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Fermentation Quality and Bacterial Diversity of *Broussonetia papyrifera* Leaves Ensiled with *Lactobacillus plantarum* and Stored at Different Temperatures

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Abstract: *Broussonetia papyrifera* has increasingly been used as a high-quality feedstuff for ruminants due to its advantageous characteristics. The storage temperature can influence the fermentation quality of silage; however, the effect of temperature on *B. papyrifera* leaves (BPL) silage has not been reported. In the present study, the fermentation quality and bacterial community of BPL, stored at 15 °C and 30 °C, were investigated during ensiling (day 3, 7, 14, 30) with or without *Lactobacillus plantarum* strain (LP) added. The pH and the coliform bacteria counts were significantly lower in silage stored at 30 than 15 °C ($p < 0.01$), while the lactic acid content increased significantly ($p < 0.05$). Adding LP decreased the dry matter loss, pH, coliform bacteria count, and ammonia-N and butyric acid contents at 30 °C. The relative abundance of *Lactobacillus* increased, while the bacterial diversity decreased in the silage stored at 30 °C when LP was added. During silage, the high abundance of *Lactobacillus* decreased gas and carbon dioxide (CO_2) production, and the lowest gas and CO_2 production were detected in silage stored at 30 °C when LP was added. In conclusion, adding LP and storing it at 30 °C could effectively improve the quality of BPL silage.

Keywords: *Broussonetia papyrifera*; temperature; fermentation quality; bacterial community

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1. Introduction

Broussonetia papyrifera, also named paper mulberry, a deciduous tree of Moraceae family [1], is found in China and other countries [2]. Many pharmacological functions of *B. papyrifera* have been reported, including antioxidant, anti-nociceptive, anti-tyrosinase, and anti-inflammatory properties [3]. In China, "targeted poverty alleviation through the planting of *B. papyrifera*" is an important anti-poverty project and more than 5 million hectares of *B. papyrifera* are currently planted [4]. In recent years, *B. papyrifera* has increasingly been used as a high-quality feedstuff for ruminants [5], due to its advantageous characteristics, including rapid growth, adaptability, resistance, and high nutrient content [6]. Wu et al. [7] reported that replacing alfalfa meals with *B. papyrifera* could improve the growth performance and flesh color of New Zealand white rabbits. Obour [8] also found that *B. papyrifera* could be a potential food resource for rearing sheep and goats. Therefore, *B. papyrifera* can be a suitable protein resource, which helps with poverty alleviation through

enhancing animal husbandry in poor areas of China. However, the *B. papyrifera* leaves (BPL) are mainly harvested during the summer wet–rainy season and are exceedingly susceptible to mold [9]. While a natural method of preservation is often inadvisable, ensiling could be an effective strategy for preserving BPL nutrients. During the ensiling process, lactic acid bacteria (LAB) produce lactic acid (LA) and acetic acid (AA) by converting water-soluble carbohydrate (WSC) under anaerobic conditions [10,11]. Consequently, the pH of the leaves rapidly decreases and inhibits other undesirable microorganisms, prolonging the duration that the BPL can be ensiled. However, *B. papyrifera* is difficult to ensile due to abundant undesirable microorganisms [4]. During ensiling, metabolisms of undesirable microorganisms always lead to carbon dioxide (CO_2) accumulation, which is not only a source of greenhouse gases emissions but also a cause of dry matter (DM) loss of silage. Therefore, effective measures are necessary to inhibit undesirable microorganisms and improve the preservation of BPL silage.

LAB inoculants are frequently added as additives to improve the fermentation quality of silage during ensiling [12]. Yan et al. [13] isolated and identified a LAB strain (from high-moisture corn silage) which could effectively improve the quality of Italian ryegrass fermented silage. According to our previous research, *Lactobacillus plantarum* strain (LP) was selected for the faster growth rate and acid production activity [14]. Some sensitive environmental factors influence the efficiency of LAB inoculants, such as storage temperature [15]. Low temperatures may cause a decrease in enzymatic activity and the slow growth rate of LAB, and the ecological composition of the microbial community may change by screening for low-temperature-resistant LAB [16]. Meanwhile, temperature also influences the fermentation quality of silage. For example, high temperatures led to poor quality silage [17] and reduced aerobic stability [18]. However, the effect of temperature on BPL silage has not been reported.

Therefore, in the present study, the effect of LP on the fermentation quality, nutritional components, and CO_2 production of BPL silage at two different storage temperatures (15 °C and 30 °C) were investigated. The bacterial diversity was analyzed through the identification of the 16S rRNA gene sequence.

2. Materials and Methods

2.1. Sampling and Silage Preparation

BPL were collected manually and sub-regionally from the Zengcheng experimental base at South China Agricultural University (Guangdong, China) on 24 September 2020. Herbicides and fertilizers were not used during the growth of *B. papyrifera*. Fresh BPL were chopped to 2–3 cm, approximately. Then, about 200 g BPL were subjected to ensiling treatments with or without LP, respectively, and then separately stored at two temperatures (15 °C and 30 °C). The LP was isolated and identified from *Moringa oleifera* silage and then the method of LAB powder preparation was undertaken, following Zhang et al. [14]. The silage treatments (with three repetitions in each treatment) were designed as follows: no inoculant at 15 °C (15CK); LP inoculant (1×10^6 colony-forming unit, CFU/g fresh matter, FM) at 15 °C (15LP); no inoculant at 30 °C (30CK); and LP inoculant (1×10^6 CFU/g FM) at 30 °C (30LP). Specifically, the appropriate amount of LAB powder was dissolved in 4 mL sterilized water and sprinkled evenly on the 200 g of chopped leaves with a micro-sprinkler and then fully mixed. Preparation of the CK treatments only involved adding 4 mL of sterilized water. In each case, the resulting silage was immediately packed separately into vacuum-sealed polyethylene silo bags (20 × 30 cm) and stored in two incubators (set at 15 °C and at 30 °C) after recording the bag weights.

2.2. Gas Production Analysis

The volume of these bags was checked using the drainage method. The difference in volume before and after silage is gas production. The concentration of CO_2 was determined by gas chromatography (Shimadzu GC-20A with a TCD detector) equipped with Molecular Sieve 5A and a Porapak N column.

