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Effect of different bacterial agents on fermentation quality and microbial community of pigeon pea silage

Jiahui Pan[1,†], Youyin Wang[1,†], Zhiyong Wang[2], Jie Cai[3], Shan Yu[3], Jian Wang[4], Xiande Liu[5], Yang Wu[6,*],Wenjun Ou[3,*] and Li Liao[1,*]

[1] College of Tropical Crops, Hainan University, Haikou 570228, Hainan, China;[2] College of Forestry, Hainan University, Haikou 570228, Hainan, China; [3] Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou, 571101, China; [4] College of Animal Science and Technology, Haikou, Hainan 570228, China; [5]School of Life Sciences, Hainan University, Haikou, China; [6]School of Life Sciences, Jinggangshan University.
[†] Both authors contributed equally
[*]Corresponding author's e-mail: wuyangfenghao@jgsu.edu.cn; Cassava6973@163.com; liaoli@hainanu.edu.cn

Despite pigeon pea being an important feed resource globally, very few reports have studied the application of bacterial agents in pigeon pea silage, thereby limiting the development and utilization of pigeon pea silage in animal husbandry. This study aimed to investigate how three bacterial agents affected the chemical composition, fermentation quality, and microbial community of the pigeon pea silage. Chopped pigeon pea was subject to the following treatments: Control [no additive], MFA [*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} fresh weight (FW)], HECBEP [*Bacillus*, *lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW] and BFFA [*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW]. After analyzing the chemical composition, fermentation quality, and microbial community of the silage post 30 days of ensiling, the bacterial agents were found to improve the quality of pigeon pea silage. Notably, the BFFA group showed higher dry matter (DM) content, lower pH, and the higher ratio of ammonia nitrogen·total nitrogen^{-1} (AN·TN^{-1}) contents as compared to the MFA, HECBEP, and control group. Additionally, the microbial analysis and Spearman correlation analysis demonstrated that the application of bacteria agent BFFA increased the microbial community richness, *Lactobacillus* abundance, Firmicutes abundance, and decreased the Proteobacteria abundance. Therefore, these results suggest that bacterial agent BFFA could be recommended to be applied in pigeon pea for high-quality silage production.

Keywords: Pigeon pea, silage, fermentation quality, bacterial agents, microbial community.

INTRODUCTION

The development of animal husbandry has been affected by rising feed prices and a shortage of forage resources. Therefore, developing new feed resources is considered a viable approach. Pigeon pea (*Cajanus cajan* L. Millsp.) is a perennial legume forage grass widely grown in the semi-arid and tropical subregions in Asia and Africa (Copani *et al*., 2016). It is rich in nutrients, including crude protein, polysaccharides, flavonoids, and stilbenes. Furthermore, pigeon pea increases the efficiency of converting metabolizable energy to net energy for livestock (Onu and Okongwu, 2006; Amaefule *et al*., 2011; Li *et al*., 2013), and could be used as a high-quality protein feed resource to cope with the feed crisis (Saxena *et al*., 2021).

However, climatic extremes have seriously limited the pigeon pea production in South China, thereby inhibiting its regular supply as livestock feed (Tres *et al*., 2020). Ensiling could be the best option for pigeon pea preservation due to its high economical and practical feasibility. During the ensiling process, epiphytic lactic acid bacteria (LAB) convert soluble carbohydrates into organic acids (mainly lactic acid) under anaerobic conditions, thereby creating an acidic environment that inhibits harmful microorganisms, thus extending the shelf life of the feed and reducing the nutritional loss (Veriato *et al*., 2018; Grant and Ferraretto, 2018). Exogenous application of chemicals or biological additives to forage grass silage is an effective technique to improve the silage quality (Ke *et al*., 2021; Li *et al*., 2021). *Bacillus* degrades cellulose, releases nutrients, and promotes the digestion and absorption of digestible nutrients (Bonaldi *et al*., 2021). Yeast can quickly achieve an anaerobic environment in the silage process and utilize lactic acid and other organic acids to promote the LAB growth (Yang *et al*., 2006). Besides, being one of the tropical

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legume forage, although pigeon pea has coarse and stemmy structures with low water-soluble carbohydrates (WSC), its high fiber content makes developing good quality pigeon pea silage more challenging (Zhu and Guo, 2010). However, there is limited information on the appropriate bacterial agents that can enhance silage quality of pigeon pea.

