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Relationships between microbial community, functional profile and fermentation quality during ensiling of hybrid *Pennisetum*

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ABSTRACT. With the increasing shortage of feed resources, hybrid *Pennisetum* (HE) shows promise as a potential animal feedstuff. The present study aimed to evaluate the fermentation quality, microbial community and functional shifts in HE during ensiling. HE was ensiled naturally (NHE) for 1, 3, 7, 15, 30 and 60 days. After 60 days of ensiling, NHE presented acetate-type fermentation with a high pH value (5.02), as well as acetic acid, butyric acid (0.33% DM), ethanol and ammonia nitrogen (14.0% TN) concentrations, while low lactic acid levels. *Enterobacter* (45.57%), *Klebsiella* (12.95%) and *Lactococcus* (10.24%) were dominant in the 3-day NHE, while *Enterobacter* (26.27%), *Clostridium*_sensu_stricto_11 (21.15%) and *Lactobacillus* (18.51%) were the most abundant genera in the 60-day NHE. Spearman's correlation heatmap revealed a positive relationship between *Clostridium_sensu_stricto*_1 and ammonia nitrogen, ethanol and butyric acid concentrations. Our analysis of functional profiles obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed significant differences between fresh and ensiled HE. The ensiling process suppressed amino acid, cofactors, vitamins and energy metabolism, but promoted metabolism associated with carbohydrates and nucleotides. The results of next-generation sequencing in combination with KEGG functional predictions revealed distinct differences in the initial and late phases of ensiling both in terms of community succession and functional shifts. Additional management measures such as delayed harvest or lactic acid bacteria inoculation are necessary to reduce nutrient loss and improve the fermentation quality of NHE.

Introduction

With the rapid development of animal husbandry, there has been an increased demand for forages. To alleviate the supply pressure of maize silage, it is necessary to find high yielding grasses that could be used in feed production. Hybrid *Pennisetum*, a cross of *Pennisetum americanum* and *P. purpureum*, is a perennial gramineous grass that is widely cultivated as a forage or energy grass (Cai et al., 2020). It is grown in humid, semi-humid and semi-arid regions of tropical and subtropical regions due to its strong resistance to adverse conditions such as drought, waterlogging, salinity and high temperatures (Tian et al., 2022). It has also been reported that hybrid *Pennisetum* can be mowed 5 to 6 times, producing 210 tons of fresh matter (FM) per hectare annually (Zhang et al., 2019); however, the high moisture content of tropical grasses, including hybrid *Pennisetum* cannot be ignored during agricultural production (Oliveira et al., 2020). While the low moisture of forage at harvests can be solved by direct water

addition, wilting of high-moisture forage additionally increases the cost of labour or drying equipment. Considering the rainy climate and seasonal surplus in these planting areas, silage development is the preferred processing method for hybrid *Pennisetum* over hay-making.

Ensilage is a lactic acid bacteria (LAB)-driven anaerobic fermentation involving complex microbial interactions (Keshri et al., 2019). Therefore, it is crucial to extensively study the microbial ecology of the silage process (Keshri et al., 2019). With the advancement of silage research, traditional culture-dependent methods have proven insufficient to fully understand the succession of microbial communities from forage grass to silage. Fortunately, recent advances in culture-independent technologies, represented by next-generation sequencing, have enabled researchers to explore changes in the microbial community involved in the ensiling process (McAllister et al., 2018). While next-generation sequencing has provided valuable insights into the microbial communities involved in silage studies, there is still a lack of knowledge regarding the functional annotation of these communities, particularly in the case of hybrid *Pennisetum*. To date, only one study has explored the effects of various additives on the bacterial community and metabolic profile of hybrid *Pennisetum* silage (Tian et al., 2022). Compared with conventional maize and alfalfa silages, there is little research on hybrid *Pennisetum* silage, which may limit its widespread use as feed. Asshauer et al. (2015) have highlighted the importance of phylogenetic and functional diversity in microbial community analyses. Therefore, the prediction of higher-order functional profiles associated with silage microbial community can provide additional valuable information and supplement 16S rRNA analysis (Kanehisa and Goto, 2000).

The present study evaluated the fermentation quality, microbial community and functional shifts in hybrid *Pennisetum* (HE) during ensiling, providing insights into the fermentation mechanisms and the regulation of tropical grass silage fermentation.

Material and methods

Silage preparation

Hybrid *Pennisetum* (Tift 23A × N_{51}) was planted in the experimental field of Baima National Agricultural-tech Zone (31′61°N, 119′18°E, altitude 25.2 m, Jiangsu, China). The trial field (30 m^2) was divided evenly into three blocks (replicates) and eight plots in each block were randomly selected for silage production. After 12 weeks of growth, HE at the vegetative stage was mowed and chopped into ~20 mm fragments by a crop chopper. After thorough mixing, the cut HE was split into two parts for silage preparation and fresh sample analysis. Specifically, approximately 500 g of the mixed material was loaded into a presterilized laboratory-scale silo (50 × 30 cm polythene plastic bag), vacuum-sealed and incubated at ambient temperature (29 ± 3 °C). The treatment silos (three per treatment) were opened and sampled after 1, 3, 7, 15, 30 and 60 days of ensiling.