2.3. Analysis of Microbial Counting and Chemical Composition

After fermentation for 3, 7, 14, and 30 day, these 48 vacuum polyethylene silo bags (2 temperatures × 2 treatments × 3 repetitions × 4 day) were opened and sampled in triplicate. The first sample was used for microbial counting and the second sample for measuring organic acid, the pH, and for determination of ammonia-N content; the last sample was used to determine the 16sRNA sequence. Specific methods for measuring microbial quantity were employed as described by Wang et al. [19]. The 20 g samples and 180 mL of sterilized water were gradually added into leach liquor, which was serially diluted from 10^{-1} to 10^{-6}. Subsequently, for selective isolation and counts of LAB and coliform bacteria, the dilutions were added, respectively, into MRS medium and Violet Red Bile Agar (VRBA) to incubate at 30 °C for 2 day. Yeasts and molds were counted by using Rose Bengal Agar medium, which was incubated at 28 °C for 2 day. After storage at 4 °C for 18 h, the second sample (20 g sample mixed with 180 mL distilled water) was then filtered with four layers of cheesecloth. The pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., Shanghai, China) was used to measure the pH, and the method of Broderick and Kang [20] was adopted to measure the ammonia-N content. High-performance liquid chromatography (HPLC) was used to determine the organic acid content, including LA, AA, and butyric acid (BA) [21].

2.4. Bacterial Community Analysis

The E.Z.N.A. stool DNA kit (Omega Biotek, Norcross, GA, USA) was used for total DNA extraction according to the manufacturer's explanatory notes. The V3–V4 hyper-variable regions of 16S rDNA were amplified with primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) [22]. A polymerase chain reaction (PCR) procedure was conducted in a 50 µL mixture (5 µL of 10 × KOD Buffer, 1.5 µL of each primer (5 µM), 1 µL of KOD polymerase, 5 µL of 2.5 mM dNTPs, and 100 ng of template DNA) [23]. According to the manufacturer's instructions, amplification products were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Subsequently, the PCR products after purification were sequenced using an Illumina platform (Guangzhou Gene Denovo Co., Ltd., Guangzhou, China). Using FLSAH (version 1.2.11, Guangzhou Gene Denovo Co., Ltd., Guangzhou, China), the pairing end cleaning readings were merged into original tags, with a minimum overlap of 10 bp and a mismatch error rate of 2%. QIIME (version 1.9.1, Guangzhou Gene Denovo Co., Ltd., Guangzhou, China) was used for noise sequence filtering and data processing. Clean reads were searched in reference databases. (http://drive5.com/uchime/uchime_download.html, accessed on 30 June 2021) The UCHIME algorithm was used to perform the reference-based chimera examination. (http://www.drive5.com/usearch/manual/uchime_algo.html, accessed on 30 June 2021) The chimeric sequence was removed and the UPARSE pipeline was used to cluster 97% effect labels with the same identity into the operation taxonomic unit (OTU). The Ribosome Database Project (RDP) classifier (version 2.2, Guangzhou Gene Denovo Co., Ltd., Guangzhou, China) was used to analyze the taxonomy assignment of the representative sequences.

2.5. Nutritional Component Analysis

The rest of the silage after sampling (approximately 100 g) was weighed and oven-dried (65 °C for 48 h) to determine DM content and calculate DM loss. By using a mini plant disintegrator, dried samples were cut into powder sufficiently fine enough to pass through a millimeter screen. Adopting the method of the Association of Official Analytical Chemists [24], crude protein (CP) was measured using a Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto-Analyzer, FOSS Analytical AB, Hoganas, Sweden). Fiber contents, including neutral detergent fiber (NDF) and acid detergent fiber (ADF), were measured as reported

by Van Soest et al. [25]. Measuring the amount of WSC was done according to the method detailed by Murphy [26].

2.6. Statistical Analysis

Data on the effects of inoculated LAB, temperature, ensiling days, and their interaction were evaluated using two-way analysis of variance and Duncan's new multiple range test with IBM SPSS 22 statistical software (IBM, Armonk, NY, USA). The raw high throughput sequences were selected and analyzed on the online platform (http://www.omicshare.com/tools, accessed on 30 June 2021) according to Wang et al. [27].

3. Results

3.1. Characteristics of Raw B. papyrifera Leaves before Ensiling

Characteristics of raw *B. papyrifera* leaves before ensiling were analyzed (Table 1). The DM content was 354 g/kg FM. The CP content was 139 g/kg DM. The content of NDF (350 g/kg DM) and ADF (122 g/kg DM) was detected, and the WSC content was 57.1 g/kg DM. For the microorganism, the LAB count of BPL was 3.92 $\log_{10}$ colony-forming unit, CFU/g FM. Counts of undesirable microorganisms, including coliform bacteria (5.15 $\log_{10}$ CFU/g FM) and yeasts (3.78 $\log_{10}$ CFU/g FM) were detected.

Table 1. Characterization of raw *B. papyrifera* leaves.

Item	Means ± SD
Dry Matter (g/kg)	354 ± 1.53
Crude protein (g/kg DM)	139 ± 1.93
Neutral detergent fiber (g/kg DM)	350 ± 17.2
Acid detergent fiber (g/kg DM)	122 ± 6.83
Water-soluble carbohydrates (g/kg DM)	57.1 ± 2.47
Lactic acid bacteria ($\log_{10}$ CFU/g FM)	3.92 ± 0.14
Coliform bacteria ($\log_{10}$ CFU/g FM)	5.15 ± 0.26
Yeasts ($\log_{10}$ CFU/g FM)	3.78 ± 0.16
Molds ($\log_{10}$ CFU/g FM)	<2.00

DM, dry matter; FM, fresh matter; CFU, colony-forming unit; SD, Standard Deviation.

3.2. Fermentation Quality and Microbial Populations of B. papyrifera Leaves Silage

Fermentation characteristics are shown in Table 2. The results showed that the DM loss during silage was low. In DM loss, the interaction of temperature and LP inoculant, ensiling days and LP inoculant had significant effects ($p < 0.01$). The addition of LP reduced the DM loss and was better at 30 °C than at 15 °C. The effect of LP addition in reducing the DM loss has become increasingly evident as the ensiling days are prolonged. The lowest DM loss in the LP treatment was recorded at 30 °C (0.62 g/kg FM). In this parameter of pH, the two-by-two interaction of all three factors, ensiling days, LP inoculant and temperature, was significant ($p < 0.01$). The pH gradually decreased over time with ensiling ($p < 0.01$), and decreased more rapidly in the CK than in LP treatment. Adding LP significantly decreased the pH ($p < 0.01$). After fermentation for 30 days, the pH was lower at 30 °C than at 15 °C in the CK treatment, and the pH of the LP treatment at 30 °C (4.49) reached the minimum of all experimental conditions. The pH of LP treatment at 15 °C also decreased to 4.57 and there were no significant differences between 30 °C and 15 °C. Overtime during ensiling, AA content gradually increased ($p < 0.01$). The addition of LP significantly increased LA content ($p < 0.01$). LA content ($p < 0.05$) was significantly higher in silage stored at 30 °C than at 15 °C. The two-by-two interaction of all three factors—ensiling days, LP inoculant, and temperature—was significant in terms of LAB, BA and ammonia-N contents ($p < 0.01$). With the ensiling days prolonged, the count of LAB significantly decreased at 30 °C ($p < 0.01$) and the count of LAB also decreased significantly ($p < 0.01$) in LP treatment at 15 °C. The addition of LP significantly decreased BA content ($p < 0.01$) at 30 °C and better than at 15 °C. The CK treatment enhanced the ammonia-N content ($p < 0.01$) significantly

