sphingomonas* (7.87%), *Aureimonas* (6.31%) and *Rhizobium* (6.01%) were the 5 genera with high relative abundance in N_{II}. From N_I to N_{II}, the relative abundance of *Pseudomonas* increased from 12.4% to 17.6%, whereas *Acinetobacter*, *Enterobacter* and *Lactococcus* decreased from 26.5%, 15.0% and 8.01% to 2.36%, 9.14% and 2.65%, respectively. With the process of anaerobic fermentation, *Acinetobacter* and *Sphingomonas* decreased to an undetectable level. Differently, after 7 days of anaerobic fermentation, the relative abundance of *Pediococcus* and *Lactococcus* in NN_I increased up to 10.1% and 22.5%, respectively, and the relative abundance of *Klebsiella* in NN_{II} increased up to 12.9%. After 30 days of anaerobic fermentation, *Lactobacillus* dominated the bacterial community of both NN_I and NN_{II}, with relative abundance accounting for 63.1% and 34.1%, respectively.

The stream graphs showed that both harvest date and storage time had a remarkable impact on the succession of bacterial communities during the anaerobic fermentation of surplus napiergrass (Fig. 2C–H). Although the variation of harvest date affected the bacterial community succession of surplus napiergrass (Fig. 2C, F), the anaerobic fermentation process had a more significant effect on the bacterial community succession (Fig. 2D, E, G, H).

Bacterial co-occurrence networks of surplus napiergrass before and after anaerobic fermentation

The co-occurrence network based on the correlation coefficient matrix, to a certain extent, can reflect the relationships between microbial members. Thus, the bacterial co-occurrence networks of fresh and fermented napiergrass based on Spearman's rank correlation were separately created at two harvest dates to clearly understand the effects of harvest date on the interrelationships of bacterial members (genera). Based on co-occurrence network analysis (Fig. 3A–D and Table 4), the number of nodes and edges in bacterial networks was ranked as follows: $N_{II} > N_I > NN_{II} > NN_I$. The genera with high closeness centrality, high mean degree and low betweenness centrality were *Lactococcus*, *Hafnia-Obesumbacterium*, *Enterococcus* and *Curtobacterium* in N_I, *Microbacterium* and *Roseomonas* in N_{II}, *Lactobacillus* and *Pediococcus* in NN_I-7, *Weissella*, *Lactobacillus*, *Enterococcus* and *Pantoea* in NN_{II}-7, *Lactobacillus* and *Lactococcus* in NN_I-30, and *Lactobacillus*, *Pedicoccus* and *Kosakonia* in NN_{II}-30 (Fig. 3E and F). Furthermore, the correlations of nodes were primarily positive (proportion of positive edges) in the bacterial networks of N_I, N_{II}, NN_{II}-7 and NN_{II}-30 but negative (proportion of negative edges) in those of NN_I-7 and NN_I-30 (Fig. 3A–F).

Correlation analysis of chemical composition and epiphytic microbiota as well as fermentation products and bacterial communities

Before anaerobic fermentation (Fig. 4A), *Acinetobacter* was negatively ($p < 0.01$) related to DM content, with correlation coefficients of -0.943. *Paenibacillus* was positively correlated with CP content ($R = 0.880$, $p < 0.05$) and WSC ($R = 0.941$, $p < 0.01$). Positive correlations were observed between CP content and *Rhizobium* ($R = 0.957$, $p < 0.01$). After anaerobic fermentation (Fig. 4B), *Lactobacillus* was positively correlated with LA ($R = 0.580$, $p < 0.05$) and LA/AA ($R = 0.776$, $p < 0.01$) concentrations, but negatively correlated with DM content ($R = -0.692$, $p < 0.05$) and pH value ($R = -0.873$, $p < 0.001$). Similarly, *Leuconostoc* was positively correlated with LA/AA ($R = 0.713$, $p < 0.01$), but negatively correlated with DM content ($R = -0.860$, $p < 0.001$). *Enterobacter*, *Klebsiella* and *Enterococcus* were positively ($p < 0.01$) correlated with DM content, with correlation coefficients of 0.783, 0.755 and 0.715, whereas negatively ($p < 0.05$) correlated with LA/AA, with correlation coefficients of -0.853, −0.790 and −0.609, respectively. There was positive correlation between *Pseudomonas* and LA concentration ($R = 0.630$, $p < 0.05$) and negative correlation between *Lactococcus* and NH_3–N concentration ($R = -0.566$, $p < 0.05$).