Chemical, microbial and fermentation parameter analyses

The chemical and microbial parameters were analysed as described in our previous study (Zhao et al., 2018). Briefly, HE or naturally ensiled HE (NHE) was oven-dried at 65 °C to a constant weight, ground and sieved through a 1 mm screen. The content of water-soluble carbohydrates (WSC) was quantified by the anthrone-sulphuric acid method (Thomas, 1977). Neutral and acid detergent fibre (NDF and ADF) contents were analysed using an Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). Total nitrogen (TN) content was determined using a Kjeltec 8200 Kjeldahl Nitrogen Analyzer (Foss Analytics, Höganäs, Sweden), and crude protein (CP) content was calculated by multiplying TN by 6.25. Buffering capacity (BC) was quantified by titration, as described by Playne and McDonald (1966). After shaking 10 g of HE or NHE in 90 ml sterile saline solution at 120 rpm, at 37 °C for 30 min, 1 ml of the resulting solution was serially diluted to count LAB (anaerobic incubation at 37 °C for 48 h), aerobic bacteria (aerobic incubation at 37 °C for 48 h), yeasts (aerobic incubation at 30 °C for 48 h), moulds (aerobic incubation at 30 °C for 48 h) and enterobacteria (aerobic incubation at 37 °C for 24 h). For the analysis of fermentation parameters, 90 ml of distilled water was added to 30 g HE or NHE and incubated at 4 °C for 30 min. After filtering through 4 layers of cheesecloth and filter paper, the pH of HE or NHE was recorded using a HI 2221 pH/mV/°C bench meter (Hanna Instruments Inc., Woonsocket, RI, USA). Ammonia-N (NH_3-N) concentration in NHE was determined colorimetrically after reaction with phenol and hypochloric acid (Broderick and Kang, 1980). The concentrations of lactic acid (LA), acetic acid (AA), propionic acid (PA), n-butyric acid (BA), isobutyric acid (IBA) and ethanol in NHE were analysed using a 1260 Infinity HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). The concentration of volatile fatty acids (VFA) was calculated as the sum of AA, PA, BA and IBA concentrations.

Next-generation sequencing and functional prediction analyses

Preparatory work for next-generation sequencing (bacterial DNA isolation and PCR amplification) was performed following the procedure reported by Zhao et al. (2022). The obtained purified amplicons were pooled at equimolar concentrations and paired-end sequenced using a MiSeq PE300 platform (Illumina Inc., San Diego, CA, USA). The resulting paired-end sequencing files were first merged using FLASH software (v 1.2.11), and the obtained raw sequence data were quality-filtered using QIIME software (v 1.9.1) to retain sequences with quality scores > 20. Operational taxonomic units (OTUs) with > 7% identity were clustered using UPARSE software (v 7.0.0), and chimeric sequences were removed using UCHIME software (v 4.1). After matching against the 132 SILVA rRNA database, the taxonomy of OTUs was assigned using RDP classifier (v 2.11). Bacterial α diversity (Shannon, Chao, Ace, Sobs and Simpson indexes) and Bray-Curtis metric β diversity was calculated using QIIME software (v 1.9.1). The principal coordinate analysis (PCoA) plot of the Bray-Curtis distance metric was constructed using the vegan R package (v 4.1.2). Spearman's correlation heatmap between fermentation products and bacterial communities was constructed using the pheatmap R package (v 1.0.12). Kyoto Encyclopedia of Genes and Genomes (KEGG) bacterial metabolic profiles for HE, NHE-3 and NHE-60 were predicted using Tax4fun developed by Asshauer et al. (2015).

Statistical analysis

A one-way ANOVA was applied to investigate the effects of ensiling time on fermentation parameters, chemical composition, microbial abundance and α diversity indices of silages based on GLM of the SAS system (ver. 9.2; SAS Institute Inc., Cary, NC, USA). The analysis of similarity (ANOSIM) with 999 permutations and Wilcoxon rank-sum test were adopted to determine whether significant differences occurred in the β diversity and KEGG metabolic profiles of the bacterial community, respectively. A significance level of $P < 0.05$ was applied.

Results

Fresh HE characteristics

In fresh HE, DM content was 19.2% FM, WSC – 6.62% DM and CP – 5.34% DM. The BC value was 63.5 mEq/kg DM. The count of LAB, aerobic bacteria, yeasts and enterobacteria was 4.71, 9.02, 4.38 and 8.91 $\log_{10}$ CFU/g FM, respectively. The mould count was below the detectable level (Table 1).

Table 1. Chemical and microbial composition of hybrid *Pennisetum* (HE)

Items	HE
pH	5.94 ± 0.07
DM, % FM	19.2 ± 1.55
WSC, % DM	6.62 ± 0.22
CP, % DM	5.34 ± 0.18
BC, mEq/kg DM	63.5 ± 3.56
NDF, % DM	67.7 ± 1.42
ADF, % DM	35.4 ± 1.07
LAB, $\log_{10}$ CFU/g FM	4.71 ± 0.07
Aerobic bacteria, $\log_{10}$ CFU/g FM	9.02 ± 0.03
Yeasts, $\log_{10}$ CFU/g FM	4.38 ± 0.06
Moulds, $\log_{10}$ CFU/g FM	<2.00
Enterobacteria, $\log_{10}$ CFU/g FM	8.91 ± 0.05

DM – dry matter, FM – fresh matter, WSC – water-soluble carbohydrates, BC – buffering capacity, NDF – neutral detergent fibre, ADF – acid detergent fibre, CP – crude protein, LAB – lactic acid bacteria, CFU – colony-forming units; data are presented as mean value ± SEM (standard error of the mean)

NHE fermentation characteristics

Ensiling time had a significant ($P < 0.05$) effect on pH, DM and WSC contents, LA, AA, PA, BA, IBA, VFA and NH_3-N levels, as well as the lactic to acetic acid ratio (LA/AA) (Table 2). The pH did not change ($P > 0.05$) during the first 7 days of ensiling, then decreased significantly ($P < 0.05$), reaching its lowest value of 4.60 on day 15 of ensiling to slowly increased in the later phase. Conversely, the concentration of LA increased significantly, reaching a maximum on day 15 of ensiling at 3.96% DM. The concentrations of AA, PA, BA, IBA and VFA increased as ensiling progressed. The LA/AA ratio fluctuated throughout the ensiling process. BA was detected in all NHE samples, with the exception of 1-day NHE. DM and WSC contents decreased ($P < 0.01$) with ensiling time, while NH_3-N concentration increased ($P < 0.001$).