at 30 °C than at 15 °C. With the ensiling days prolonged, the ammonia-N content increased gradually. The addition of LP significantly reduced the ammonia-N content ($p < 0.01$) at 15 °C and at 30 °C, respectively. With the addition of LP, the count of coliform bacteria was also significantly decreased ($p < 0.01$) at 15 and at 30 °C. In the CK treatment, coliform counts were slightly higher than at 15 °C in silage at 30 °C before fermentation for 14 days. However, after fermentation for 14 days, the coliform count gradually decreased. These observations showed a significant decrease in pH and coliform counts and a significant increase in LA content in silage at 30 °C. Although higher temperatures result in a slight increase in BA and ammonia-N content, the addition of LP effectively reduces their levels. The strongest effect was observed for silage stored at 30 °C after adding LP.

Table 2. The effect of *Lactobacillus plantarum* (LP) on the fermentation quality and microbial population of *B. papyrifera* leaves silage at different temperatures (15 °C, 30 °C).

Item	Days	15 °C		30 °C		SEM	*p*-Value					
		CK	LP	CK	LP		T	D	L	T × D	T × L	D × L
Ensiling Characteristics												
Dry Matter Loss (g/kg DM)	3	0.80 ^{b,B}	1.09 ^{a,A}	0.92 ^{a,b,B}	1.04 ^{a}	0.041	0.780	<0.01	<0.01	0.676	<0.01	<0.01
	7	0.76 ^{B}	0.75 ^{B}	0.94 ^{B}	0.71							
	14	1.06 ^{a,b,A,B}	1.03 ^{a,b,A,B}	1.21 ^{a,A,B}	0.75 ^{b}							
	30	1.32 ^{a,b,A}	1.00 ^{b,c,A,B}	1.50 ^{a,A}	0.62 ^{c}							
pH	3	7.28 ^{a,A}	4.83 ^{b,A}	6.97 ^{a,A}	5.16 ^{b,A}	0.146	<0.01	<0.01	<0.01	0.01	<0.01	<0.01
	7	6.87 ^{a,B}	4.69 ^{c,A,B}	6.55 ^{b,B}	4.56 ^{c,B}							
	14	6.47 ^{a,C}	4.62 ^{c,B}	5.92 ^{bC}	4.54 ^{d,B}							
	30	6.35 ^{a,D}	4.57 ^{c,B}	5.50 ^{b,D}	4.49 ^{c,B}							
Lactic acid (g/kg DM)	3	ND	ND	ND	ND	1.29	0.04	0.152	<0.01	0.56	0.14	0.50
	7	19.6 ^{b}	37.8 ^{a}	29.1 ^{a,b}	39.0 ^{a,A,B}							
	14	25.6 ^{b}	36.8 ^{a}	27.3 ^{b}	37.3 ^{a,B}							
	30	27.0 ^{b}	39.0 ^{a}	33.9 ^{a,b}	40.6 ^{a,A}							
Acetic acid (g/kg DM)	3	ND	11.0	9.65 ^{B}	10.6	0.50	0.17	<0.01	0.69	0.12	<0.01	<0.01
	7	9.51	11.5	10.4 ^{B}	10.7							
	14	10.5	11.5	12.3 ^{B}	11.6							
	30	13.2 ^{b}	13.6 ^{b}	21.3 ^{a,A}	12.8 ^{b}							
Butyric acid (g/kg DM)	3	1.72 ^{b,c,D}	1.53 ^{c,B}	2.89 ^{a,C}	2.00 ^{b}	1.69	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	7	2.30 ^{b,C}	1.71 ^{c,B}	4.33 ^{a,B}	2.08 ^{b,c}							
	14	3.22 ^{b,B}	2.07 ^{c,A}	4.89 ^{a,A}	2.17 ^{c}							
	30	4.19 ^{b,A}	2.11 ^{c,A}	5.01 ^{a,A}	2.16 ^{c}							
Ammonium nitrogen (g/kg DM)	3	0.31 ^{b,C}	0.29 ^{b,A,B}	0.49 ^{a,D}	0.32 ^{b,C}	0.037	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	7	0.44 ^{b,B}	0.27 ^{c,A}	0.78 ^{a,C}	0.36^{bBC}							
	14	0.51 ^{b,B}	0.32 ^{c,B}	0.93 ^{a,B}	0.43 ^{b,c,B}							
	30	0.69 ^{b,A}	0.38 ^{c,A}	1.19 ^{a,A}	0.63 ^{b,A}							
Microbial populations												
Lactic acid bacteria (log_{10} CFU/g FM)	3	7.63 ^{c,B}	9.73 ^{a,A}	8.27 ^{b,A}	9.59 ^{a,A}	0.141	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	7	8.26 ^{d,A}	9.49 ^{a,A}	8.52 ^{c,A}	8.89 ^{b,B}							
	14	7.44 ^{c,B}	9.28 ^{a,A}	7.36 ^{c,B}	8.27 ^{b,C}							
	30	8.58 ^{a,A}	8.44 ^{a,B}	7.23 ^{b,B}	6.36 ^{b,D}							
Coliform bacteria (log_{10} CFU/g FM)	3	7.72 ^{a}	6.26 ^{b,A}	8.24 ^{a,A}	5.80 ^{b,A}	0.181	<0.01	<0.01	<0.01	<0.01	0.05	0.34
	7	7.89 ^{b}	5.97 ^{c,A}	8.35 ^{a,A}	<2.00 ^{d,B}							
	14	7.88 ^{a}	<2.00 ^{b,B}	6.56 ^{a,B}	<2.00 ^{b,B}							
	30	7.37 ^{a}	<2.00 ^{b,B}	<2.00 ^{b,C}	<2.00 ^{b,B}							
Yeasts (log_{10} CFU/g FM)	3	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-	-	-
	7	<2.00	<2.00	<2.00	<2.00							
	14	<2.00	<2.00	<2.00	<2.00							
	30	<2.00	<2.00	<2.00	<2.00							
Molds (log_{10} CFU/g FM)	3	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-	-	-
	7	<2.00	<2.00	<2.00	<2.00							
	14	<2.00	<2.00	<2.00	<2.00							
	30	<2.00	<2.00	<2.00	<2.00							

$^{(a–d)}$ Means different superscript letters in the same row ($p < 0.05$). $^{(A–D)}$ Means different superscript letters in the same column ($p < 0.05$). CK, the control; SEM, standard error of means; T, temperature (15 °C, 30 °C); D, ensiling days; L, with or without LP inoculant; T × D, the interaction of temperature and ensiling days; T × L, the interaction of temperature and LP inoculant; D × L, the interaction of ensiling days and LP inoculant; ND, not detected.

