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Dynamic fermentation quality and bacterial community structure of paper mulberry silage from three regions of China

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Abstract

Background Paper mulberry has been regarded as a potential protein resource for relieving the forage supply crisis, and ensiling has become the most important method for preserving it. An in-depth analysis of the fermentation characteristics of paper mulberry silage could provide a theoretical basis for producing high-quality silage. In this study, we aimed to investigate the dynamic fermentation quality and bacterial community of paper mulberry silages harvested from different regions in China.

Results The results showed an increased trend in ammonia nitrogen (NH_3-N) concentration, despite a decrease in pH with prolonged ensiling days. Furthermore, fermentation patterns varied among paper mulberry silages from three regions. Paper mulberry from Zhuozhou, Hebei (HZ) showed the highest dry matter (DM) content and a slight decrease in pH during ensiling. While the lowest DM content was observed in Hechi, Guangxi (GH), which exhibited abnormal fermentation in the silage. In particular, silage from Lankao, Henan (HL) exhibited the best fermentation quality, with lower pH and NH_3-N concentration, and higher lactic acid concentration than others ($P < 0.05$). The bacterial richness and evenness also declined with prolonged ensiling. Among all samples, *Enterobacter* was the most abundant in all silages, with a trend of increasing and then decreasing during the fermentation process. *Pseudocitrobacter* dominated in GH silage with abnormal fermentation. Although the bacteria community during ensiling varied widely among silages from different regions, *Enterobacter cloacae* and *Lactobacillus plantarum* were the main differential bacteria in silage quality of paper mulberry.

Conclusion Ensiling days and regions had significant effects on the fermentation patterns and bacterial community of paper mulberry silage which might be due to the differences in DM content. Notably, silage quality showed a close relationship with *Enterobacter cloacae* and *Lactobacillus plantarum*. Inhibiting the proliferation of *Enterobacter* and *Pseudocitrobacter* could be critical for improving the fermentation quality of paper mulberry silage.

Keywords Paper mulberry, Silage, Bacterial community, Fermentation quality, Regions

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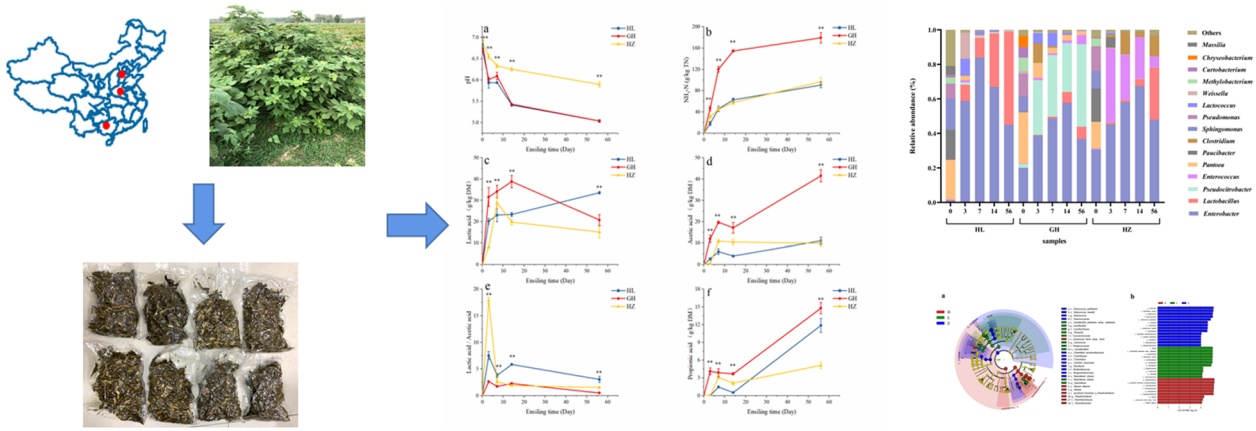
Graphical Abstract



Introduction

The shortage of feed resources, especially fresh forage, has limited the development of animal husbandry in China. In this regard, exploiting more high-quality forage resources for animal production is essential. Paper mulberry (*Broussonetia papyrifera* L.), a type of woody forage, exhibits strong adaptability to various environments and is widely cultivated throughout the world [1]. Based on its high crude protein and bioactive substance, paper mulberry has been increasingly used as a feed to promote animal growth and disease resistance [2, 3]. Therefore, paper mulberry could be a protein feed resource to alleviate the feed supply crisis.

Efficient forage storage is a vital farming strategy. Ensiling is considered as a traditional method for preserving fresh forage because of its maximum storage duration, good palatability, and high nutrition value [4]. During ensiling process, lactic acid bacteria (LAB) proliferation can ferment water-soluble carbohydrates (WSC) to lactic acid and other acids under anaerobic condition, thereby preserving the silage nutrition by lowering the pH and inhibiting the growth of undesirable microorganism [5, 6]. In addition, the competition between LAB and spoilage microorganisms, as well as the competition and collaboration among LAB species, also influence the fermentation progress [3].

The structure, function, and bacterial community succession during ensiling were determinants of the silage fermentation quality [7, 8]. Nevertheless, raw material characteristics and epiphytic microorganisms primarily influenced the microbial community and silage quality [6]. In addition, the climate condition is an essential factor that affects the characteristics and microbial distribution of fresh forage [9]. In recent years, paper mulberry has been widely extensively cultivated in various regions with different climate conditions in China. Thus, an in-depth analysis of the bacterial community of paper mulberry silage from different regions would be essential to better understand the dynamic fermentation profiles of this valuable forage resource. Previous studies have demonstrated that inoculating epiphytic LAB could improve the fermentation quality of paper mulberry in Southwest China [1, 10]. However, there is a lack of data on the dynamic fermentation characteristics and bacterial community of paper mulberry silage across different climate zones in China.

This study aimed to investigate the dynamic fermentation quality and shared and distinct features of bacterial community in paper mulberry silage from different regions. The findings of this study may provide valuable references for the production of high-quality silage.

Materials and methods

Site description

The experiment was carried out in three different regions in China. The paper mulberry originated from experimental fields, namely Zhongke Huagou Biotechnology Co., Ltd., Lankao, Henan (34° 48′ N, 114° 53′ E, 90 m above sea level), Ranquan Agricultural Science and Technology Co., Ltd., Hechi, Guangxi (24° 17′ N, 108° 31′ E, 250 m above sea level) and Teaching Experiment Field of China Agricultural University, Zhuozhou, Hebei (39° 28′ N, 115° 510′ E, 42 m above sea level).