The length of ensiling had a significant ($P < 0.001$) impact on the counts of LAB, yeasts and enterobacteria (Table 3). The LAB count slowly ($P > 0.05$) increased to reach the maximum at 6.14 $\log_{10}$ CFU/g FM in the first 30 days of ensiling, and then decreased significantly ($P < 0.05$). In contrast, the yeast count declined steadily to low levels ($P < 0.05$), while the enterobacteria count first decreased and then started to raise.

Table 2. Changes in the chemical composition and fermentation quality of natural ensiling of hybrid *Pennisetum*

Items	Ensiling time, days						SEM	*P*-value
	1	3	7	15	30	60		
pH	5.72^{A}	5.36^{AB}	5.39^{AB}	4.60^{B}	4.73^{AB}	5.02^{AB}	0.120	0.032
LA, % DM	0.48^{C}	0.74^{C}	1.03^{BC}	3.96^{A}	3.68^{A}	2.65^{AB}	0.354	<0.001
AA, % DM	0.31^{D}	0.74^{CD}	0.97^{BCD}	1.53^{ABC}	1.96^{AB}	2.22^{A}	0.177	<0.001
LA/AA	1.55	1.02	1.11	2.69	1.97	1.31	0.180	0.047
PA, % DM	0.02^{B}	0.04^{B}	0.04^{B}	0.05^{B}	0.09^{AB}	0.17^{A}	0.013	<0.001
BA, % DM	0.00^{C}	0.03^{BC}	0.08^{BC}	0.12^{BC}	0.16^{B}	0.33^{A}	0.028	<0.001
IBA	0.01^{B}	0.02^{B}	0.10^{AB}	0.10^{AB}	0.13^{AB}	0.25^{A}	0.022	0.003
VFA, % DM	0.34^{D}	0.82^{CD}	1.19^{BCD}	1.80^{ABC}	2.35^{AB}	2.98^{A}	0.232	<0.001
Ethanol, % DM	0.07^{C}	0.38^{BC}	1.08^{ABC}	1.74^{AB}	2.12^{A}	2.04^{A}	0.218	0.003
DM, % FM	19.6^{A}	18.8^{AB}	18.4^{ABC}	18.0^{ABC}	17.0^{BC}	16.9^{C}	0.262	0.004
WSC, % DM	4.99^{A}	3.95^{B}	2.94^{C}	2.52^{CD}	1.99^{DE}	1.58^{E}	0.277	<0.001
NH_3-N, % TN	4.63^{C}	5.31^{C}	7.45^{BC}	8.22^{BC}	10.1^{AB}	14.0^{A}	0.802	<0.001

DM – dry matter, FM – fresh matter, LA – lactic acid, AA – acetic acid, LA/AA – ratio of lactic to acetic acid, PA – propionic acid, BA – n-butyric acid, IBA – isobutyric acid, WSC – water-soluble carbohydrates, NH_3-N – ammonia nitrogen, TN – total nitrogen, SEM – standard error of the mean; $^{A–E}$ – means with different letters differ significantly at $P < 0.05$

Table 3. Changes in microbial abundance in natural ensiling of hybrid *Pennisetum*

Items	Ensiling time, days						SEM	*P*-value
	1	3	7	15	30	60		
LAB, $\log_{10}$ CFU/g FM	4.93^{C}	5.35^{BC}	5.39^{BC}	5.90^{AB}	6.14^{A}	5.30^{C}	0.103	<0.001
Aerobic bacteria, $\log_{10}$ CFU/g FM	6.38^{B}	4.94	<2.00	ND	ND	<2.00	–	–
Yeasts, $\log_{10}$ CFU/g FM	5.42^{AB}	5.67^{A}	4.43^{B}	4.31^{BC}	3.26^{CD}	3.13^{D}	0.234	<0.001
Moulds, $\log_{10}$ CFU/g FM	2.70	<2.00	ND	ND	ND	<2.00	–	–
Enterobacteria, $\log_{10}$ CFU/g FM	6.87^{A}	6.75^{AB}	5.99^{AB}	4.32^{C}	5.70^{B}	5.74^{B}	0.212	<0.001

LAB – lactic acid bacteria, CFU – colony-forming units, FM – fresh matter, SEM – standard error of the mean; $^{A–D}$ – means with different letters differ significantly at $P < 0.05$

HE and NHE bacterial community

Figure 1 presents the bacterial α-diversities in fresh HE and NHE. The Shannon, Chao1, Ace and Sobs indices were highest in fresh HE, followed by NHE-60 and finally NHE-3, while the Simpson index showed the opposite trend. The coverage index for each sequenced sample was more than 99.5%. Figure 2 displays the Bray-Curtis distance metric PCoA plot, revealing the differences in bacterial β-diversity. HE, NHE-3 and NHE-60 symbols are located in different quadrants.

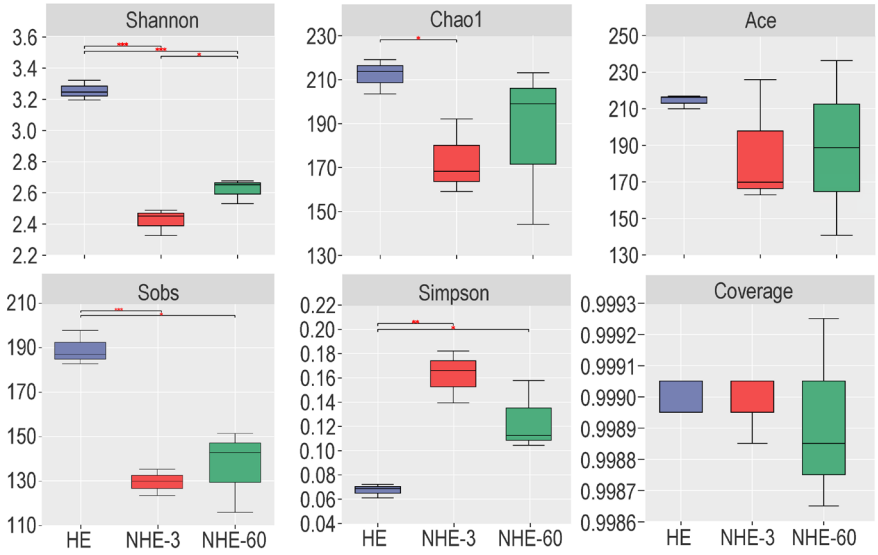

Figure 1. Bacterial α-diversities (Shannon, Chao, Ace, Sobs, Simpson and Coverage indices) of fresh HE and NHE
HE – hybrid Pennisetum, NHE – natural ensiling of HE; * $P < 0.05$, ** $0.001 < P \leq 0.01$, *** $P \leq 0.001$