3.3. Bacterial Community of B. papyrifera Leaves Silage

The dynamics of the bacterial community during silage fermentation are crucial because silage fermentation is essentially a competition between LAB and undesirable microbes. As shown in Figure 1, the principal coordinates analysis (PCoA) was used to an-

alyze the beta diversity of the bacterial community (employ unweighted UniFrac distances (A) and weighted UniFrac distances (B)). The results showed that there were differences between the bacterial species for the different fermentation days (Figure 1A). There was a significant difference between the LP treatment and the CK treatment (Figure 1B). As shown in Table 3, the parameters recorded indicated the alpha diversity of bacterial communities. Goods_coverage can be used to evaluate the amount of sequencing data based on the coverage of low abundance OTU in the sample. In the present study, goods_coverage values all exceed 0.99, implying sufficient volumes of sequencing data for robust analysis of the bacterial community. The Shannon and Simpson indexes were used to indicate the abundance and evenness of bacteria in each treatment. After fermentation for 7 days, the Shannon (from 1.78 to 1.63) and Simpson (from 0.60 to 0.50) indexes decreased in LP treatment at 15 °C. Furthermore, the effect (Shannon, from 2.09 to 0.98, and Simpson, from 0.62 to 0.17) was decreased more obviously in LP treatments at 30 than at 15 °C. In the later stages of silage, the Shannon (from 2.84 to 1.77) and Simpson (from 0.75 to 0.54) indexes of the CK treatments at 15 °C were higher than during the early stages. A similar trend was observed in the Shannon (from 3.26 to 2.28) and Simpson (from 0.79 to 0.61) indexes of the CK treatments at 30 °C.

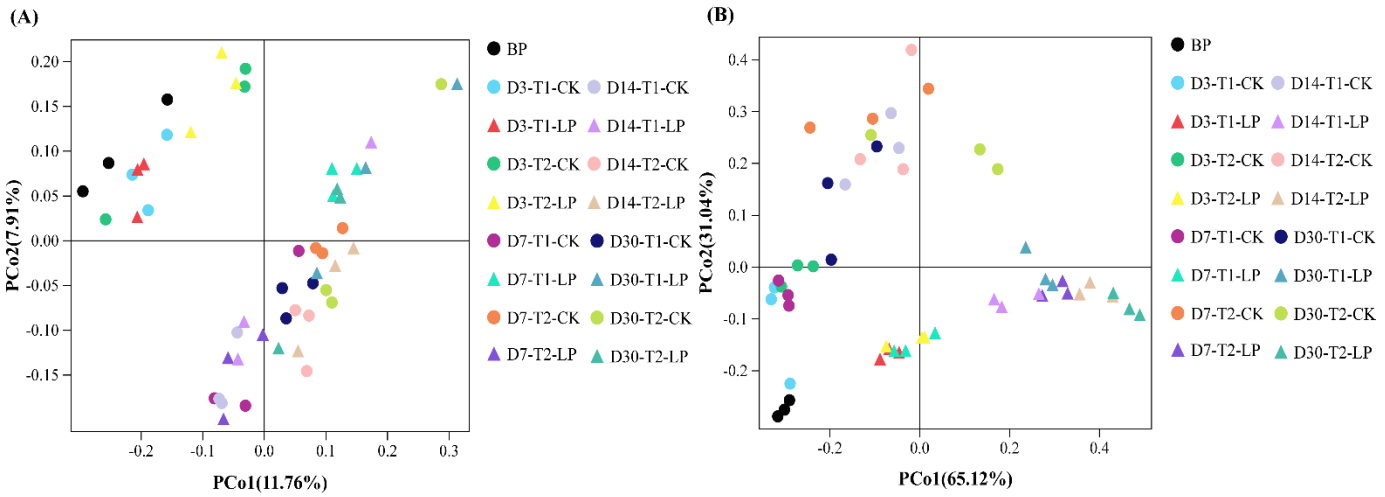

Figure 1. Principal Coordinates Analysis (PCoA) of bacterial communities for B. papyrifera leaves silage (days 3, 7, 14, 30) treated with LP inoculant and stored at 15 °C (T1) and at 30 °C (T2). Algorithm based on unweighted Unifrac metric (**A**) and weighted Unifrac metric (**B**). BPL, B. papyrifera leaves; CK, the control; LP, Lactobacillus plantarum; D3, D7, D14, D30, after 3, 7, 14, 30 days ensiling, respectively.

Figure 2 showed stacked plots of the bacterial community at the phylum and genus levels. At the level of the phylum (Figure 2A), the bacterial communities of all treatments were similar with *Firmicutes* (2.70–89.72%), *Cyanobacteria* (3.89–69.13%), and *Proteobacteria* (5.77–51.42%) as the main groups. With the addition of LP, the proportion of *Firmicutes* increased significantly (from 32.6% to 89.7%). The relative abundance of *Firmicutes* was also higher in treatments at 30 than at 15 °C (except the CK treatment for 14 days). Especially in LP treatments at 30 °C (after fermentation for 30 days), the effect was most obvious (>89%). *Cyanobacteria* and *Proteobacteria* occupied a relatively high proportion of the total bacterial count in the early stages of silage, and gradually reduced in the later periods.

At the level of the genus (Figure 2B), *Lactobacillus* (1.13–88.84%), *Lactococcus* (0.20–15.94%), *Enterococcus* (0.07–2.93%), *Weissella* (0.01–3.07%), and *Methylobacterium* (0.44–2.25%) were detected in the silage. The addition of LP made *Lactobacillus* the dominant genus: from 32.1% to 62.4% (15 °C) and from 36.4% to 88.8% (30 °C). With regards to the increase of *Lactobacillus* in the CK treatment, the effect at 30 °C (from 2.57% to 32.0%) was also greater than at 15 °C (from 1.12% to 11.0%). The relative abundance of *Lactococcus* in the LP treatments (0.20–0.74%) was lower than in the CK treatments (1.07–15.94%). Figure 3 showed the species abundance heatmap at the genus level. The relative abundance of *Methylobacterium*

(0.44–2.25%) was lower. The abundance of *Pantoea* (0.71–3.02%) in the CK treatments was recorded. The relative abundance of *Pantoea* (0.11–0.28%) in LP treatments was extremely low. In the process of silage, a few Clostridia were found such as *Lachnoclostridium_5* and *Clostridium_sensu_stricto_1*. However, adding LP reduced the relative abundance of *Lachnoclostridium_5* (from 1.68% to 0.05%) and *Clostridium_sensu_stricto_1* (from 2.84% to 0.01%). The relative abundance of *Clostridium* was lower in silage stored at 30 than at 15 °C when LP was added. In addition, after fermentation for 7 days, the relative abundance of other bacteria such as *Ralstonia*, *Hydrogenophaga*, and *Prevotella_7* also decreased to an extremely low level. From all observations, LP played an extremely important role during silage. The addition of LP increased *Lactobacillus* to an extremely high abundance level and inhibited other bacteria, such as *Clostridium* in BPL silage. The BPL silage stored at 30 °C performed better.