According to the climate zoning of China, HL has a temperate monsoon climate, with an average temperature of 14.30 °C, an average humidity of 46.00%, and an average annual rainfall of 648.20 mm. GH has a subtropical monsoon climate, with a temperature averaging 17.00 °C, an average humidity of 76.00%, and an average annual rainfall of 1479.60 mm. HZ has a temperate continental monsoon climate, featuring a mean temperature of 11.60 °C, an average humidity of 28.00%, and an average annual rainfall of 354.00 mm (Additional file 1: Table S1).

Silage preparation

The paper mulberry trees (hybrid *Broussonetia Papyrifera* L., Zhongke No. 1) were cultivated on April 2017 and applied with no herbicides and fertilizers. All paper mulberry samples were harvested at the first cutting between June and August 2018 at the height with about 1.2 m. After being manually harvested with a pruning shear (KOMAX, Zhenmei Co., Ltd., Zhejiang, China), all collected paper mulberry were manually direct cut into 2 cm length with a fodder chopper (600 mm, Shandong Anke Hardware Tools Co., Ltd., Shandong, China) and mixed well on the site. The homogeneous materials were packed into plastic bags, with 400 g per bag (22 × 32 cm, Cangzhou Hualiang Packaging and Decoration Co. Ltd., Dongguan, China), and then the bags were vacuum-sealed. Fifteen bags were made for each region and kept at room temperature (about 25 ℃). Three bags were randomly selected and sampled to analyze fermentation parameters, nutritive components, microbial population based on plate culture, and bacterial communities after 0, 3, 7, 14, and 56 days of ensiling, respectively.

Fermentation quality, nutritive components and microbial population analysis

Samples (20 g) were mixed with 180 mL sterile water and placed at 4 ℃ for 24 h. They were filtrated to measure pH value with glass electrode pH meters (PHS-3C, INESA, Shanghai, China). Lactic acid, acetic acid, propionic acid, and butyric acid were determined by high-performance liquid chromatography (HPLC) (column: Shodex RS Pak KC-811; Showa Denko K.K., Kawasaki, Japan; detector: DAD, 210 nm, SPD-20A; Shimadzu Co., Ltd., Kyoto, Japan; eluent: 3 mmol L^{-1} $HClO_4$, 10 mL min^{-1}; temperature: 50 °C) [2]. NH_3-N concentration was analyzed by the phenol-hypochlorite colorimetric method [11]. The fresh and silage samples were dried for 48 h in an oven at 65 °C to measure the DM content. Dried samples were crushed and passed through a 1-mm sieve to determine nutrients. The total content of nitrogen (TN) was determined by the Kjeldahl method (FOSS Kjeltec™ 2300), then the content of crude protein (CP) was calculated by the AOAC method [12]. The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by Fann's fiber analysis method, and the hemicellulose content was the difference between NDF and ADF [13]. The content of WSC was determined by anthrone-sulfuric acid colorimetry [14]. The buffering capacity (BC) was measured by titration (15).

The count of the microbial population was determined by the plate culture method [2]. The 20 g of sample were homogenized with 180 mL distilled water, and then the supernatant was serially diluted. LAB was counted on Man Rogosa Sharpe (MRS) agar medium and cultured in an anaerobic incubator at 30 ℃ for 48 h. Rose Bengal agar medium was used to count molds and yeasts, cultured at 28 ℃ for 48 h. Coliform bacteria were counted on eosin–methylene blue agar medium and cultured at 37 ℃ for 48 h.

Bacterial community analysis

Bacterial communities of samples were analyzed using the third-generation Pacific Biosciences (PacBio) single-molecule real-time sequencing (SMRT). The total bacterial DNA extraction was performed from the samples [16]. The full-length 16S rDNA gene was amplified by PCR using primers (27F: 5'-AGRGTTTGATYNTGG CTCAg-3'; 1492R: 5'-TASGGHTACCTTGTTAS-GACTT-3'). The polymerase chain reaction (PCR) was performed under the following conditions: pre-denaturation at 95 ℃ for 5 min, (denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 90 s) for 30 cycles, and final extension at 72 ℃ for 7 min. After being purified and quantified, the PCR products were sequenced on a PacBio Sequel platform. Mothur 3 software and Silva database were used to compare

Table 1 Characteristics of raw materials prior to ensiling

Items	HL	GH	HZ
pH	6.90 ± 0.04	6.72 ± 0.02	6.90 ± 0.01
DM (g/kg)	257.54 ± 2.52	203.78 ± 4.36	305.68 ± 3.46
CP (g/kg DM)	240.48 ± 4.79	122.95 ± 1.26	171.72 ± 3.63
WSC (g/kg DM)	63.08 ± 2.48	28.80 ± 1.21	54.07 ± 1.43
NDF (g/kg DM)	288.07 ± 4.06	417.75 ± 5.81	414.23 ± 5.82
ADF (g/kg DM)	153.27 ± 2.87	303.09 ± 4.45	262.69 ± 4.98
Hemicellulose (g/kg DM)	134.80 ± 1.10	114.66 ± 2.76	151.54 ± 3.35
BC (mEq/kg DM)	614.05 ± 6.60	589.52 ± 5.20	634.60 ± 6.54
LAB (lg cfu/g FM)	4.24 ± 0.12	5.68 ± 0.21	4.94 ± 0.38
Coliform bacteria (lg cfu/g FM)	4.44 ± 0.31	6.52 ± 0.42	6.56 ± 0.10
Yeasts (lg cfu/g FM)	4.64 ± 0.34	5.29 ± 0.15	4.60 ± 0.06
Molds (lg cfu/g FM)	4.52 ± 0.01	4.08 ± 0.32	4.35 ± 0.22

DM: dry matter; CP: crude protein; WSC: water-soluble carbohydrates; NDF: neutral detergent fiber; ADF: acid detergent fiber; BC: buffering capacity; LAB: lactic acid bacteria; ± standard error of the mean

representative sequences to obtain classification information [17]. Alpha diversity analysis was conducted to study the species diversity of individual samples. Mothur was applied in calculating the co-ordinates of different principal components, and R language was used to draw the bacterial principal co-ordinates analysis (PCoA) diagram at the OTU (operational taxonomic units) level. The relative abundances of bacterial communities were analyzed as well. The free online platform (http://huttenhower.sph.harvard.edu/galaxy) was used for analyzing community structure difference (linear discriminant analysis effect size, LEfSe). R language was adopted for random forest analysis and mapping.