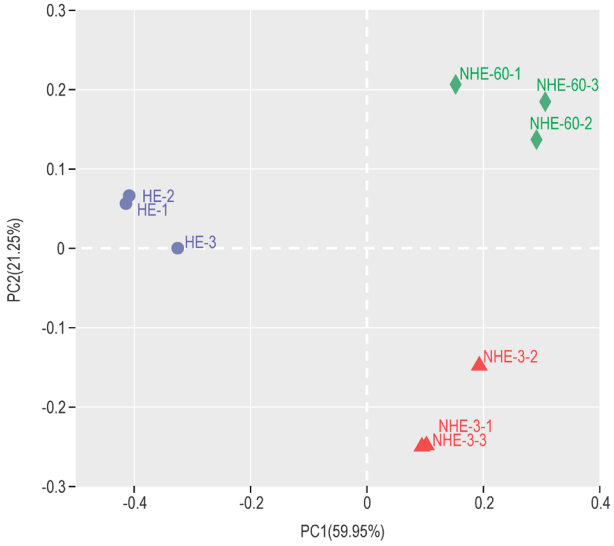

Figure 2. Bacterial β-diversity of HE and NHE, calculated using principal coordinates analysis (PCoA) plot based on the Bray-Curtis distance metric
HE – hybrid Pennisetum, NHE – natural ensiling of HE

At the phylum level (Figure 3A), Proteobacteria and Firmicutes were the most abundant phyla in fresh HE, accounting for 47.3% and 46.9% of the relative abundance of bacterial community, respectively. After 3 days of ensiling (Figure 3C), the relative abundance of Proteobacteria increased, while that of Firmicutes and Actinobacteriota decreased. After 60 days of ensiling (Figure 3E), Firmicutes became the dominant phylum (62.6%), followed by Proteobacteria (37.1%).

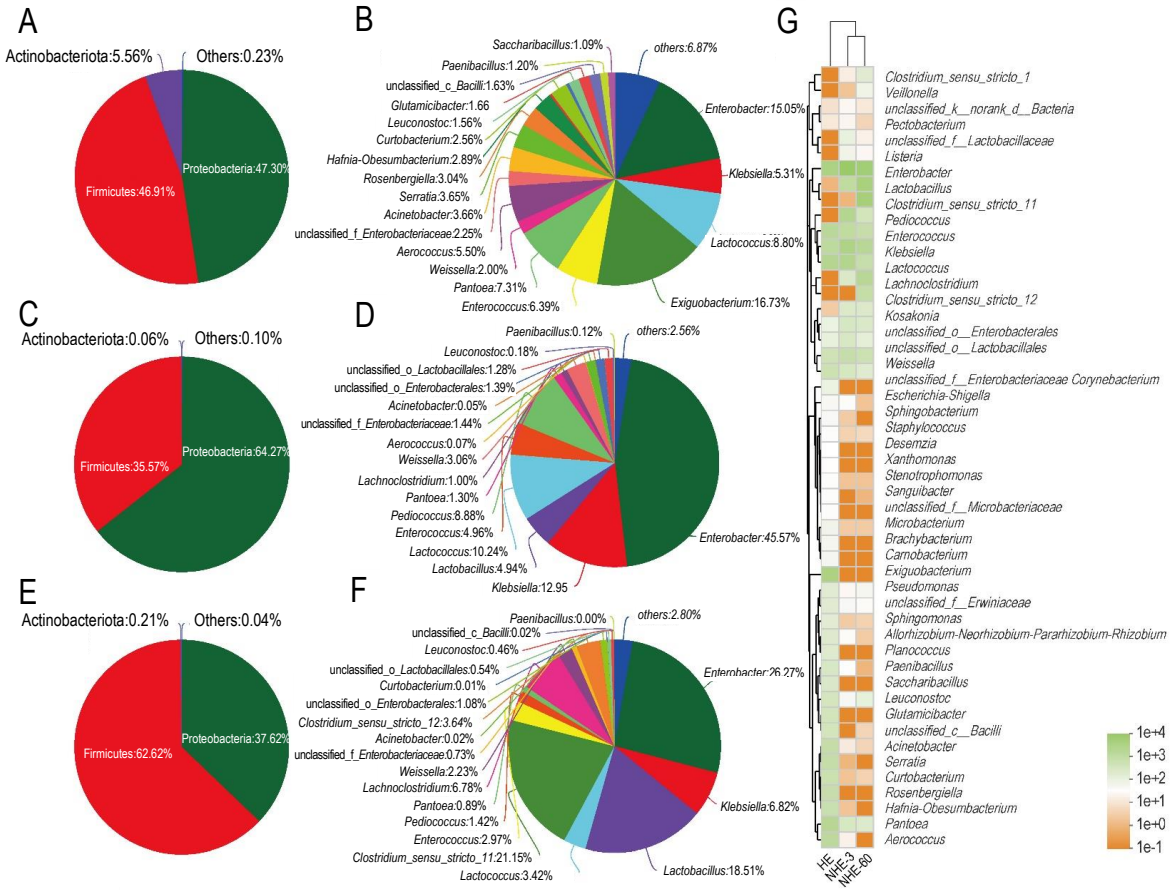

Figure 3. Relative abundance of bacterial community at phylum (A, C, E) and genus (B, D, F) levels. Community heatmap analysis at genus (top 50) level in HE and NHE (G)
HE – hybrid Pennisetum, NHE – natural ensiling of HE