Table 3. Alpha diversity of bacterial community of *B. papyrifera* leaves silage ensiled with or without LP.

Item	Days	15 °C		30 °C		SEM	*p*-Value					
		CK	LP	CK	LP		T	D	L	T × D	T × L	D × L
Sobs	3	236 ^{A}	241 ^{B,C}	258 ^{A}	297 ^{A}	6.62	<0.01	<0.01	<0.01	<0.01	<0.01	0.245
	7	178 ^{d,B}	314 ^{a,A}	247 ^{b,B}	208 ^{c,B}							
	14	176 ^{c,B}	214 ^{b,c,C}	241 ^{a,b,B}	276 ^{a,A,B}							
	30	230 ^{b,A}	281 ^{a,A,B}	256 ^{a,b,A}	257 ^{a,A,B}							
Shannon	3	1.77 ^{b,B}	1.72 ^{b}	2.28 ^{a,B}	2.09 ^{a,b,A}	0.100	0.681	0.066	<0.01	<0.01	<0.01	<0.01
	7	2.16 ^{b,B}	1.78 ^{b,c}	2.71 ^{a,A,B}	1.57 ^{c,B}							
	14	3.16 ^{a,A}	1.70 ^{c}	2.62 ^{b,A,B}	1.29 ^{c,B}							
	30	2.84 ^{a,A}	1.63 ^{b}	3.26 ^{a,A}	0.98 ^{c,C}							
Simpson	3	0.54 ^{b,B}	0.57 ^{a,b,A}	0.61 ^{a,B}	0.62 ^{a,A}	0.0253	<0.01	0.023	<0.01	<0.01	<0.01	<0.01
	7	0.60 ^{b,B}	0.60 ^{b,A}	0.77 ^{a,A}	0.45 ^{c,B}							
	14	0.80^{aA}	0.54^{bAB}	0.77^{aA}	0.34^{cC}							
	30	0.75 ^{a,A}	0.50 ^{b,B}	0.79 ^{a,A}	0.17 ^{c,D}							
Chao	3	342 ^{a,B}	296 ^{b,C}	353 ^{a}	367 ^{a,A}	9.54	0.584	<0.01	0.037	0.191	<0.01	<0.01
	7	244 ^{b,C}	395 ^{a,A,B}	353 ^{a}	266 ^{b,B}							
	14	259 ^{b,C}	451 ^{a,A}	369 ^{a}	371 ^{a,A}							
	30	415 ^{a,A}	361 ^{b,B,C}	357 ^{b}	373 ^{b,A}							
Ace	3	351 ^{a,B}	307 ^{b,B}	346 ^{a,b}	372 ^{a,A,B}	9.23	0.018	<0.01	<0.01	<0.01	0.027	<0.01
	7	241 ^{b,C}	386 ^{a,A}	354^{a}	251 ^{b,C}							
	14	235 ^{b,C}	332 ^{a,A,B}	353 ^{a}	406 ^{a,A}							
	30	405 ^{a,A}	355 ^{b,A,B}	360 ^{b}	358 ^{b,B}							
Goods_coverage	3	0.999	0.999	0.999	0.999	0	0.783	0.252	0.455	0.204	0.020	<0.01
	7	0.999	0.999	0.999	1.000							
	14	1.000	0.999	0.999	0.999							
	30	0.999	0.999	0.999	0.999							

$^{(a–d)}$ Means different superscript letters in the same row ($p < 0.05$). $^{(A–D)}$ Means different superscript letters in the same column ($p < 0.05$).

3.4. Gas Production of *B. papyrifera* Leaves Silage

Figure 4 shows the gas and CO_2 production of BPL silage. After fermentation for 14d, the gas production gradually declined, except for the CK treatment at 15 °C. In the LP treatment at 30 °C, the gas or CO_2 production was decreased after fermentation for 3 days and lower than other treatments. The CO_2 production in the other treatments all decreased after peaking at 7 days.

During silage, the gas production was closely related to the activity of bacteria. In the present study, through Pearson's analysis, the correlations between gas and CO_2 production, and bacteria are listed in Table 4. Our results indicated that there was a significantly negative correlation between *Lactobacillus* and gas (−0.355) and CO_2 (−0.297) production ($p < 0.05$). *Serratia* (−0.306), *Aureimonas* (−0.37) and *Pseudomonas* (−0.299) also indicated a significantly negative correlation with CO_2 production ($p < 0.05$). *Enterococcus* showed a significantly positive correlation (0.331) with gas production in this study ($p < 0.05$). Two species of Clostridia (*Lachnoclostridium_5* and *Clostridium_sensu_stricto_1*) had no significant correlation with CO_2 production. *Lachnoclostridium_5* (0.533) and *Clostridium_sensu_stricto_1* (0.319) were significantly positively correlated with gas production. *Escherichia-Shigella* also showed a significantly ($p < 0.01$) positive correlation (0.397) with gas production.