Statistical analysis

Statistical difference was determined by Duncan's multiple range method. The two-way ANOVA was performed on data regarding fermentation quality, nutrient components, microbial population based on plate culture, and α diversity of bacterial communities of all samples using Statistical Package for the Social Sciences (SPSS version 24.0, SPSS Inc., Chicago, IL, USA), with a factorial design with three regions and four or five ensiling days:

$$Yij = \mu + Gi + Tj + (G \times T)ij + eij,$$

where *Y*ij represents the dependent variable; μ is overall mean; *G*i is the effect of region of paper mulberry; *T*j is the effect of ensiling day; (*G*×*T*)ij is the effect of

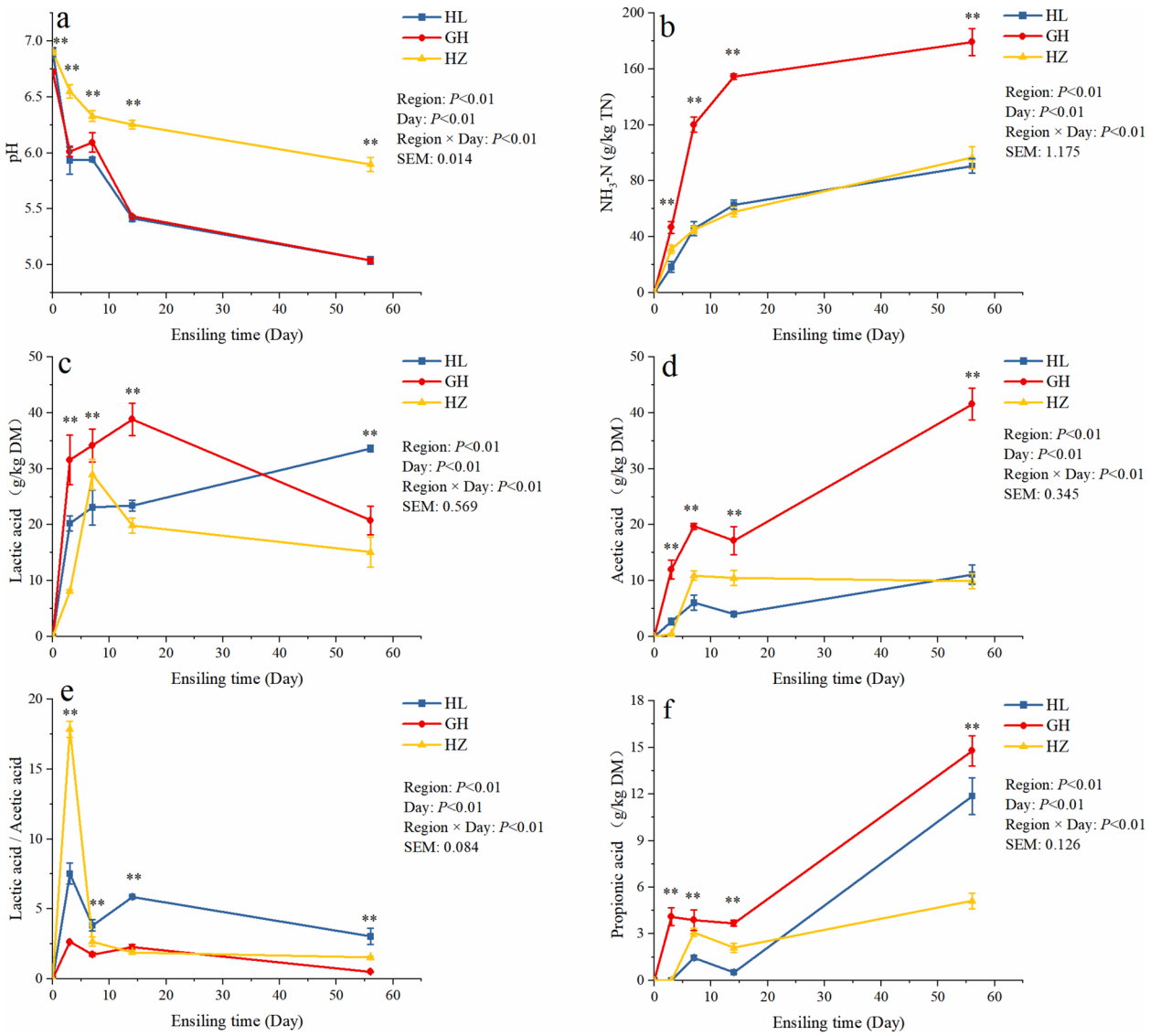


Fig. 1 Fermentation characteristics of paper mulberry silage during ensiling. **a** change of pH; **b** change of NH_3-N concentration; **c** change of lactic acid concentration; **d** change of acetic acid concentration; **e** change of lactic acid/acetic acid; **f** change of propionic acid concentration; ** stands for significant difference ($P < 0.01$) in treatments on each ensiling day

Table 2 Dynamic changes of nutritive components and microbial population of paper mulberry silage