At the genus level (Figure 3B), *Exiguobacterium* (16.7%), *Enterobacter* (15.1%) and *Lactococcus* (8.80%) were the main genera in fresh HE. After 3 days of ensiling (Figure 3D), the relative abundance of *Enterobacter*, *Klebsiella*, and *Lactococcus* increased from 15.1%, 5.31% and 8.80% to 45.6%, 13.0% and 10.2%, respectively. After 60 days of ensiling (Figure 3F), *Enterobacter*, *Clostridium_sensu_stricto_*11 and *Lactobacillus* dominated the bacterial community in NHE, with relative abundance of 26.3%, 21.2% and 18.5%, respectively. As ensiling progressed, the relative abundance of *Exiguobacterium*, *Aerococcus*, *Acinetobacter*, *Serratia*, *Rosenbergiella*, and other bacterial genera rapidly fell below detectable limits (< 1%). However, a non-negligible relative abundance of *Klebsiella* (6.82%) was still detected in NHE-60.

Correlation analysis of fermentation parameters and bacterial community

Spearman's correlation heatmap (Figure 4) demonstrated positive correlations between the pH value and the relative abundance of *Klebsiella* or *Enterobacter* ($P < 0.05$). The relative abundance of *Lactococcus* was negatively correlated with NH_3-N, ethanol, IBA, PA and BA levels ($P < 0.01$), but positively correlated with WSC content ($P < 0.05$). On the other hand, the relative abundance of *Clostridium_sensu_stricto_*1 was positively correlated with NH_3-N, ethanol, IBA, PA and BA concentrations, while being negatively correlated with the count of aerobic bacteria ($P < 0.05$). Moreover, the relative abundance of all detected *Clostridium* (*Clostridium_sensu_stricto_*1, 11 and 12) was negatively correlated with DM content.

KEGG functional prediction for the bacterial community

The predicted KEGG functional categories for the bacterial community is presented in Figure 5. At pathway level 1, metabolism was the most common functional category. At pathway level 2, carbohydrate metabolism and amino acid metabolism were the major functional categories of metabolism. The extent of carbohydrate metabolism and amino acid metabolism differed significantly ($P < 0.05$) between HE, NHE-3 and NHE-60. In particular, NHE-60 showed the highest level of carbohydrate metabolism, followed by NHE-3 and fresh HE. Conversely, fresh HE demonstrated the highest amino acid metabolism and NHE-60 had the lowest.

Carbohydrate metabolism and amino acid metabolism were further analysed at pathway level 3 (Figure 6). For carbohydrate metabolism, the

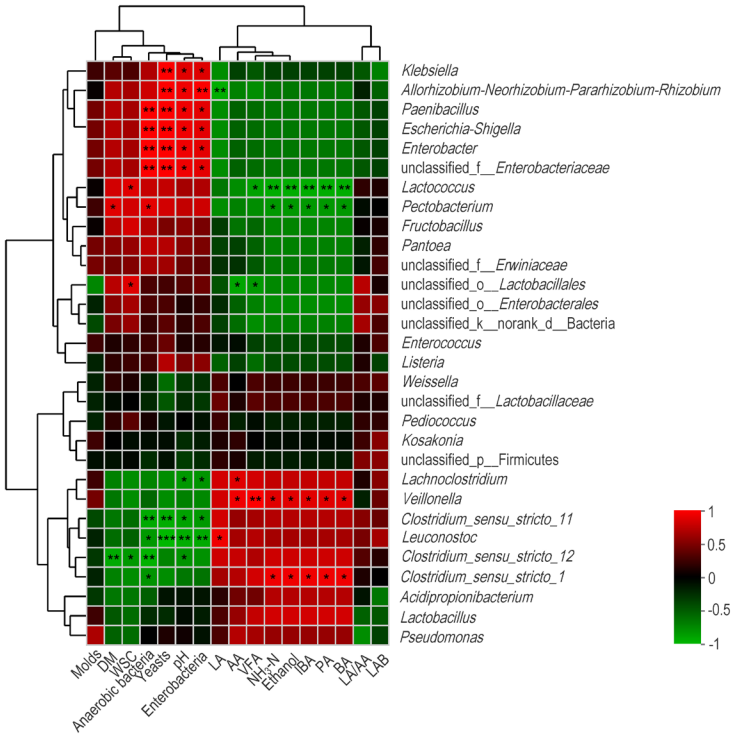

Figure 4. Spearman's correlation heatmap of fermentation parameters and top 30 genera in NHE. Red squares refer to positive correlations ($0 < r < 1$), whereas green squares indicate negative correlations ($-1 < R < 0$)

DM – dry matter, WSC – water-soluble carbohydrates, LA – lactic acid, AA – acetic acid, VFA – volatile fatty acid, NH_3-N – ammonia nitrogen, IBA – isobutyric acid, PA – propionic acid, BA – n-butyric acid, LA/AA – the ratio of lactic to acetic acid, LAB – lactic acid bacteria; * $P < 0.05$, ** $0.001 < P \leq 0.01$, *** $P \leq 0.001$