To fill this gap, this study aimed to investigate the effects of three different bacterial agents on the fermentation quality and microbial community of pigeon pea silage. Additionally, the correlation between fermentation parameters and microbial community diversity of microbial agent-treated pigeon pea silage was also determined using high-throughput sequencing and Spearman correlation analysis. Therefore, the results of this study are valuable for increasing the capacity to produce high-quality pigeon pea silage.

MATERIALS AND METHODS

***Experimental conditions*:**Pigeon peas (*Cajanus cajan* L. Millsp) were planted in Haikou (110º 32′ E, 20º 3′ N), which is located in the north of Hainan province with a tropical maritime climate. The average annual temperature in Haikou is 23.8°C, the average annual rainfall was 1,664 mm, the average relative humidity is 85%, and the average annual rain days were ~150 d. The average annual sunshine hours are long and the average annual rainfall was ~1,000 mm. The average annual sun hours are 2,239.3 h. The main soil type was sandy shale brick red soil (Qin *et al*., 2020).

Experimental treatment: The experiment was conducted by planting seeds of 'No. 2' pigeon pea at 4–8 cm in a monopoly with (0.5 × 0.5 m) plant spacing, along with adoption of conventional field management with consistent water and fertilizer conditions. After three months of planting, the plot was mowed at a height of 20 cm above the ground. Pigeon pea stems and leaves were cut to ~2 cm with a guillotine for silage use. The chopped pigeon pea stems and leaves were divided into four groups. Then, the stems and leaves were silaged alone as the control group, while the other three groups were supplemented with MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW; provided by Weifang Shengyi Biological Company), HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW; provided by the Institute of Biotechnology, Chinese Academy of Tropical Agriculture) and BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW; provided by Hainan Shengxu Biotechnology Ltd.), with the source, composition, and concentration of bacteria agents being listed in Table 1. These bacterial agents were added to the samples according to their recommendations, and were all diluted in deionized water and then applied in liquid forms (10 mL). The control group received an equal volume of deionized water. The silage was packed into (30 × 20 cm) polyethylene silage bags (~150g) and sealed by vacuum packing machine. A total of 12 bags (4 treatments × 3 replicates) were prepared and stored under shade at room temperature (27 ± 3°C). They were finally opened on the 30th day of silage for determining their chemical composition, fermentation quality, and microbial community.

Analysis of chemical composition: The harvested pigeon pea stems and leaves were used for the determination of chemical composition before and after making silage. After 30 d of ensiling, they were taken in open bags (Yuan *et al*., 2022), and the dry samples of silage and control treatment were all dried at 65°C for 48 h to measure the dry matter (DM) content. These were then ground with a hammer crusher to pass through a 0.425 mm screen for chemical composition determination. The WSC content was determined using anthrone sulfuric acid colorimetry (Li *et al*., 2018). The total nitrogen (TN) content was determined by a nitrogen analyzer, with crude protein (CP) being calculated as TN × 6.25 (AOAC, 1990). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed as described previously (Van Soest *et al*., 1991).

Analysis of fermentation quality: First, 20 g of the sample was weighed in a 250 mL flask, and 70 mL of distilled water was added such that the samples were completely submerged, then sealed with a film, and stored at 4°C for 24 h. The extract was filtered through four layers of filter paper. The pH of the filtrate was measured using a pH meter and subsequently stored at -20°C for additional analysis. Ammonia nitrogen (AN) was determined using the colorimetric method of phenol-sodium hypochlorite and the ratio of ammonia nitrogen·total nitrogen^{-1} (AN·TN^{-1}) was calculated as described previously (Jobim *et al*., 2007). Lactic acids (LA), acetic acids (AA), propionic acids (PA), and butyric acids (BA) were determined using high-performance liquid chromatography (LC-20A column, Shodex, Shimadzu, Japan; oven temperature, 30°C; flow rate, 1 mL·min^{-1}; SPD, 210 nm) (Qiu *et al*., 2021).

***Determination of microbial community*:** After 30 days, pigeon pea silage samples of all groups were taken in triplicates, and were placed in 50 mL sterile cryopreserved tubes and stored at −80°C for DNA extraction. The total DNA

Table 1. The source, composition, and concentration of bacteria agents.