Discussion

Effects of harvest date on the characteristics of surplus napiergrass

The harvest date is reported to be the most important factor affecting forage quality at harvest [16]. In this work, with the delay of harvest date, the CP content decreased, while the DM, NDF and ADF content increased, which was in line with the study of Silva et al. [17]. The increase in DM content could be explained by the deposition of cell walls (structural carbohydrates) produced by photosynthesis. While the increase in NDF and ADF content could be attributed to the decrease in leaf–stem ratio, since the cell wall components in the stem are higher than that in the leaf [18, 19]. Meanwhile, the increased proportion of NDF and ADF led to a relative decrease in CP and WSC content [18, 20]. Queiroz et al. [21] indicated that the decrease of CP and WSC

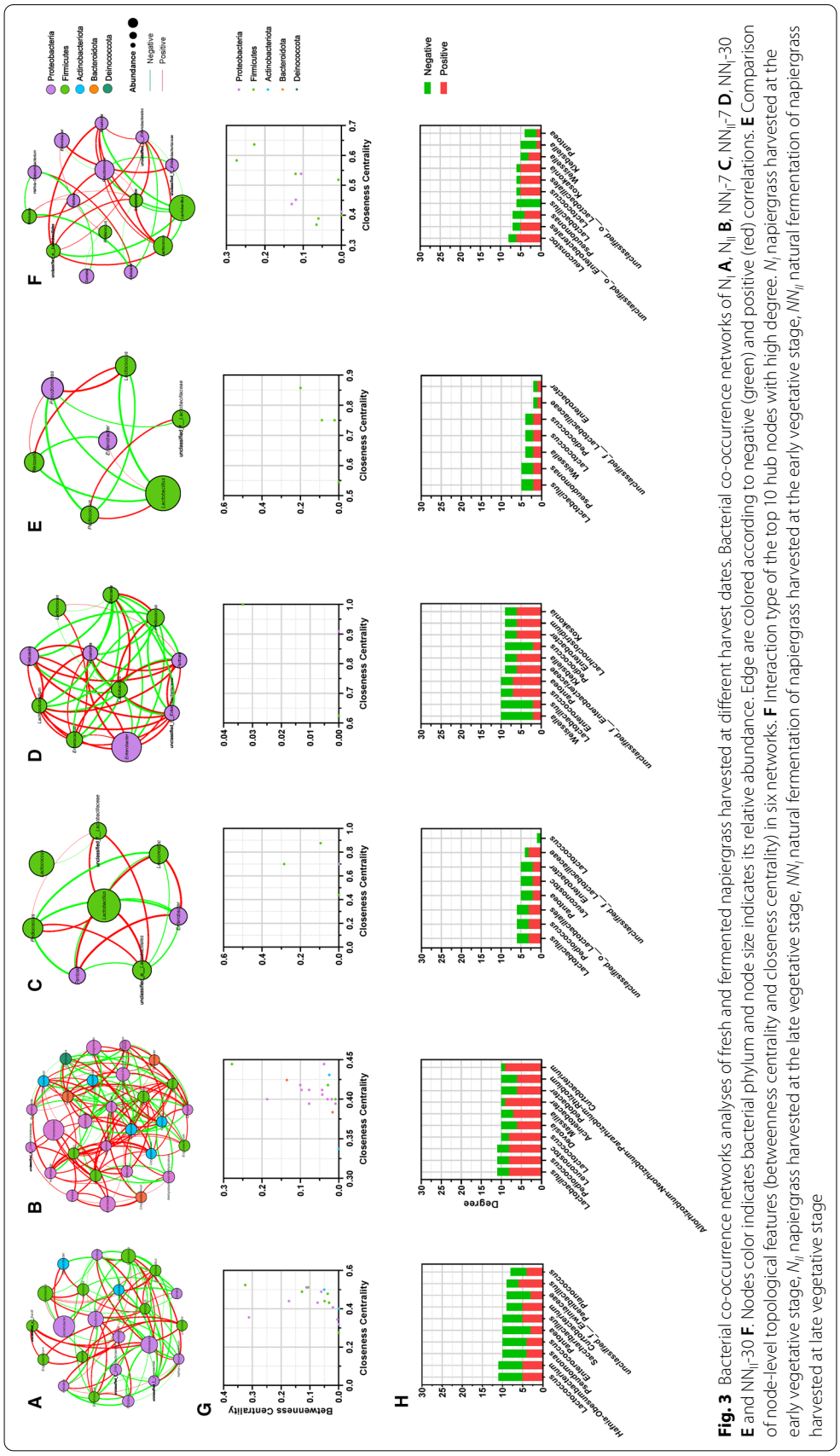


Fig. 3 Bacterial co-occurrence networks analyses of fresh and fermented napiergrass harvested at different harvest dates. Bacterial co-occurrence networks of N_I **A**, N_II **B**, NN_I-7 **C**, NN_{II}-7 **D**, NN_I-30 **E** and NN_{II}-30 **F**. Nodes color indicates bacterial phylum and node size indicates its relative abundance. Edge are colored according to negative (green) and positive (red) correlations. **G** Comparison of node-level topological features (betweenness centrality and closeness centrality) in six networks. **F** Interaction type of the top 10 hub nodes with high degree. N_I napiergrass harvested at the early vegetative stage, N_{II} napiergrass harvested at the late vegetative stage, NN_I natural fermentation of napiergrass harvested at the early vegetative stage, NN_{II} natural fermentation of napiergrass harvested at late vegetative stage

Table 4 Network topological characteristics of fresh and fermented napiergrass

Items and treatments	Fresh		Fermented			
	N_I	N_{II}	NN_I-7	NN_{II}-7	NN_I-30	NN_{II}-30
Node	23	29	8	11	7	15
Edge	83	126	19	49	13	36
Average degree	7.22	8.69	4.75	8.91	3.71	4.80
Clustering coefficient	0.785	0.773	0.843	0.952	0.767	0.634

N_I napiergrass harvested at the early vegetative stage, N_{II} napiergrass harvested at the late vegetative stage, NN_I natural fermentation of napiergrass harvested at the early vegetative stage, NN_{II} natural fermentation of napiergrass harvested at the late vegetative stage