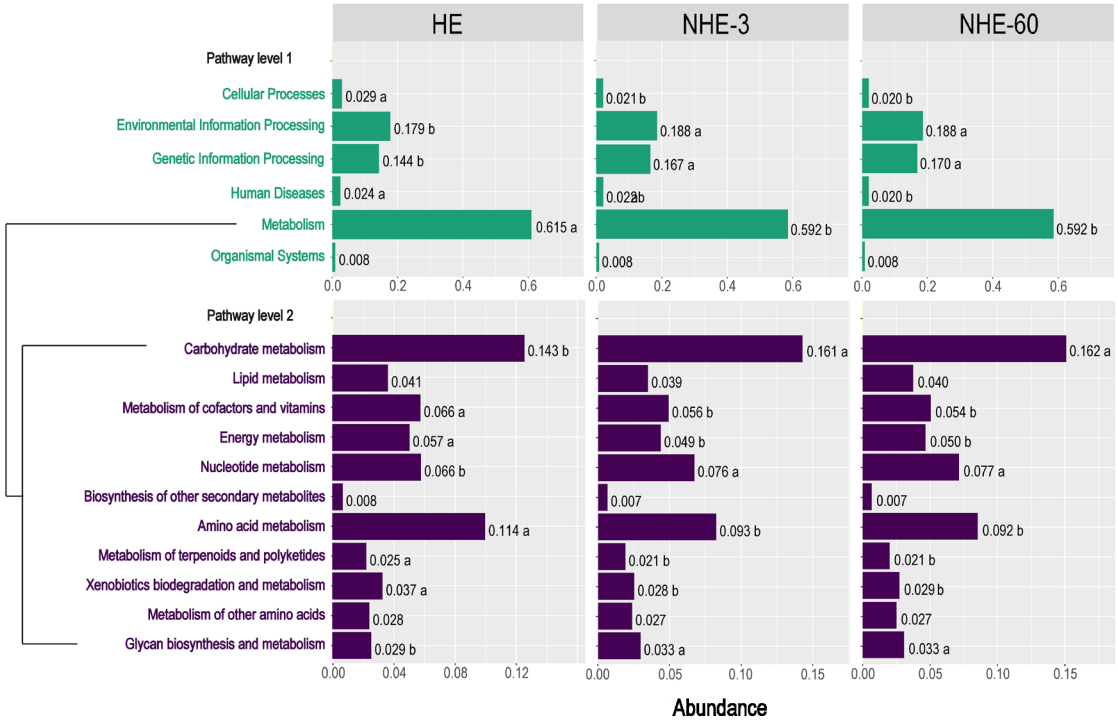

Figure 5. KEGG function profiles predicted based on the 16S rRNA gene sequencing at pathway level 1 and pathway level 2 (A). Means with different letters differ significantly at $P < 0.05$

HE – hybrid Pennisetum, NHE – natural ensiling of HE

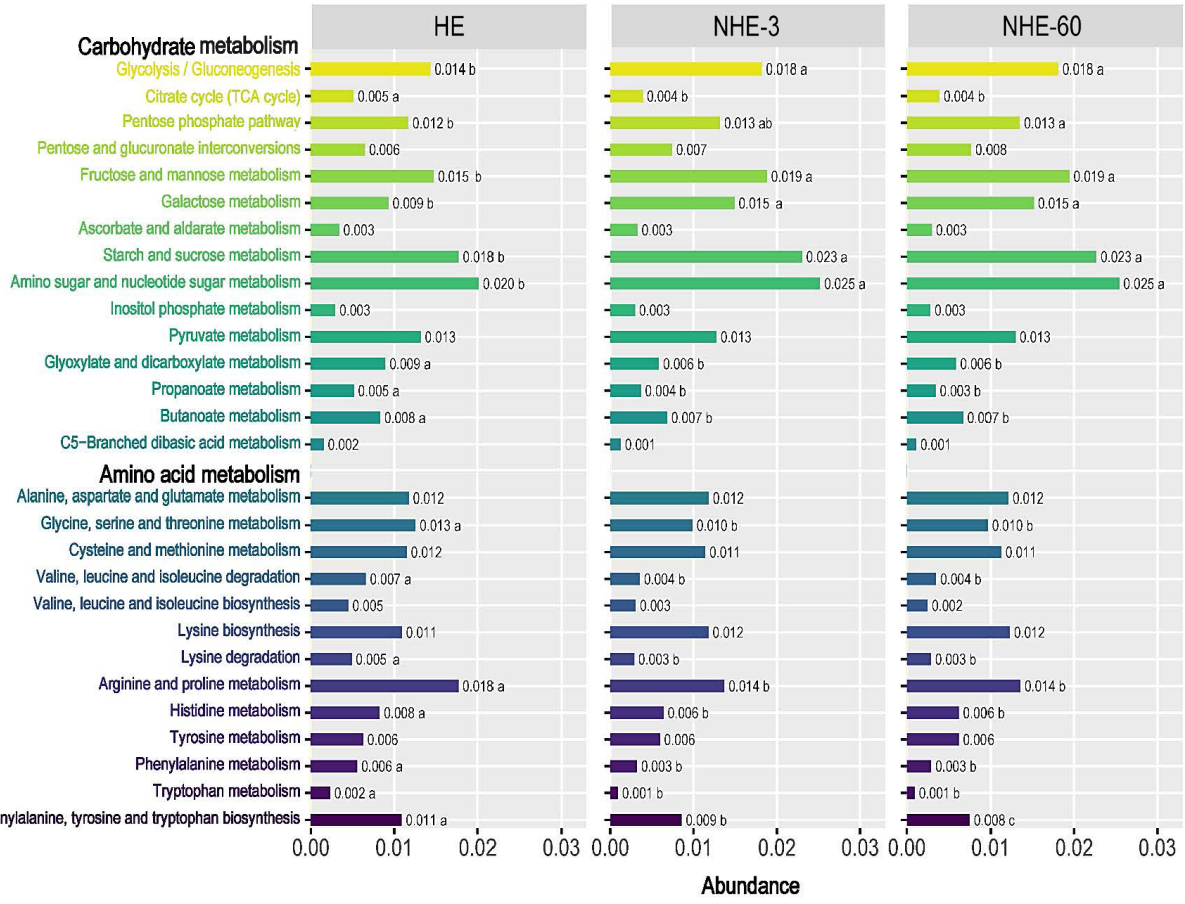

Figure 6. Carbohydrate metabolism and amino acid metabolism predicted based on 16S rRNA gene sequencing at pathway level 3. Means with different letters differ significantly at $P < 0.05$

HE – hybrid *Pennisetum*, NHE – natural ensiling of HE

abundance of glycolysis/gluconeogenesis, pentose phosphate pathway, fructose, mannose, galactose, starch, sucrose, amino sugar and nucleotide sugar metabolism was significantly ($P < 0.05$) higher in NHE compared to HE, while that of citrate cycle (TCA cycle), glyoxylate, dicarboxylate, propanoate and butanoate metabolism was significantly ($P < 0.05$) reduced in NHE in relation to HE. With respect to amino acid metabolism, the abundance of glycine, serine, threonine, arginine, proline, histidine, phenylalanine and tryptophan metabolism, valine, leucine, isoleucine and lysine degradation, as well as phenylalanine, tyrosine and tryptophan biosynthesis was significantly ($P < 0.05$) lower in NHE compared to HE.