Items	DM (g/kg)	CP (g/kg DM)	WSC (g/kg DM)	NDF (g/kg DM)	ADF (g/kg DM)	Hemicellulose (g/kg DM)	Lactic acid bacteria (lg cfu/g FM)	Coliform bacteria (lg cfu/g FM)	Yeasts (lg cfu/g FM)	Molds (lg cfu/g FM)
HL										
D 3	253.16c	189.72	57.10a	385.84	210.94	174.90a	8.43c	6.80e	4.29d	2.85c
D 7	249.91c	174.50	19.57c	405.66	226.04	179.62a	9.70a	7.37de	6.75a	NDd
D 14	244.07c	190.21	12.64d	443.7	259.91	183.79a	9.21ab	7.35de	NDe	NDd
D 56	243.98c	183.79	10.24def	337.87	248.77	89.10c	7.74d	NDg	NDe	NDd
GH										
D 3	199.27d	115.91	9.57ef	403.80	251.13	152.67ab	8.86bc	8.65ab	6.16ab	6.13a
D 7	198.07d	110.01	9.62ef	384.06	269.81	114.25bc	8.86bc	9.01a	4.60 cd	NDd
D 14	184.15d	114.37	8.26f	398.22	251.30	152.75ab	8.69bc	7.80 cd	NDe	NDd
D 56	186.95d	113.26	8.22f	321.67	247.95	73.73c	7.70d	5.75f	NDe	NDd
HZ										
D 3	282.47b	189.88	24.49b	416.69	257.09	159.60ab	8.48c	8.18abcd	5.33bc	2.70c
D 7	344.75a	195.00	11.21de	409.51	222.32	187.20a	9.25ab	8.41abc	5.13 cd	3.70b
D 14	345.80a	196.26	11.25de	330.46	249.97	80.49c	9.11abc	8.92ab	NDe	NDd
D 56	300.28b	172.82	10.42def	381.99	277.04	174.75a	8.77bc	8.09bcd	NDe	NDd
Day effect										
D 3	244.97	165.17	30.39	402.11	239.72	162.39	8.59	7.88	5.26	3.89
D 7	264.24	159.84	13.47	399.75	239.39	160.36	9.27	8.27	5.49	1.23
D 14	258.01	166.95	10.71	390.79	253.73	139.01	9.00	8.02	ND	ND
D 56	243.74	156.62	9.63	347.18	257.92	112.52	8.07	4.61	ND	ND
Region effect										
HL	247.78	184.55a	24.89	393.27	236.41	156.85	8.77	5.78	2.76	0.71
GH	192.11	113.39b	8.92	376.94	255.05	123.35	8.52	7.80	2.69	1.53
HZ	318.32	188.49a	14.34	384.67	251.60	150.51	8.90	8.40	2.61	1.60
SEM	2.330	2.121	0.244	10.589	7.363	4.671	0.060	0.076	0.089	0.053
P-value										
Region	< 0.01	< 0.01	< 0.01	0.82	0.56	0.02	0.05	< 0.01	0.80	< 0.01
Day	0.01	0.31	< 0.01	0.25	0.74	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Region × Day	< 0.01	0.45	< 0.01	0.40	0.70	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

DM: dry matter; CP: crude protein; WSC: water-soluble carbohydrates; NDF: neutral detergent fiber; ADF: acid detergent fiber; FM: fresh material; ND: not detected; SEM: standard error of the mean

[a –g]Different lowercase letters in the same column indicate significant difference ($P < 0.05$)

interaction between region and ensiling day; and *e*ij is the residual error term. The level of statistical significance was set to $P < 0.05$.

Results and discussion

Characteristics of raw materials before ensiling

The characteristics of raw materials before ensiling are shown in Table 1. The pH values of paper mulberry materials were consistent with a prior report [18]. Different DM, CP, WSC, NDF, and ADF contents of paper mulberry in three regions might be affected by the cultivated environment, such as soil quality (nutrient elements and microbial communities) and climate (temperature, light and rainfall), which affected the process of paper mulberry growth and development, and then influenced the DM and nutrient contents [19]. The CP content in HL and HZ ranged from 158.91 to 240.48 g/kg DM, which was comparable to conventional silage such as alfalfa (160–230 g/kg DM) [2], indicating that paper mulberry is a good protein source in animal feed. The WSC content of raw materials were generally regarded as an important factor in predicting the difficulty of ensiling. However, only the WSC content of paper mulberry in HL (63.08 g/kg DM) met the recommended minimum (60–80 g/kg DM), showing a limited substrate for LAB in GH and HZ [2, 20]. The NDF and ADF contents in HL were the lowest among three regions, suggesting that the lignification degree of the paper mulberry might be influenced by planting condition. Additionally, the BC values of paper mulberry in three regions (589.52–634.60 mE/kg DM) were higher than that of alfalfa, which was not conducive to ensiling paper mulberry [15, 21]. Nevertheless, paper mulberry in HL had the highest contents of CP (240.48 g/kg DM) and WSC (63.08 g/kg DM) and the lowest count of epiphytic coliform bacteria (4.44 lg cfu/g FM), suggesting its potential for better fermentation.

Table 3 Alpha diversity of bacterial community in fresh and ensiled paper mulberry

Items	ACE	Chao1	Shannon
HL			
D 0	76.66a	77.93a	4.08a
D 3	14.78e	14.17f	2.24ef
D 7	25.98de	19.08f	1.86g
D 14	11.94e	11.50f	2.00fg
D 56	15.64e	14.61f	1.73g
GH			
D 0	49.68c	50.44bc	3.63b
D 3	47.27c	38.73cde	2.97c
D 7	44.14bc	48.22bc	2.43de
D 14	50.01c	54.54bc	2.42de
D 56	42.20bcd	41.03bcd	2.28ef
HZ			
D 0	68.63b	57.86b	3.92b
D 3	29.30cde	26.32def	2.71cd
D 7	23.26e	19.75f	2.40de
D 14	24.18e	22.73ef	2.41de
D 56	22.61e	22.33ef	2.60d
Day effect			
D 0	64.99	62.08	3.87
D 3	30.45	26.41	2.64
D 7	31.13	29.02	2.23
D 14	28.71	29.59	2.28
D 56	26.82	25.99	2.20
Region effect			
HL	29.00	27.46	2.38
GH	46.66	46.60	2.75
HZ	33.60	29.80	2.81
SEM	1.481	1.400	0.025
P-value			
Region	< 0.01	< 0.01	< 0.01
Day	< 0.01	< 0.01	< 0.01
Region × Day	< 0.01	< 0.01	< 0.01

[a –g] Different lowercase letters in the same column indicate significant difference ($P < 0.05$)

Fermentation quality, nutritive components and microbial population of paper mulberry silage