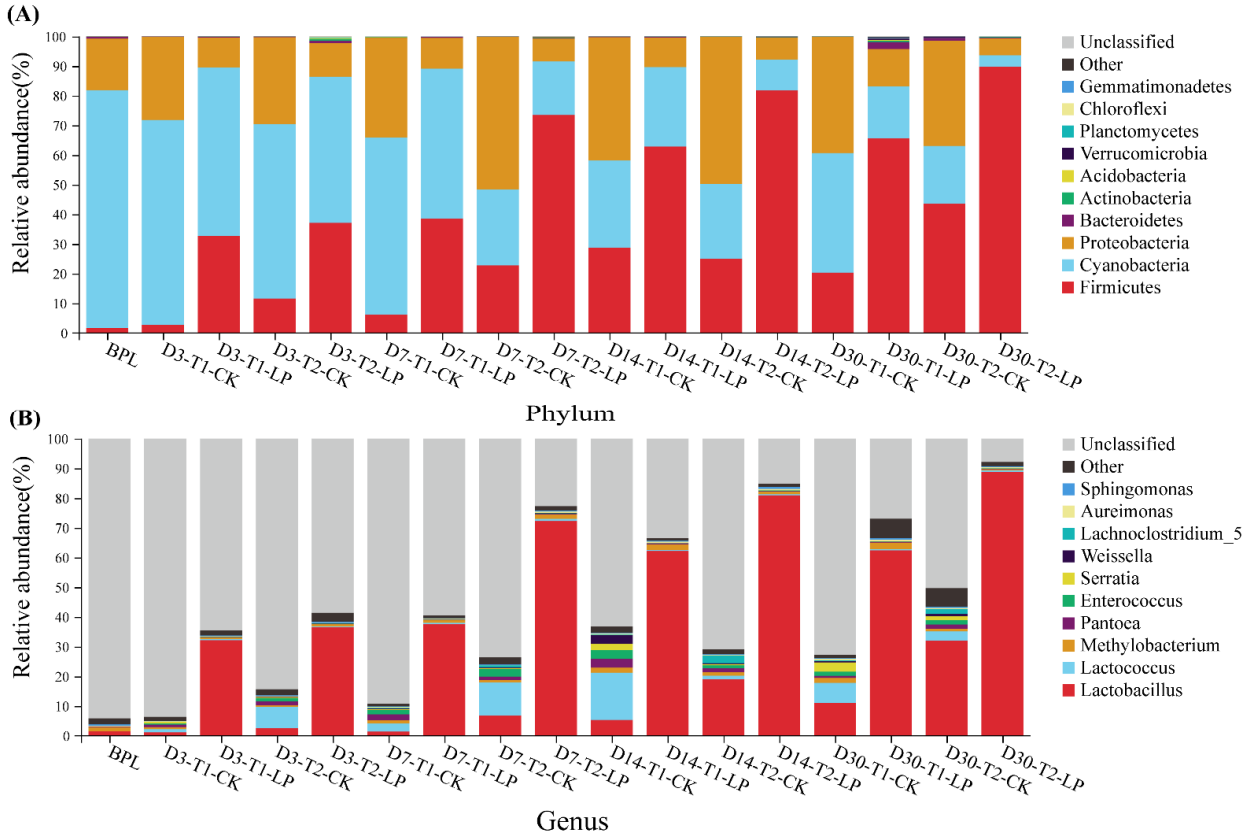

Figure 2. Relative abundance of bacterial communities by phylum (**A**) and genus (**B**) for B. papyrifera leaves silage (days 3, 7, 14, 30) treated with LP inoculant and stored at 15 °C (T1) and at 30 °C (T2). BPL, B. papyrifera leaves; CK, the control; LP, Lactobacillus plantarum; D3, D7, D14, D30, after 3, 7, 14, 30 days ensiling, respectively.

Table 4. Correlation and significance analysis of gas production, CO_2 production and bacterial community of *B. papyrifera* leaves silage.

Genus	Gas Production		CO_2 Production	
	Correlation	Significance	Correlation	Significance
Lactobacillus	−0.355	<0.05	−0.297	<0.05
Lactococcus	0.177	0.229	0.12	0.426
Methylobacterium	0.102	0.492	−0.235	0.116
Pantoea	0.178	0.225	0.035	0.819
Enterococcus	0.331	<0.05	0.189	0.209
Serratia	0.189	0.199	−0.306	<0.05
Weissella	0.092	0.533	−0.091	0.546
Lachnoclostridium_5	0.533	<0.01	0.136	0.366
Aureimonas	−0.224	0.125	−0.37	<0.05
Sphingomonas	−0.139	0.345	−0.277	0.062
Clostridium_sensu_stricto_1	0.319	<0.05	0.005	0.974
Escherichia-Shigella	0.397	<0.01	0.251	0.092
Ralstonia	−0.22	0.133	0.072	0.633
Hydrogenophaga	−0.157	0.285	0.174	0.246
Aerococcus	−0.152	0.304	−0.065	0.67
Roseomonas	−0.078	0.596	−0.097	0.521
Acinetobacter	−0.197	0.18	−0.277	0.063
Pseudomonas	−0.119	0.419	−0.299	<0.05
Bacillus	0.182	0.216	0.029	0.85
Prevotella_7	−0.031	0.836	0.089	0.556

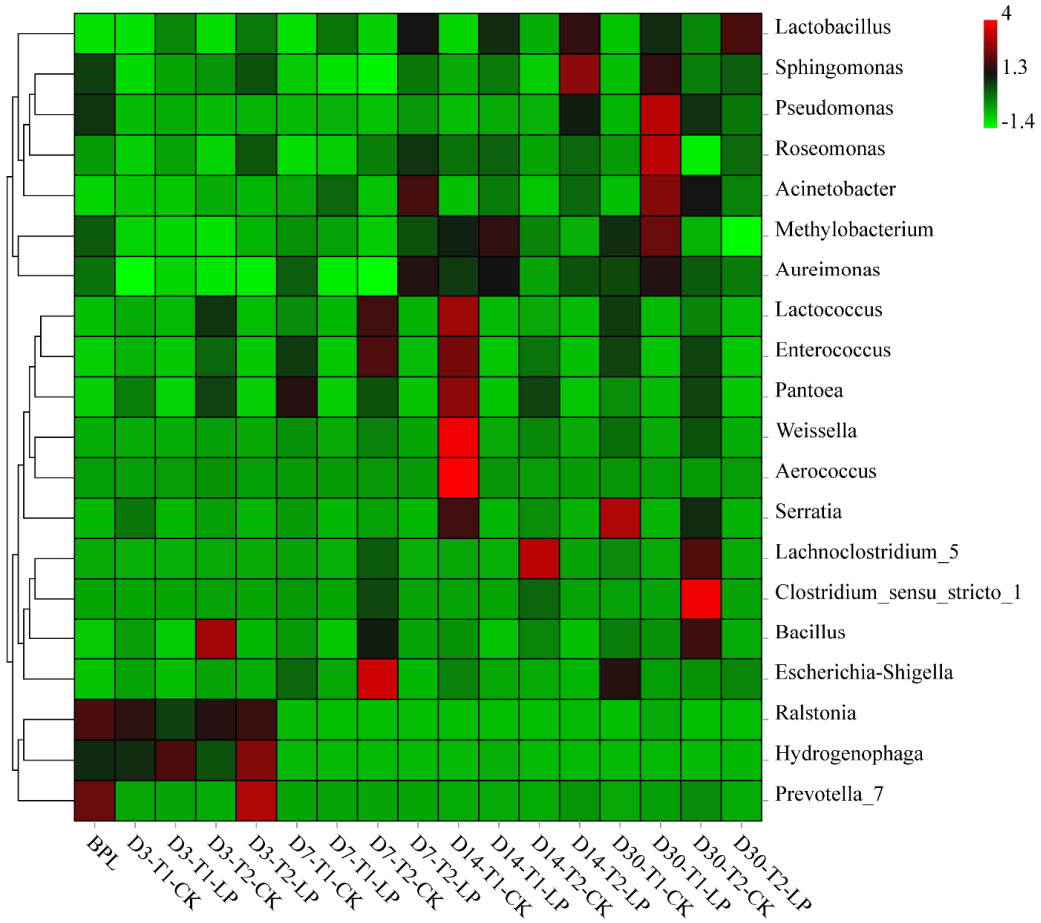

Figure 3. Heatmap of prominent bacterial genera (20 most abundant genera) for B. papyrifera leaves silage (days 3, 7, 14, 30) treated with LP inoculant and stored at 15 °C (T1) and at 30 °C (T2). BPL, B. papyrifera leaves; CK, the control; LP, Lactobacillus plantarum; D3, D7, D14, D30, after 3, 7, 14, 30 days ensiling, respectively.