Discussion

Initial levels of available sugar and epiphytic LAB are key determinants of the final quality of silage (McDonald et al., 1991). Although the WSC content (> 5% DM) and BC (63.5 mEq/kg DM) of fresh HE met the requirement of a well-preserved silage (McDonald et al., 1991) in the current study, low DM content (< 20% FM), insufficient epiphytic LAB count (< 5.0 $\log_{10}$ CFU/g FM) and high abundance of aerobic bacteria and enterobacteria (> 8.0 $\log_{10}$ CFU/g FM) presented challenges for HE silage production.

The rate of pH decline during ensiling is an indicator of silage preservation quality, and a slower decline in pH increases the risk of silage failure. In this study, the pH of NHE remained high throughout ensiling due to the insufficient number of LAB (< 5.0 $\log_{10}$ CFU/g FM) in HE and low LA production in NHE. As reported in many studies, the concentration of AA in this experiment also increased during ensiling, possibly due to the activity of acetate-producing microbes in the early stage of ensiling, and the transformation of homolactic fermentation to heterolactic fermentation in the late stage of ensiling (Esen et al., 2022). The LA/AA ratio for all time points remained below 3.0, indicating that the fermentation pattern of NHE in this study was dominated by acetate-type fermentation, which was also reflected in the high concentrations of AA, PA, VFA and ethanol.

PA, BA and NH_3-N are known to be detrimental to silage quality (Kung et al., 2018), but we recorded acceptable PA levels (< 1% DM) (Agarussi et al., 2019) in NHE throughout the ensiling process. However, the final BA concentration in NHE (0.33% DM) exceeded the maximum limit (0.2% DM) for well-preserved silage (McDonald et al., 1991), indicating the occurrence of distinct clostridial fermentation. The unacceptable BA concentration in NHE could be due to its low DM content, as BA is often detected in silages with DM less than 30% (van Niekerk et al., 2010). Moreover, high NH_3-N concentrations (> 10% TN) were also detected at the end of ensiling, indicating the occurrence of intensive proteolysis in NHE. Protein degradation and ammonia generation during ensiling is a complex biochemical process involving the activity of plant proteases, clostridia, enterobacteria, etc., and the degree of protein and amino acid degradation in silage depends on the pH decline rate (van Niekerk et al., 2010). Thus, the slow pH decrease in NHE in the initial phase of ensiling could not effectively inhibit protein degradation, resulting in high NH_3-N concentration. Furthermore, the high moisture content of HE may have also contributed to the high NH_3-N concentration in the resulting silage, as it has been previously reported that it can promote plant protease activity (He et al., 2020).

The slow proliferation of LAB during the initial phase of ensiling was associated with an insufficient abundance of epiphytic LAB (< 5.0 $\log_{10}$ CFU/g FM). As anticipated, the number of strict aerobes (aerobic bacteria and moulds) decreased rapidly to negligible levels in the anaerobic silo environment, while the high abundance of yeasts and enterobacteria during the whole ensiling process was connected to the consistently high pH value.

The coverage index for each sequenced sample was above 99%, suggesting that most of the bacterial community had been accurately captured. Prior research has suggested that pH decline can reduce microbial diversity in the environment (Mendez-Garcia et al., 2015). Similar results were also observed in this study, namely, lower pH was associated with a lower α-diversity of bacteria, likely due to the inactivation of acid-intolerant aerobic bacteria (Dong et al., 2020).

The differences in bacterial community composition on the PCoA plot are directly visualized as distances between symbols. HE, NHE-3 and NHE-60 symbols were clearly distributed in different quadrants, indicating large differences in bacterial community composition in fresh HE before and after ensiling. These differences could be attributed to the inactivation of aerobic and acid-intolerant epiphytic bacteria during ensiling, as mentioned earlier.

The increase in the number of Proteobacteria and decrease in Firmicutes after 3 days of ensiling is an uncommon phenomenon, which has been mainly associated with the proliferation of *Enterobacter* and *Klebsiella* (Wang et al., 2021; Zhang et al., 2022). This phenomenon could be explained by the early

stage ensiling conditions, characterized by a higher pH (> 5.0) and a micro-oxygen environment (oxygen remaining after sealing), which favoured the growth of Proteobacteria over Firmicutes, leading to a brief period of Proteobacteria dominance. Although Ogunade et al. (2018) found that Proteobacteria and Firmicutes were the dominant members of the silage bacterial community, it was observed that Proteobacteria preferred a neutral environment (Brenner et al., 2005). The observed succession from Proteobacteria to Firmicutes before and after ensiling could be ascribed to the inhibition of *Acinetobacter*, *Curtobacterium*, etc., as well as the prevalence of *Lactobacillus* and *Clostridium_sensu_stricto*_11 and *Clostridium_sensu_stricto*_12. Overall, the ensiling microenvironment benefits Firmicutes as they thrive under anaerobic and acidic conditions (Zhao et al., 2017); however, the dominant bacterial genera in well-preserved silage should be composed of *Lactococcus*, *Pediococcus*, *Weissella* and *Lactobacillus*. The genera *Lactococcus, Pediococcus*, and *Weissella* are essential for silage fermentation, because although they are less tolerant to acid than *Lactobacillus*, they establish an initial acidic environment suitable for the growth of the latter genus, which is crucial for successful silage fermentation (Muck, 2013; Graf et al., 2016). Nevertheless, during the ensiling process, the bacterial community in NHE shifted from being dominated by *Enterobacter* and *Klebsiella* (day 3) to being mainly composed of *Enterobacter* and *Clostridium_sensu_stricto*_11 (day 60). *Enterobacter* is known for its ability to degrade protein and produce NH3-N, while *Klebsiella* is a ubiquitous pathogen commonly found in water, faeces, and soil (Zhang et al., 2020; Zheng et al., 2022). Their presence in silage can lead to a disruption of its aerobic stability and emergence of various animal diseases, such as mastitis (Zadoks et al., 2011). Several species of clostridia, including *Clostridium botulinum*, *C. perfringens* and *C. butyricum* have been found to produce botulinum toxins, posing a health threat to livestock (Popoff, 2018). Hence, the abundant presence of these genera at the end of ensiling, which could be related to the high silage pH (> 5.0), was undesirable and unacceptable. It was reported that a pH value of 4.35 was required to effectively suppress *Enterobacter* activity in 25% DM silage (Pedroso et al., 2010). Clostridia have the ability to form spores under adverse conditions such as nutritional limitations, extreme temperature, pH changes, and UV penetration, remaining dormant until external conditions become appropriate, such as a silage pH > 5.0, triggering their reactivation (Doyle et al., 2015).