Dynamic changes of the fermentation quality of paper mulberry silage in different regions are shown in Fig. 1 and Additional file 1: Table S2. The pH, lactic acid/acetic acid, concentrations of NH_3-N, lactic acid, acetic acid, and propionic acid were significantly affected by the interaction of region and ensiling days ($P < 0.01$). As reported previously [2], rapid pH decline and lactic acid concentration increase were observed during the first 7 or 14 days of ensiling. The final pH values in HL and GH silages (5.0) were significantly lower than those in the HZ silage (5.9) ($P < 0.05$). However, the pH of all silages at the end of fermentation did not decrease to the ideal level (4.5), possibly due to the weak fermentation capacity of LAB on the surface of paper mulberry during natural fermentation. NH_3-N concentration is an important factor for monitoring protein degradation in silage [22]. After 56 days of ensiling, the NH_3-N concentration in GH silage was over 100 g/kg TN (179.39 g/kg TN), which was significantly higher than that in HL and HZ silages ($P < 0.05$), indicating a higher protein degradation in GH [23]. The NH_3-N concentration increased with prolonged ensiling days, even though the pH decreased, which was consistent with a previous report [2], indicating that the decrease in pH was not sufficient to inhibit the activity of protein-degrading bacteria. The acetic acid concentration in GH significantly enhanced at the late stage. Moreover, the lactic acid/acetic acid of GH silage was lower than 1 after 56 days of ensiling, indicating the abnormal

fermentation, which might be related to the changes of microbial structure and metabolism caused by the high humidity and temperature during ensiling [24]. After ensiling for 56 days, the pH and NH_3-N concentration of HL silage were the lowest, while its lactic acid concentration and lactic acid/acetic acid ratio were the highest among silages from the three regions ($P < 0.05$). Butyric acid concentrations were not detected in any of the silages (data were not shown), indicating that butyric acid fermentation did not occur during ensiling.

Dynamic changes of nutritive components and the microbial population of paper mulberry silage in different regions are shown in Table 2. The contents of DM and WSC were significantly affected by the interaction of region and ensiling days ($P < 0.01$). During ensiling, microorganisms such as LAB grow and ferment WSC to produce organic acid at a specific moisture level [25]. Excessive moisture of raw forage can lead to suboptimal fermentation and reduced feed quality with production of unwanted compounds such as butyric acid and ammonia-N, while insufficient moisture can lead to incomplete fermentation and poor nutritional value. Therefore, maintaining the appropriate moisture level and DM content is crucial for achieving optimal fermentation and preserving the quality of the silage [23, 26–28]. This study showed that the DM content of paper mulberry in GH was the lowest among that of the three regions ($P < 0.05$), resulting in abnormal fermentation. The high DM content of paper mulberry in HZ limited the activity of LAB, leading to slow accumulation of lactic acid and the reduced silage pH. The moderate DM content of paper mulberry from HL was conducive to LAB acid production, resulting in better fermentation quality with lower pH and a higher lactic acid concentration compared to GH and HZ silages ($P < 0.05$). After fermentation initiation, the WSC contents decreased in all samples, indicating utilization by LAB and other microorganisms to produce organic acids. The CP content was better preserved in HZ silage, which could be attributed to the low moisture in such silages that inhibiting the activity of protein-degrading microorganisms. Besides, although the region and ensiling days did not show significant ($P > 0.05$) effects on NDF and ADF contents, hemicellulose content was significantly influenced by the interaction of region and ensiling days ($P < 0.05$), with significant decomposition in HL and GH silages at the end of fermentation ($P < 0.05$).

The interaction of region × day had significant effects on the counts of LAB, coliform bacteria, yeasts, and mold ($P > 0.01$) (Table 2). The counts of all culturable microorganisms initially increased and then decreased during the fermentation process. Generally speaking, the growth

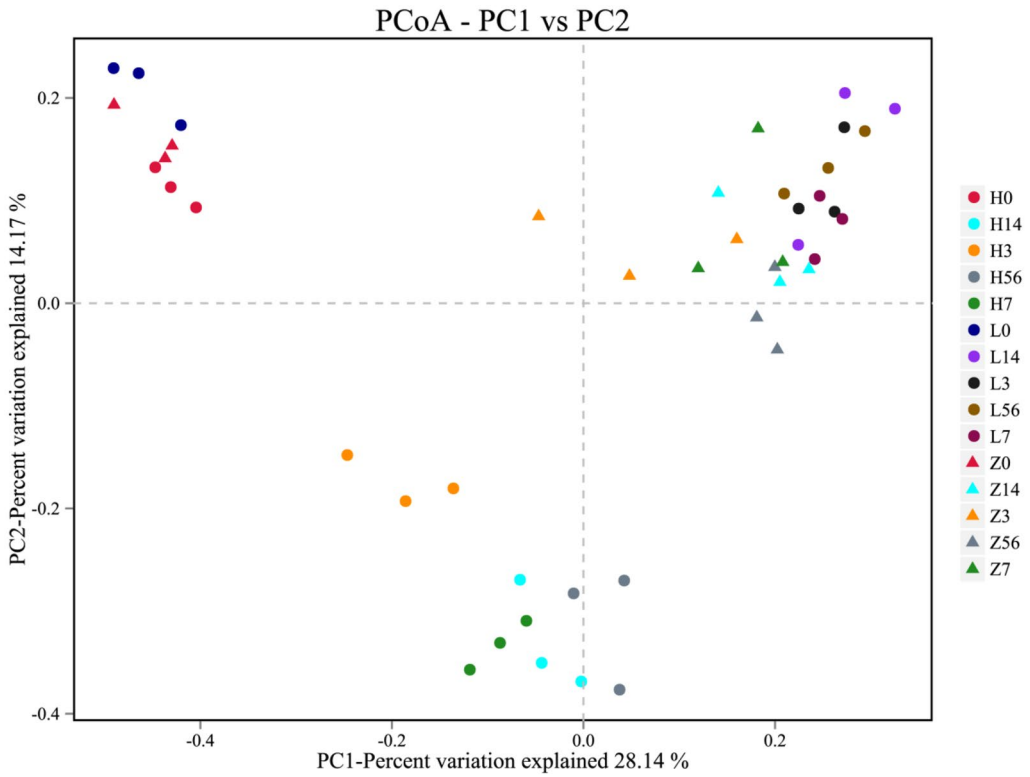


Fig. 2 Principal co-ordinates analysis (PCoA) analysis based on OTU level of paper mulberry silage. L, HL; H, GH; Z, HZ; 0, before ensiling; 3, 7, 14 and 56 means ensiling days

of LAB was inhibited by the low pH and fermentation products when it reached numbers of around 10 lg cfu/g FM during ensiling [29]. The LAB count increased rapidly in the first 7 days of fermentation and then decreased slowly. Some coliform bacteria can metabolize lactic acid to produce acetic acid, which could lead to a reduction in lactic acid content and an increase in acetic acid content [30]. The lactic acid concentrations of HZ and GH silages initially increased and then decreased, possibly due to the presence of coliform bacteria in high numbers during the late fermentation stage.