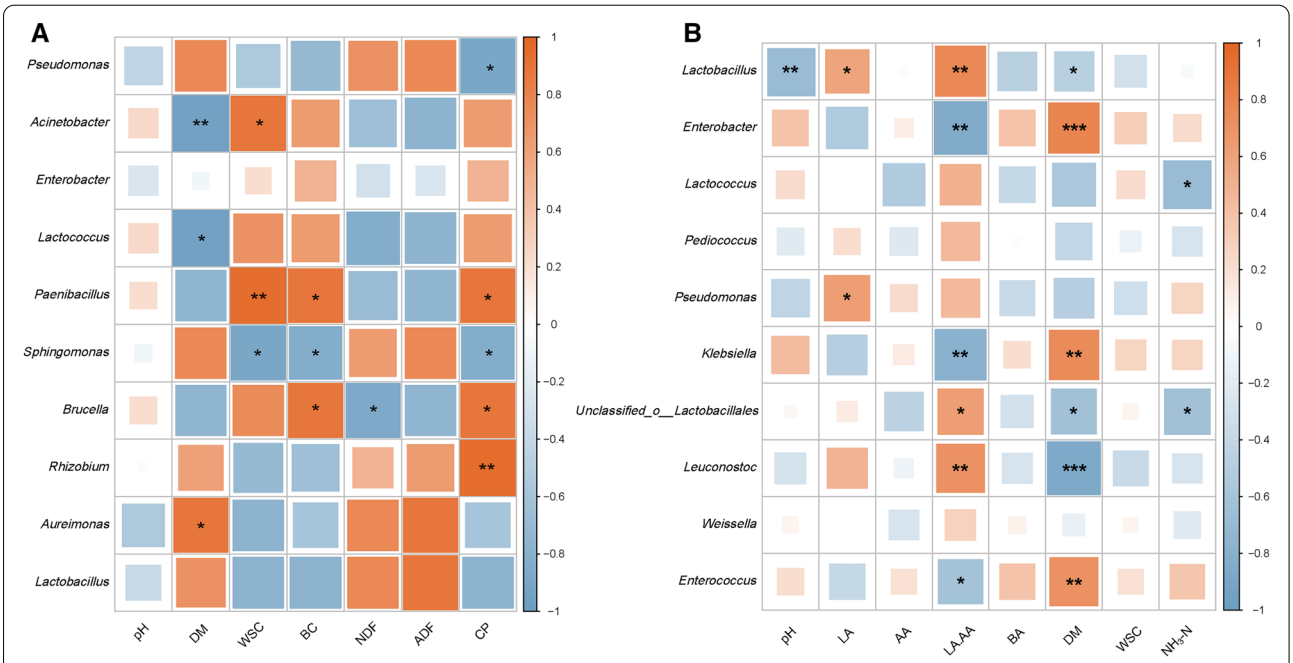


Fig. 4 Spearman's correlation heatmap of **A** chemical composition and epiphytic microbiota (top 10 genera) in fresh napiergrass and **B** fermentation parameters and bacterial community (top 10 genera) in fermented napiergrass. Orange squares represent positive correlation ($0 < r < 1$), whereas blue squares represent negative correlation ($-1 < r < 0$). *, significant at $p < 0.05$; **, significant at $0.001 < p \leq 0.01$; ***, significant at $p \leq 0.001$. *BC* buffering capacity, *CP* crude protein, *DM* dry matter, *ADF* acid detergent fiber, *WSC* water soluble carbohydrates, *NDF* neutral detergent fiber, *PA* propionic acid, *NH_3–N* ammonia–nitrogen, *AA* acetic acid, *LA* lactic acid, *LA/AA* the ratio of lactic to acetic acid

content during forage growth might be the result of the 'dilution effect' caused by the increasing proportion of cell walls. Moreover, these variations could also be attributed to the plant varieties, geographical location, climate, harvest season and fertilization. The CP content of forage grass is known to influence its BC [22], and the decrease in CP content may explain the decline in BC.

As Macarisin et al. [23] reported, the bacterial number and diversity can be sharply impacted by the development stages of leaves and plants. Throughout the growth cycle of forage grass, the external environment (e.g., solar radiation, temperature and rainfall) and internal environment (e.g., plant morphology, moisture content and leaf thickness) have been changing, and they are reported to impact microbial colonization [24]. In this work, the number of LAB and enterobacteria decreased with the increase of maturity, which may be caused by the decrease of water and WSC content on the napiergrass surface at the late harvest date. Recent studies showed that sugar and volatile organic compounds secreted by forage play an important role in determining the microbial population of forage grass [25, 26]. It is known that microbes including LAB are enriched on sugar-rich plants. Thus, the lower WSC content in N_{II} could explain its decreased number of epiphytic LAB. Moreover, the nutrient release from aging tissue and leaves in N_{II} was considered to be beneficial to microbial growth [27], which could explain the higher aerobic bacteria and yeast numbers in N_{II}.

Effects of harvest date and store time on the fermentation quality of surplus napiergrass after anaerobic fermentation

During the anaerobic fermentation of surplus napiergrass, the pH increased with the advancement of harvest date, which was in line with the result of Faria et al. [28]. Similarly, Abdelhadi and Tricarico [29] also found that the silage pH increased as the stage of forage maturity increased. The above results were considered to be related to the decrease of WSC and water content during the growth of forage. According to McDonald et al. [22], as forage matured, the decrease of water activity (a_w) and WSC content in fresh material lowed the production of LA and other organic acids during anaerobic fermentation, thereby increasing the pH value. Hence, a higher pH value and lower LA concentration were observed in NN_I than that in NN_{II}. The LA/AA of NN_I and NN_{II} were always greater than 2 throughout the anaerobic fermentation, indicating that all anaerobic fermentation of napiergrass presented lactic acid-type (homolactic) fermentation. As in most studies, AA concentration increased with the extension of store time, and this could be related to the activities of AA-producing microorganisms. The BA produced by clostridia is easily found in the anaerobic fermentation of material with DM less than 30% [7]. However, negligible BA (< 2 g/kg DM) was detected in NN_I and NN_{II}, indicating that no serious clostridial fermentation occurred in this study.