Items	Production sources	Principal components	Total number of active bacteria
MFA	Weifang Shengyi Biological Company	*Bacillus*, *Lactobacillus*, yeast	≥3.0×10^{9}CUF·mL^{-1}·g^{-1}
HECBEP	Institute of Biotechnology, Chinese Academy of Tropical Agriculture	*Bacillus*, *Lactobacillus*, yeast, enzymes	≥3.0×10^{9}CUF·mL^{-1}·g^{-1}
BFFA	Hainan Shengxu Biotechnology Ltd.	*Bacillus*, *Lactobacillus*, yeast	≥3.0×10^{9}CUF·mL^{-1}·g^{-1}

of the sample microorganisms was extracted using the E.Z.N. ATM Mag-bound Soil DNA Kit (OMEGA, USA), with the DNA quality being subsequently verified by 1% agarose gel electrophoresis. The PCR amplification and bioinformatics analysis of all microbial DNA samples were conducted by Biotech Bioengineering Co., Ltd., (Shanghai, China). The primers 341F (5'-ATGCGTAGCCGACCTGAGA-3') and 805R (5'-CGTCAGACTTTCGTCCATTGC-3') were chosen to amplify the V3-V4 region of the 16S rRNA (Wang *et al*., 2020). The PCR product was recovered and purified using the Axyprep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and subsequently quantified by an Invitrogen Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were prepared using the Illumina Tru Seq TM DNA Sample Prep Kit. The DNA were paired-end sequenced (2×300 bp) on an Illumina MiSeq PE300 platform at Biotech Bioengineering Co., Ltd., Shanghai, China.

The FLASH (version 1.2.11) was used to process the raw sequence. The QIIME (v 1.9.1) quality control process was used to obtain high-quality sequences. Identification and deletion of chimeric sequences were done using the UCHIME platform (v 4.1). The sequences were then grouped into operational taxonomic units (OTUs) at a 97% similarity level. The α-diversity and β-diversity were analyzed by Mothur (version 1.30.2). The Spearman correlation heatmap was constructed by R language package and the data visualization was performed, including Venn diagram, community composition map, and correlation analysis.

***Statistical analysis*:** The experimental data were tested for statistical significance by one-way analysis of variance (ANOVA) with SPSS 25.0 (Chicago, IL, USA) for chemical composition and fermentation quality parameters. Multiple comparisons of means were calculated using the Duncan's method, and the results were expressed as mean ± standard deviation, with $p < 0.05$ being considered significant. Values of the deterioration constants (a, b, and c) in the numerical exponential model were determined using a non-linear dynamic model (SAS, Cary, NC, USA). A total of 11 indicators (DM, WCS, CP, NDF, ADF, pH, LA, AA, PA, BA, and AN/TN) were used in each treatment group for comprehensively evaluating their silage quality using fuzzy mathematics affiliation function theory (Bonfim-Silva *et al*., 2014).

$$R(Xi) = \frac{x_i - x_{min}}{x_{max} - x_{min}} \quad [1]$$

$$R(Xi) = 1 - \frac{x_i - x_{min}}{x_{max} - x_{min}} \quad [2]$$

The equation 1 is for the affiliation function, while the equation 2 is for the inverse affiliation function. Where, *Xi* is the current index measurement value, x_{max} and x_{min} are the maximum and minimum values of the index; where NDF, ADF, pH and AN/TN are inverse affiliation functions, and the others are positive affiliation functions.

RESULTS

***Chemical composition of fresh pigeon pea*:** To obtain the basic information on chemical components of the pigeon pea silage, we initially analyzed the main component parameters in the pigeon pea silage before adding the bacterial agents (as the control). The chemical composition of fresh pigeon pea is presented in Table 2.

Table 2. Chemical composition of fresh pigeon pea.

Items	Pigeon pea stem and leaf
Dry matter (% FM)	35.17±0.25
Water-soluble carbohydrates (% DM)	2.38±0.46
Crude protein (% DM)	16.96±0.41
Neutral detergent fiber (% DM)	73.09±1.48
Acid detergent fiber (% DM)	36.90±1.94

Fresh matter=FM; dry matter=DM.

***Chemical composition and fermentation quality of pigeon pea silage after adding different bacterial agents*:** To further investigate the positive impact of application of bacterial agents on the chemical composition and fermentation quality of pigeon pea silage, we added three bacterial agents (MFA, HECBEP, and BFFA) to the pigeon pea stems and leaves in the process of making silage. The chemical composition of the silages is shown in Table 3. Significant changes were observed in the levels of DM, CP, NDF, and ADF. However,

Table 3. Chemical composition after addition of different bacterial agents in the pigeon pea silage.