Alpha diversity and PCoA of raw materials and silage samples

The structure of bacterial community of silage plays an important role in fermentation. In this study, SMRT technology was utilized to characterize the bacterial community structure. As shown in Table 3, region, ensiling days, and their interaction significantly influenced the bacterial alpha diversity (ACE, Chao 1, and Shannon) ($P<0.01$). The ACE, Chao 1, and Shannon indexes decreased as the ensiling time extended, indicating a decline in bacterial richness and evenness. After 56 days of ensiling, the Shannon index of HL silage was lower than that of GH and HZ ($P<0.05$), suggesting a uniform community structure. The results of PCoA (Fig. 2) demonstrated that bacterial communities of raw materials were located in the second quadrant (Fig. 2), which was distinguishable from silages. The bacterial communities of GH silages were notably different from those in the other two regions, which might have been caused by the increase of harmful bacteria and thus led to abnormal fermentation.

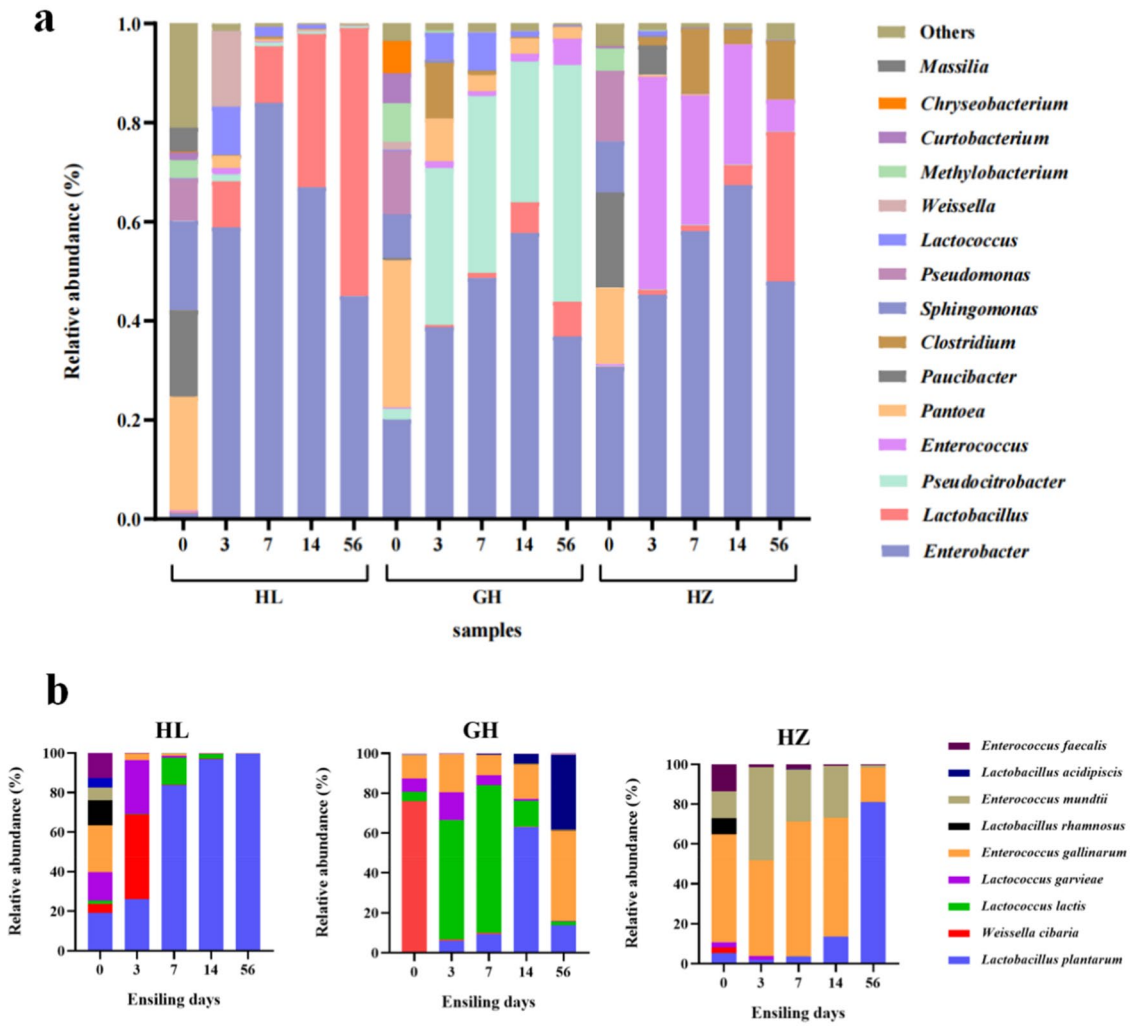


Fig. 3 Relative abundance of bacteria and lactic acid bacteria of paper mulberry silage from different regions. **a** bacteria community; **b** lactic acid bacteria community; 0, before ensiling; 3, 7, 14 and 56 means ensiling days