The significantly higher DM content of NN_{II} than that of NN_I could ascribe to the higher DM content in N_{II} than that in N_I. Based on the fermentation parameters, such as pH, LA/AA and BA, NN_{II} seems to be effectively preserved. However, the high NH_3–N concentration (150 g/kg TN) detected in NN_{II} suggested that the anaerobic fermentation of NN_{II} had severe protein degradation and nutrient loss. As an indicator of protein degradation, NH_3–N concentration has long been used to evaluate the fermentation quality of anaerobic fermentation. Although the appropriate DM content and low BC of N_{II} could benefit its subsequent LA fermentation, the insufficient WSC (< 50 g/kg DM) content and LAB number (< 5.0 log_{10} CFU/g FM) finally determined the worse fermentation quality of NN_{II}. The degradation degree of forage protein depends on the decline rate of pH during anaerobic fermentation [7]. The rapid pH decline of NN_I in the initial phase of anaerobic fermentation effectively inhibited the degradation and deamination of protein, resulting in the acceptable NH_3–N concentration (< 100 g/kg TN) of NN_I. Furthermore, the high proteolysis degree in aging and dead tissues of mature forage (N_{II}) might also contribute to the high NH_3–N concentration in NN_{II} [28].

The higher number of LAB in NN_I was related to the higher WSC content and epiphytic LAB of N_I. As O_2 depleted during anaerobic fermentation, the number of aerobic bacteria and molds in both harvest dates rapidly decreased to an undetectable level. In addition, the negligible yeasts and enterobacteria in NN_I could be explained by its low pH value and high LA concentration and LAB number. Furthermore, the decline of yeasts in NN_I was also associated with the massive proliferation of LAB, which reduced the niche available for yeasts.

Effects of harvest date and store time on the bacterial community of surplus napiergrass before and after anaerobic fermentation

The Coverage index of all sequenced samples was above 99%, indicating that the sequencing depth was sufficient for reliable analysis of microbial community. Bacterial alpha diversity of surplus napiergrass, characterized by Shannon, Chao1, Ace, Sobs and Simpson indexes, increased with the delay of harvest date. The anaerobic fermentation process further decreased its bacterial alpha diversities, which could be due to the deactivation of acid- and anaerobic-intolerant epiphytic bacteria [30]. Mendez-Garcia et al. [31] found that low pH is the main reason for the decrease of microbial diversity in acidic environments. The adequate WSC content (> 50 g/kg DM) and LAB number (> 5.0 log_{10} CFU/g FM) of N_I ensured the LAB proliferation and rapid acidification during anaerobic fermentation, thereby reducing the bacterial alpha diversity of NN_I.

The PCoA plots were plotted to visualize the differences in bacterial community composition between treatments as distances between symbols. The well separation of symbols N_I and N_{II} showed great differences in the composition of bacterial community for surplus napiergrass harvested at these two harvest dates, and this discrepancy could be associated with climate, the physio-biochemical characteristics of forage grass, or other factors [32]. Meanwhile, the separated clustering between the fresh and fermented samples was, as abovementioned, due to the deactivation of acid- and anaerobic-intolerant epiphytic bacteria during anaerobic fermentation. In addition, the separation among N_I, NN_I-7 and NN_I-30 suggested that the composition of bacterial community in NN_I was distinctly different at different store times. The decrease of the distance from the symbols of N_{II} and N_{II}-7 to N_{II}-7 and N_{II}-30 showed that, as ensiling proceeded, the bacterial community composition of NN_{II} tended to be similar.