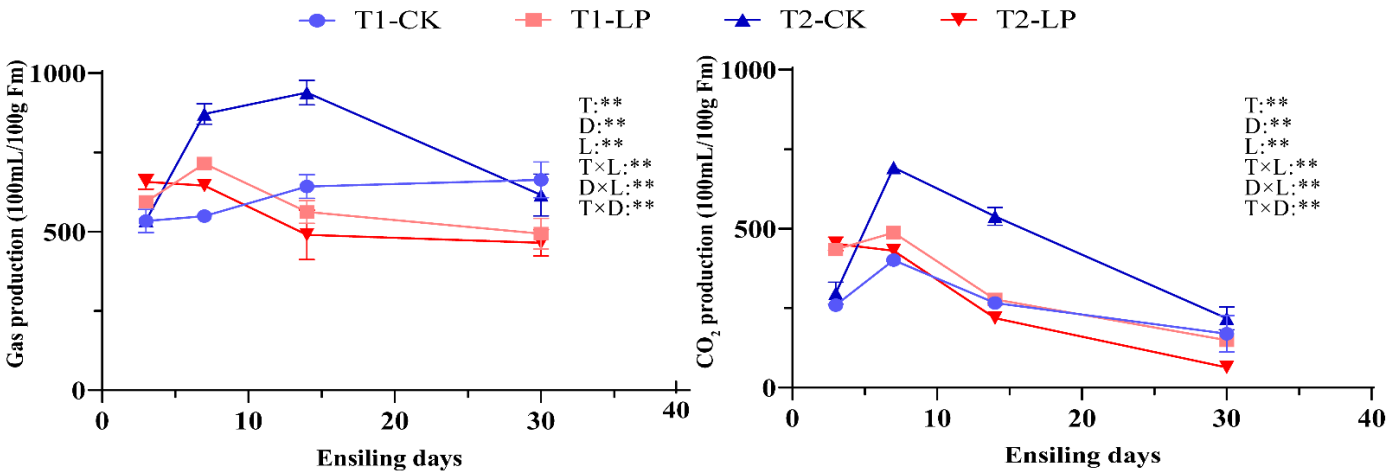

Figure 4. CO_2 production and gas production for B. papyrifera leaves silage treated with LP inoculant and stored at 15 °C (T1) and at 30 °C (T2) after fermentation for 3, 7, 14, and 30 days, respectively. CK, the control; LP, Lactobacillus plantarum; T, temperature; D, ensiling days; L, with or without LP inoculant; T × D, the interaction of temperature and ensiling days; T × L, the interaction of temperature and LP inoculant; D × L, the interaction of ensiling days and LP inoculant. **, a significant difference ($p < 0.01$).

4. Discussion

As shown in Table 1, the characteristics of the raw BPL before ensiling showed its nutritional value and the suitability of the raw material BPL for silage. In general, a DM content of 300–350 g/kg FM was most suitable for silage [28]. In the present study, the DM content of raw BPL was higher than that described by Dong et al. [4] and suitable for silage. As described by Peng et al. [6], the CP content of BPL can reach more than 200 g/kg DM, which is close to that of alfalfa. The report of Dong et al. [4] showed that the CP content of BPL (294 g/kg DM) was much higher than that of perennial ryegrass. In our study, the CP content was less than in previous studies. These differences are possibly caused by the different growth periods of the leaves and the climatic conditions [18]. The WSC content was lower than the silage standard (60–70 g/kg DM) for well-fermented silage [29]. The LAB count of raw BPL was below the standard for good silage (>5.0 $\log_{10}$ CFU/g FM) [30]. Undesirable coliform bacteria and yeasts of the microorganisms likely caused the loss of nutrients during silage. Thus, the characteristics of raw BPL indicated that measures should be taken to achieve a high-quality fermentation. Adding LAB inoculants possibly aids in inhibiting undesirable microorganisms.

Generally, the respiratory effect of plant cells and the activities of microorganisms were the main reasons for the DM loss in the fermented silage. During BPL silage, the relatively low DM loss might imply good preservation of nutrients [10]. The addition of LP and storing at 30 °C can effectively reduce the DM loss (Table 2). It is known that the influence of pH on silage quality is vitally important. In the present study, temperature, LP inoculant, and ensiling days all had significant effects on pH. The pH was lower at 30 than at 15 °C in the CK treatment. The addition of LP significantly reduced the pH at 15 or at 30 °C and the pH gradually decreased with the ensiling days prolonged. The pH was 4.49 in the LP treatment at 30 °C. It has been reported that when the pH is lower than 4.5, the growth of undesirable microorganisms in silage is inhibited [31]. The BA content is undesirable in silage. Muck [32] showed that a BA content >5 g/kg DM implied greater *Clostridium* activity, which reduced consumption of the silage by livestock. High ammonia-N content is also undesirable in silage not only because it results in nutritional loss from the resultant forage but also because it may cause excess nitrogen emissions during animal production [33]. Although the BA and ammonia-N contents increased in the CK treatment at 30 °C, the probable explanation was that higher temperatures led to higher microbial metabolic activity [10]. Due to the inhibition of undesirable microorganisms, the addition of LP was effective in reducing the BA and ammonia-N content, which were better at 30 °C than at 15 °C. It is worth mentioning that the count of LAB decreased in silage stored at 30 °C as the ensiling days were prolonged. The possible reason is that LA content increased, and the pH decreased rapidly, leading to a decrease in the activity of acid-intolerant LAB. Similarly, with the ensiling days prolonged, the count of LAB also decreased gradually due to the antagonism between LAB and rapid acidification [34]. Further, the result indicated that adding LP and storing it at 30 °C was more beneficial to inhibit coliform bacteria. The possible reason is that the lower pH leads to the inhibition of the activity of undesirable microorganisms. From these observations, in silage stored at 30 °C, the overall pH and the count of coliform bacteria decreased significantly, while LA content increased significantly. Although higher temperature would slightly lead to increases in BA and ammonia-N contents, the addition of LP could effectively reduce their content. The strongest effect was observed for silage stored at 30 °C after adding LP.