In NHE, *Klebsiella* and *Enterobacter* were positively correlated with pH value, suggesting that these two genera were acid-intolerant. Their high relative abundance throughout the ensiling process was attributed to the consistently high pH value in NHE. Rapid acidification is the key to inhibiting the growth of these genera in silage. On the other hand, the positive correlation between *Lactococcus* and WSC content, and the negative correlation between *Lactococcus* and undesirable fermentation parameters was expected. *Lactococcus*, as an early colonizer in silage fermentation, effectively converts WSC into LA, inducing initial acidification, thereby inhibiting the activity of undesirable microorganisms. A higher abundance of *Lactococcus* leads to faster acidification, resulting in less production of NH_3-N, ethanol, PA, BA and IBA. In general, during ensiling, NH_3-N is generated by plant protease, enterobacteria, and/or clostridia. However, in this study, a positive correlation was found between *Clostridium* and NH3-N concentration, indicating that *Clostridium* rather than *Enterobacter* could be primarily responsible for NH_3-N generation in the late phase of ensiling. Further research is needed to explore the potential association between plant protease and NH3-N generation in the initial phase of ensiling. Additionally, the negative correlation between all detected *Clostridium* and DM content further confirmed the fact that clostridia prefer high moisture habitats.

The ensiling process creates an anaerobic and acidic environment that effectively inhibits the metabolism of harmful microbes. As expected, the ensiling process effectively suppressed bacterial metabolism in fresh HE. In this study, we found that the bacterial metabolism involved in ensiling was primarily associated with carbohydrates, amino acids, cofactors, vitamins, energy and nucleotides, which was consistent with the results of Bai et al. (2021). The fundamental principle of ensilage production is based on the conversion of available carbohydrates in forage to SCFAs (mainly lactate) by LAB under anaerobic conditions. This could explain the observed increase in carbohydrate metabolism after ensiling. Amino acids are essential substrates for organisms (including microorganisms), as they play a significant role in primary metabolism and protein synthesis. The higher intensity of amino acid metabolism in fresh HE may be due to the presence of complex and diverse epiphytic microorganisms, while the lower amino acid metabolism in NGJ was due to its low pH. It has been reported that the acidic conditions during ensiling can suppress the amino

acid metabolism induced by undesirable microorganisms (Flythe and Russell, 2004). The degradation of vitamins in fresh forage during ensiling has been previously reported (Liu et al., 2019), which could explain the decrease in cofactors and vitamin metabolism observed in fresh HE after ensiling. Pessione et al. (2010) observed that energy metabolism of LAB was essential to stimulate LA production during ensiling. However, in this study, the ensiling process did not enhance the energy metabolism in NHE. This could be related to the unsatisfactory quality (high BA and NH_3-N) of the resulting silage, as stimulation of energy metabolism has been previously described in quality silage (Xu et al., 2021). Nucleotides are recognized for their role in synthesizing nucleic acids and providing energy for cellular processes (Kilstrup et al., 2005). Interestingly, differences in energy metabolism during ensiling showed an opposite trend to that of nucleotide metabolism. Therefore, a thorough assessment of the functional annotation and metabolic pathways utilised by microbial communities before and after ensiling by other omics approaches such as proteomics, metagenomics and metabolomics is needed.

To further elucidate the key bacterial functions in HE and NHE, the study specifically analysed important carbohydrate and amino acid metabolic pathways. Metabolism of most sugar components increased with ensiling, confirming that LAB could utilise various carbon sources for anaerobic fermentation. Among these, high intensity of fructose, mannose, galactose, starch, sucrose, amino sugar and nucleotide sugar metabolism in NHE could be primarily associated with *Enterobacter* and *Clostridium*. The pentose phosphate pathway is important for microorganisms in terms of acetate generation. Thus, the high relative abundance of *Enterobacter* might explain the high activity of the pentose phosphate pathway since this genus is able to utilise this route. Amino acid metabolism during ensiling plays a significant role in explaining the formation of NH_3-N. In this study, the unacceptable NH_3-N levels (> 10% TN) could be due to the degradation of phenylalanine, tyrosine and tryptophan, as reflected in the apparent suppression of phenylalanine, tyrosine and tryptophan biosynthesis.

Conclusions

Fresh HE contains low DM content, insufficient number of LAB and high counts of aerobic bacteria and enterobacteria. As a result, additional management measures such as delayed harvest or LAB inoculation are required to reduce BA and NH_3-N production and improve its fermentation quality. The ensiling process had a significant impact on the fermentation parameters, bacterial community, and functional profiles of NHE. Although functional predictions based on 16S rRNA gene sequencing may require further validation, combining it with high-throughput sequencing can offer new insights into overall silage quality assessment and anaerobic fermentation mechanisms.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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