This obvious succession of bacterial community from Proteobacteria to Firmicutes before and after anaerobic fermentation can ascribe to the suppression of aerobic genera (*Sphingomonas*, *Acinetobacter*, etc.) and the bloom of LAB (mainly *Lactobacillus*, *Pediococcus*, *Weissella*, and *Lactococcus*). Anaerobic conditions favor the growth of

Firmicutes, because this genus is common in anaerobic fermentation [33]. *Pediococcus*, *Weissella* and *Lactococcus* are generally considered early colonizers during anaerobic fermentation [34, 35] due to their weaker tolerance to acid compared with *Lactobacillus*. However, the initial acid environment established by these genera is particularly suitable for the proliferation of *Lactobacillus* [36], which explained the overwhelming dominance of *Lactobacillus* in both NN_I and NN_{II} after 30 days of anaerobic fermentation. As expected, the relative abundance of *Enterobacter* in NN_I decreased continuously during anaerobic fermentation, while it is worth noting that the relative abundance of *Enterobacter* in NN_{II} increased first and then decreased, and this could be related to the high pH value of 7-day NN_{II}. *Enterobacter* are commonly considered undesirable microbes because this genus can metabolize LA and WSC into AA and amino acids into ammonia [37, 38]. Although studies have shown that under anaerobic fermentation of material with 25% DM, the pH value below or equal to 4.35 can effectively inhibit the activity of *Enterobacter* [39, 40], a certain abundance of *Enterobacter* was still detected in NN_I even its pH value was low. This finding might be attributed to the existence of several acid-resistant strains of *Enterobacter*. This genus has been reported to protect itself and grow well in some adverse environments, i.e., low pH conditions [41].

Different from *Lactobacillus* and *Enterobacter*, *Pseudomonas* has not been widely studied in anaerobic fermentation. As aerobic and non-fermentative bacteria, *Pseudomonas* is generally thought to be difficult to survive under anaerobic and acidic conditions. However, after an initial inhibition, a high relative abundance of *Pseudomonas* was observed in 30-day NN_I and NN_{II}. These results are unexpected but consistent with the findings of Dong et al. [34]. Previous studies have reported that, under certain conditions, *Pseudomonas* can grow anaerobically using acetate and nitrate as electron acceptors [42]. Overall, according to the stream graphs, store time seems to have a greater influence on the bacterial community compositions of napiergrass than harvest date.

Effects of harvest date and store time on the bacterial co-occurrence networks of surplus napiergrass before and after anaerobic fermentation

Co-occurrence networks among bacterial genera for fresh and fermented napiergrass were constructed to clearly understand the effects of harvest date on the correlation and interaction of the epiphytic and anaerobic fermentative microbiome. Based on the number of nodes and edges, the complexity of bacterial networks in fresh napiergrass increased from the early vegetative stage to the late vegetative stage (Table 4, Fig. 3A, andB). Differently, anaerobic fermentation decreased the complexity of bacterial networks, with the simplest bacterial correlation structures in 30-day NN_I (Table 4, Fig. 3C–F). High fermentation quality was accompanied by low network complexity, which is consistent with Bai et al. [43].

As reported by Banerjee et al. [44], the negative correlation of co-occurrence networks indicates a possible competition for resources and common predators, while the positive correlation indicates symbiotic or cooperative relationships within microbial taxa. The lower proportion of negative correlations in the network of N_{II} (35.7%) than those in the network of N_I (47.0%) manifested that, with the growth of napiergrass, the competition among bacterial taxa in its epiphytic microbiota weakened. Co-occurrence network analyses have been increasingly applied to interrogate community stability based on topological properties [45, 46]. According to the findings of previous studies, microbial networks with lower positive correlations (or higher negative correlations) among members are more stable [47, 48]. Thus, the lower proportion of positive correlations in the network of NN_I-7, NN_{II}-7, NN_I-30 and NN_{II}-30 (47.4%, 53.1%, 46.2% and 55.6%) than those in the networks of N_I and N_{II} (53.0% and 64.3%) reflected that the bacterial networks of fermented samples were more stable than those of fresh samples.