As shown in Table 3, the abundance and evenness of bacteria were higher slightly in the CK treatment than that in the LP treatment, which might indicate the increase of bacterial activity. However, the addition of LP decreased the abundance and evenness of bacteria, and the effect was more obvious at 30 than at 15 °C. The temperature at 30 °C might have more contribution to the bioactivity of LAB. The higher proportion of LAB might be due to LAB occupying a dominant niche in the microbial community and the lower pH inhibited the undesirable microorganisms. As the number of dominant bacteria increased, the diversity of the microbial community decreased [35]. The treatments of silage

additives greatly changed the structure of bacterial communities, which was also reported by He et al. [23].

Our result (Figure 2A) was similar to a study concerning alfalfa silage that reported about 74% of *Firmicutes*, followed by *Proteobacteria*, with the lowest abundance of *Cyanobacteria* [35]. At genus levels (Figure 2B), the temperature influenced the relative abundance of *Lactobacillus*, and the addition of LP had a positive influence. Typically, the complicated microbial community in raw materials was gradually displaced by LAB and the microbial diversity decreased rapidly—one of the markers for successfully fermented silage [10]. In general, the abundance of *Lactobacillus* in woody plants before silage was low, while the abundance of undesirable bacteria was high [4]. In our study, *Lactobacillus* was the dominant genus, followed by *Lactococcus*, and the proportion of other bacteria was low in the LP treatments. The study by Kung et al. [36] showed that adding *Lactobacillus* to silage could improve DM recovery and the milk yield of dairy cattle. Other studies showed that the addition of *Lactobacillus* to silage could inhibit yeast growth and improve the aerobic stability of silage [37]. According to Pahlow et al. [38], *Lactococcus*, *Enterococcus*, *Weissella*, and *Lactobacillus* are the main lactic acid-producing bacteria in silage, in general. The abundance of *Lactobacillus* in the present silage was the highest. Therefore, the changes in LA content and pH in Table 2 could be greatly interpreted. In the present silage (Figure 3), *cocci* such as *Lactococcus* and *Enterococcus* may have been inhibited by the higher abundance of *bacilli* (*Lactobacillus*). Meanwhile, *Methylobacterium*, *Pantoea*, and *Clostridium* were detected in silage. *Methylobacterium* is usually found in plants with a strictly aerobic state and is one of the dominant species in fresh legumes [39]. Liu et al. [40] considered that *Pantoea* might compete with LAB for nutrition and produce ammonia-N content. The presence of *Clostridium* is not desirable in silage. The degradation of LA by *Clostridium* leads to an increase in the pH of silage and decreases the nutritional value of forage through the dissimilation of amino acids, resulting in the loss of fermentation and livestock consumption [41]. On the whole, during the BPL silage period, the abundance of *Pantoea* and *Clostridium* was low.

Reducing the production of greenhouse gas (CO_2) during BPL silage is not only conducive to the preservation of silage nutrition but also conducive to the development of environmentally friendly livestock husbandry. The reason for the reduction of CO_2 and gas production (Figure 4) might be that the anaerobic and low pH environment inhibited the respiration of plant cells and the activity of atherogenic microorganisms during silage. Therefore, we investigated the correlations between gas and CO_2 production, and bacteria (Table 4). *Lactobacillus*, which showed significantly negative correlation with CO_2 and gas production, was the dominant genera in our study. The reason for the decrease in CO_2 and gas production might be that organic acids produced by lactic acid bacteria inhibited other gas-producing bacteria. Further, LP is the most common homofermenter in silage. The homofermentative bacteria produce two moles of LA from one mole of glucose. Heterofermentative bacteria produce one mole of LA, one mole of CO_2 and one mole of ethanol or one mole of AA from glucose [11]. Therefore, we inferred that the increase of CO_2 and gas production in early silage was related to the activity of heterofermentative bacteria and other gas-producing bacteria, such as *Enterococcus*, *Clostridium*, and *Escherichia-Shigella*. A study by Pang et al. [42] indicated that adding *Enterococcus faecium* in three different forages increased gas production. *Clostridium* could produce BA and CO_2 by converting lactic acid and acetic acid [38]. However, the high abundance of LP (homofermentative inoculant) turned into the dominant bacteria and inhibited the CO_2-producing bacteria. The study of Muck [11] expected that the DM recovery rate of the homofermentative inoculant in silage increases by 2% to 3%, on average. With the ensiling days prolonged, the CO_2 production gradually decreased due to the high proportion of *Lactobacillus* and the reduction of CO_2-producing bacteria (Figure 4 and Table 4). Similarly, the proportion of *Lactobacillus* in LP treatment was higher than that in CK treatment, which led to the lower CO_2 production. In CK treatment, the CO_2 production was higher at 30 °C than at 15 °C, which might be due to the high activities of CO_2-producing bacteria. However, with the decrease of pH and the increase of *Lactobacillus* abundance, CO_2 production also

decreased. After fermentation for 30 days, the CO_2 production was at a lower value at 15 °C and at 30 °C (no significant difference). In addition, other bacteria, such as *Serratia*, *Aureimonas* and *Pseudomonas*, which are negatively correlated with CO_2 production, also played a role. As described by Upendar et al. [43], when the surrounding CO_2 concentration was 10%, *Serratia* could fix 94.06–98.32% of CO_2. The study found that *Pseudomonas* had significant CO_2 assimilation (3500%) [44]. Therefore, the CO_2 and gas production decreased significantly during the later stage of silage. All conclusions indicated that storing at 30 °C when LP had been added could reduce gas and CO_2 production. It might be beneficial for the reduction of DM loss in BPL silage.

5. Conclusions

LP could improve the fermentation quality of *B. papyrifera* leaves silage, and more so at 30 °C than at 15 °C. The results indicated pH, ammonia nitrogen and butyric acid contents were lower when LP had been added. After adding LP, the DM loss, coliform bacteria count, and CO_2 production decreased more at 30 °C than at 15 °C. The temperature and the presence or absence of LP also greatly affected the bacterial community. The relative abundance of other bacteria was lower while that of *Lactobacillus* was higher in the silage at 30 °C when LP had been added. The high abundance of *Lactobacillus* could reduce the CO_2 production during silage, implying a reduction of DM loss.

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Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found here: the sequences were deposed in the Sequence Read Archive (SRA) with an accession number PRJNA767512.

Conflicts of Interest: The authors declare no conflict of interest.

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