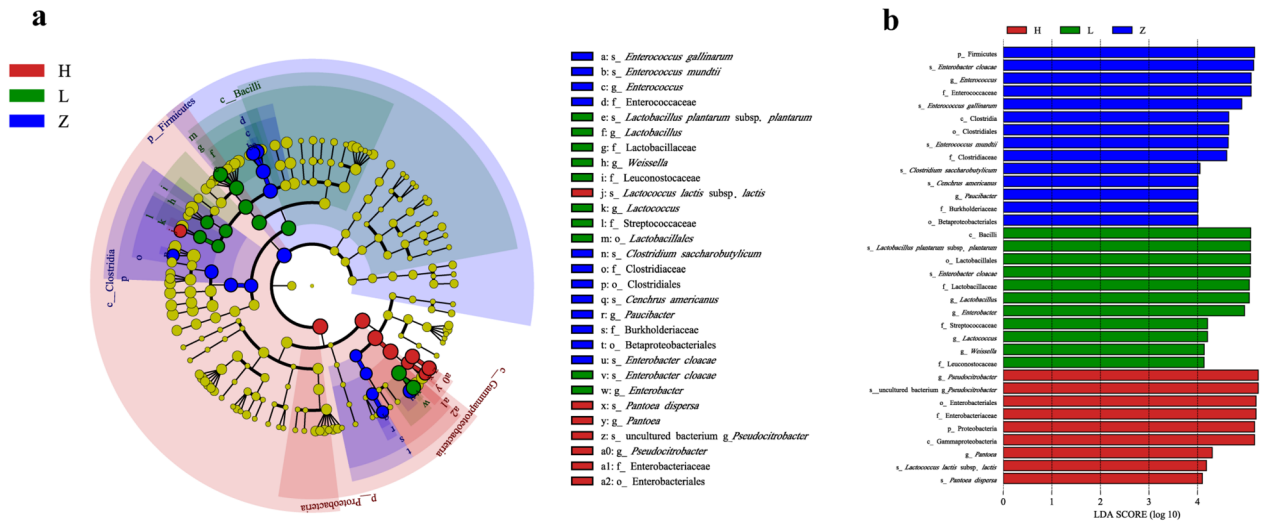


Fig. 4 Linear discriminant analysis effect size (LEfSe) of bacteria community of paper mulberry silage. L, HL; H, GH; Z, HZ. **a** Cladogram showing the taxonomic differences; **b** identified taxonomy was significantly different based on LDA score larger than 4.0

Relative abundance of bacterial community of raw materials and silage samples

The bacterial compositions and significance analysis of three regions at different fermentation stages are shown in Fig. 3a and Additional file 1: Table S3, respectively. Before ensiling, the main epiphytic microorganisms were *Pantoea* (15.04–29.76%), *Sphingomonas* (8.82–18.06%), *Paucibacter* (17.36–19.18%), *Pseudomonas* (8.49–14.24%), and *Enterobacter* (20.09–30.70%), which differed from a previous study that reported *Pseudomonas, Pantoea, Serratia, Erwinia* and *Rahnella* as the dominant epiphytic bacteria in fresh paper mulberry [18]. The variation might be due to the different sampling sites. After ensiling, *Enterobacter* (36.86–47.98%) became the dominant genus in all silages, with a significant increase and subsequent gradual decrease during ensiling. The low pH and anaerobic environment during ensiling might have limited their growth. Previous study have shown that *Enterobacter* could compete with LAB for available substrates during ensiling and reduce feed quality [31]. Therefore, the high abundance of *Enterobacter* suggested a poor quality of natural fermentation in paper mulberry silage. The abundance of *Lactobacillus* increased gradually with the ensiling time extension. Specifically, *Lactobacillus* abundances in HL and HZ silages were up to 54.11% and 30.13% after 56 days of ensiling, respectively. *Lactobacillus* was one of the dominant bacteria genera in HL silage, which might be related to their better fermentation quality. During ensiling, the abundance of *Pseudocitrobacter* in GH silage gradually increased, reaching 47.79% after 56 days of ensiling. *Pseudocitrobacter* belongs to Enterobacteriaceae family and was the dominant genus in GH, though its role in silage remains unreported.

It was suggested that climate might affect the distribution of LAB [32]. As shown in Fig. 3 b and Additional file 1: Table S4, the LAB compositions of samples from the three regions were quite different. The main dominant LAB in the paper mulberry of HL were *Enterococcus gallinarum* (23.81%), *Lactobacillus plantarum* (19.05%), *Enterococcus faecalis* (18.58%), *Lactococcus Garvieae* (14.29%) and *Lactobacillus rhamnosus* (12.70%). *Weissella cibaria* (75.63%) was the dominant LAB in the paper mulberry of GH and *Enterococcus gallinarum* (54.06%) and *Enterococcus mundtii* (13.51%) were the dominant LAB in the paper mulberry of HZ. Before ensiling, the number and types of LAB attached to the surface of raw materials play a crucial role in the final effect of fermentation. The presence of an abundance of lactic acid cocci (*Weissella*, *Leuconostocs*, *Lactococci,* and *Enterococci*) may lead to incomplete fermentation [33], while abundant *Lactobacilli* contribute to full fermentation [33, 34]. In our study, the culture method showed that the LAB count of raw materials was the lowest in HL silage (Table 1). However, the fermentation quality was the opposites to expectation. The possible reason might be that paper mulberry from HL had a higher proportion of *Lactobacilli* (*Lactobacillus plantarum* and *Lactobacillus rhamnosus*) than paper mulberry from the other two regions, which favored late fermentation. During ensiling, the abundance of *Lactobacillus plantarum* gradually increased, reaching 99.76% of LAB after 56 days of ensiling. *Lactobacillus plantarum* were widely used as promoters of silage fermentation [15]. Therefore, the