According to Berry and Widder [49], the keystone taxa in the bacterial community can be identified by the combined scores of low betweenness centrality, high closeness centrality and high mean degree. Correspondingly, *Lactococcus*, *Hafnia-Obesumbacterium*, *Enterococcus* and *Curtobacterium* in N_I, *Microbacterium* and *Roseomonas* in N_{II}, *Lactobacillus* and *Pediococcus* in NN_I-7, *Weissella*, *Lactobacillus*, *Enterococcus* and *Pantoea* in NN_{II}-7, *Lactobacillus* and *Lactococcus* in NN_I-30, and *Lactobacillus*, *Pedicoccus* and *Kosakonia* in NN_{II}-30 were identified as the keystone taxa (Fig. 3G, H). It should be noted that the keystone taxa in this work were not necessarily the ones with the highest relative abundance. Similarly, previous studies found that although keystone taxa have considerable effects on bacterial communities and functions, their abundance is not proportional to their effects [43, 50, 51].

Relationships between chemical composition and epiphytic microbiota as well as fermentation products and bacterial communities

In fresh napiergrass, the *Acinetobacter* was negatively correlated with DM content, indicating that this genus prefers moist habitats. Indeed, *Acinetobacter* can be readily found in water, soil and sewage [14]. In addition, the significant negative correlation between WSC content and *Paenibacillus* could be due to the fact that this genus can utilize various sugars for growth [52, 53]. While the

significant positive correlation observed between CP content and *Rhizobium* could be explained by their nitrogen-fixing capacity. It is commonly known that rhizobia can convert inorganic nitrogen from the atmosphere into organic nitrogen for use by host plants. In the anaerobic fermentation of napiergrass, there were positive correlations between *Lactobacillus* and LA concentration or LA/AA but negative correlation between *Lactobacillus* and pH value or BA concentration, which further confirmed that this genus had strong acid resistance and played a crucial role in the decline of pH in late anaerobic fermentation [36]. The significant negative correlation between NH_3–N concentration and *Lactococcus* suggested that the abundant distribution of *Lactococcus* in the initial phase of anaerobic fermentation could reduce the production of NH_3–N. The positive correlation between LA concentration and *Pseudomonas* was attributed to the increased relative abundance of *Pseudomonas* on day 30 of anaerobic fermentation. Higher relative abundances of *Pseudomonas* were observed in 30-day anaerobic fermentation with high LA concentrations. Both *Serratia* and *Hafnia-Obesumbacterium* belong to *Enterobacteriaceae* and have been reported to have proteolytic activity [54], which might explain the positive correlation between these two genera and NH_3–N concentration in this work.

Conclusions

Although surplus napiergrass is considered as 'waste', it is still in good condition with low BC but high WSC and CP content. Harvest date and store time, especially store time had remarkable effects on fermentation quality, microbial community and co-occurrence networks during the anaerobic fermentation of napiergrass. The higher LA concentration and LA/AA in NN_I were related to the higher WSC content in N_I, the higher abundance of *Lactococcus* and *Pediococcus* in initial anaerobic fermentation and the higher abundance of *Lactobacillus* in late anaerobic fermentation. The NN_I and NN_{II} both displayed lactate-type fermentation, but the latter had an unacceptable NH_3–N concentration. Therefore, anaerobic fermentation of N_I for 15 days or more is recommended for the cleaner production of surplus napiergrass. Anaerobic fermentation improved the quality and add-value of surplus napiergrass in terms of fermentation characteristics, bacterial community and functional profiles, and can be a clean solution for leftover forage grass.

Abbreviations

N_I: Napiergrass harvested at the early vegetative stage; N_{II}: Napiergrass harvested at the late vegetative stage; NN_I: Natural fermentation of N_I; NN_{II}: Natural fermentation of N_{II}; DM: Dry matter; LAB: Lactic acid bacteria; WSC: Water-soluble carbohydrates; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; TN: Total nitrogen; CP: Crude protein; BC: Buffering capacity; NH_3–N: Ammonia–nitrogen; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; CFU: Colony-forming units; FM: Fresh material; OTUs: Operational taxonomic units; RDP: Ribosomal database project; PCoA: Principal coordinates analysis; G: Harvest date; D: Store time; G × D: The interaction of harvest date and store time; LA/AA: The ratio of lactic to acetic acid.

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Author contributions

J–Z: methodology, formal analysis, visualization, writing—original draft preparation. X–Y: validation, methodology, software. J–L: investigation, formal analysis. S–W and Z–D: methodology, visualization. T–S: conceptualization, project administration, resources, data curation, supervision, funding acquisition, writing—review and editing. 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

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Competing interests

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