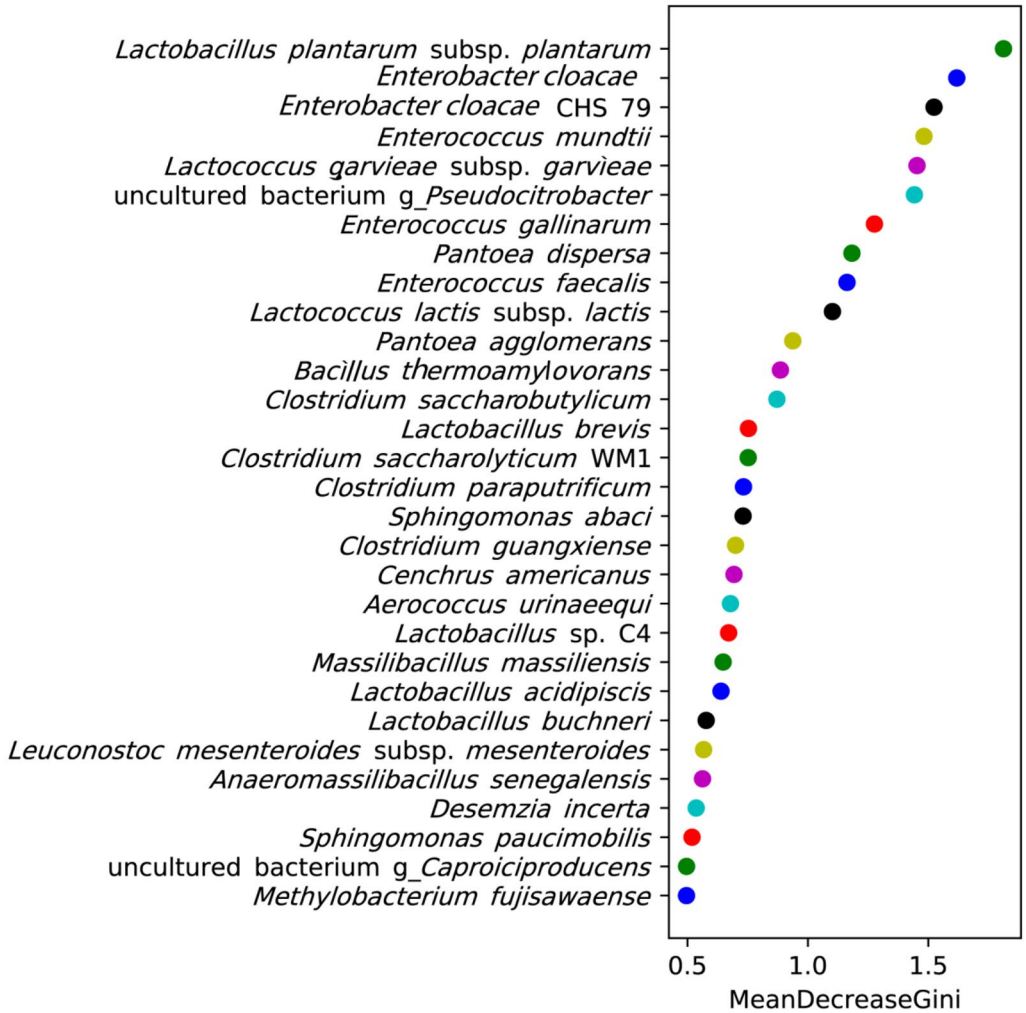


Fig. 5 Random forest analysis of bacterial community of paper mulberry silage from different regions. The y-axis, from top to bottom, displays the genera ranked by their relative importance based on mean decrease accuracy in the classification of groups

excellent fermentation quality of HL silage might be related to the high abundance of *Lactobacillus plantarum.* During the fermentation process of HZ silage, *Enterococcus gallinarum* and *Enterococcus mundtii* were gradually replaced by *Lactobacillus plantarum*. *Enterococcus* is considered early colonizer as they are outcompeted by acid-tolerant *Lactobacillus* [16, 33, 34]. However, the *Lactobacillus* abundance of HZ silage was less than 30% in the bacterial community, which might be related to the poor fermentation quality. In the first 7 days, *Lactococcus lactis* (59.86–73.91%) became the dominant LAB in GH silage, and then their abundance declined gradually. At the end of fermentation, *Lactococcus lactis* were replaced by *Enterococcus gallinarum* (45.36%) and *Lactobacillus acidipiscis* (37.52%).

Variation analysis of bacterial community of silage samples from different regions

To further elucidate the differences in microbial communities, LEfSe analysis was conducted [35, 36]. As shown in Fig. 4, the region was a significant factor affecting bacterial communities of silage. Specifically, *Lactobacillus, Lactococcus,* and *Weissella* were identified as the main differential bacteria in HL silage, while *Pantoea, Pseudocitrobacter,* and *Lactococcus* were the main differential bacteria in GH silage. Moreover, *Enterococcus* and *Clostridium* were the main differential bacteria in HZ silage.

In addition, a random forest analysis of the bacterial communities revealed that certain characteristic species

had a significant impact on the differences observed between samples. As shown in Fig. 5, *Enterobacter cloacae* and *Lactobacillus plantarum* were identified as top species that significantly influenced the bacterial community differences in paper mulberry silage from different regions, suggesting that these two species played a major role in shaping the natural fermentation of paper mulberry. Notably, *Enterobacter cloacae* is known to produce biogenic amines by excreting amino acid decarboxylases, and its growth can be inhibited by promoting the dominance of *Lactobacillus plantarum* [37]. Therefore, the mutual exclusion of *Enterobacter cloacae* and *Lactobacillus plantarum* in paper mulberry silage might further impact the fermentation quality.

Conclusion

The fermentation patterns of paper mulberry from different regions (HL, GH and HZ) and the changes in bacterial composition during ensiling were varied based on the characteristics of raw materials such as DM content. Although prolonging the ensiling days decreased pH, NH_3-N concentration was increased. *Enterobacter* was the most abundant in all silages, while *Pseudocitrobacter* dominated the silage with abnormal fermentation. Furthermore, the silage quality was primarily affected by the increased abundance and activation of *Enterobacter cloacae* and *Lactobacillus plantarum*. Paper mulberry with 257.54 g/kg DM content from HL exhibited the best fermentation quality after 56 days of ensiling with the lowest pH and NH_3-N concentration and the highest *Lactobacillus plantarum* abundance among all silages. It is suggested that the reduced proliferation of *Enterobacter* and *Pseudocitrobacter* would be beneficial to produce high-quality paper mulberry silage.

Abbreviations

ADF	Acid detergent fiber
ANOVA	Analysis of variance
BC	Buffering capacity
cfu	Colony forming unit
CP	Crude protein
DM	Dry matter
FM	Fresh matter
GH	Hechi, Guangxi
HL	Lankao, Henan
HPLC	High-performance liquid chromatography
HZ	Zhuozhou, Hebei
LAB	Lactic acid bacteria
LEfSe	Linear discriminant analysis effect size
MRS	Man Rogosa Sharpe
NDF	Neutral detergent fiber
NH_3-N	Ammonia nitrogen
OTU	Operational taxonomic units
PacBio	Pacific Biosciences
PCoA	Principal co-ordinates analysis
SEM	Standard error of the mean
SMRT	Single-molecule real-time sequencing
TN	Total nitrogen
WSC	Water-soluble carbohydrates

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00414-7.

Additional file 1: **Table S1.** Climate conditions in the cultivation area of paper mulberry. **Table S2.** Dynamic changes in fermentation quality of paper mulberry silage. **Table S3.** Significance analysis of relative abundance on the genus of silages from different regions.

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Author contributions

LG designed the study and wrote the manuscript. LG, XW and YL performed the experiments. LG, YW, YX and XL conducted the statistical and bioinformatics analysis. KN and FY contributed to conceptualization and funding acquisition, and were involved in the revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors listed have read the complete manuscript and have approved submission of the paper.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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