Items	Treatment			
	Control	MFA	HECBEP	BFFA
Dry matter (% FM)	34.06±0.24 a	32.57±0.87 b	33.22±0.49 a	34.33±0.73 a
WSC (%DM)	2.64±0.52	2.33±0.36	2.26±0.09	2.31±0.39
CP (%DM)	11.60±0.34 b	13.01±0.68 a	14.08±0.66 a	11.95±0.97 b
NDF (% DM)	50.90±2.73 a	45.52±1.61 b	43.85±2.20 b	50.44±2.14 a
ADF (% DM)	37.71±1.41 a	34.15±1.93 ab	32.15±1.67 b	37.17±2.87 a

Values are means ± standard deviation, means with different letters within columns differ from each other ($p < 0.05$). Fresh matter=FM; dry matter=DM; crude protein=CP; water-soluble carbohydrates=WSC; neutral detergent fiber=NDF; acid detergent fiber=ADF. Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW).

three inoculant treatments did not exhibit any significant variations for WSC. Specifically, the DM contents of the BFFA-treated samples and control group were significantly higher than that of MFA-treated samples. The CP content of HECBEP-treated samples was significantly higher than that of control and BFFA-treated samples, while that of MFA-treated samples was intermediate and did not differ significantly from either the HECBEP or BFFA-treated samples. The NDF content of the MFA and HECBEP-treated samples was significantly reduced as compared to the control group and BFFA-treated samples. The HECBEP-treated samples had significantly lower ADF content than the control group and BFFA-treated samples. Table 4 shows the fermentation quality of the silages. Furthermore, the pH of BFFA-treated samples was significantly lower than in all other treatments. The LA content was significantly elevated in the HECBEP-treated samples as compared to the MFA and HECBEP-treated samples. Additionally, the LA/AA of the HECBEP-treated samples and controls were significantly higher than the MFA and HECBEP-treated samples. Lower AN/TN was observed in BFFA-treated samples.

***Effects of different bacterial agents on the number of OTUs in pigeon pea silage*:** To evaluate the impact of the application of bacterial agents on the microbial diversity of pigeon pea silage, cluster analysis of operational OTUs was performed on the silage and a total of 175 OTUs were identified from the treatment groups. Among them, 96 OTUs were shared among all the silage treated with bacterial agents, with two of being unique to the control group, six to the MFA group, seven for the HECBEP group, and finally six for the BFFA group (Fig. 1).

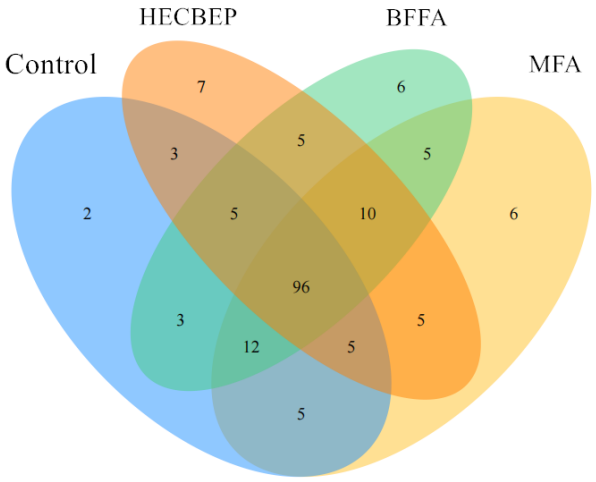

Figure 1. Venn diagram of the species distribution in pigeon pea silage.
Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW).

Table 4. Fermentation quality after addition of different bacterial agents in the pigeon pea silage.

Items	Treatment			
	Control	MFA	HECBEP	BFFA
pH	5.29±0.05 a	4.75±0.05 b	5.22±0.08 a	4.41±0.07 c
LA (% DM)	1.30±0.31 ab	0.85±0.19 b	1.71±0.33 a	0.86±0.18 b
AA (% DM)	1.68±0.25	1.93±0.05	1.91±0.21	1.82±0.31
PA (% DM)	0.02±0.01	0.03±0.01	0.03±0.01	0.03±0.01
BA (% DM)	0.19±0.02	0.16±0.14	0.14±0.12	0.21±0.06
LA/AA	0.77±0.10 a	0.44±0.09 b	0.89±0.09 a	0.48±0.11 b
AN/TN	1.57±0.23 ab	2.10±0.50 a	1.38±0.54 ab	1.35±0.36 b

Values are means ± standard deviation, means with different letters within columns differ from each other ($p < 0.05$). Dry matter=DM; Lactic acid=LA; acetic acid=AA; propionic acid=PA; butyric acid=BA; Ammonia nitrogen=AN; Total nitrogen=TN. Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW).

Table 5. Alpha diversity indices of pigeon pea silage.

Items	Treatment			
	Control	MFA	HECBEP	BFFA
Number of sequences	38943.67	42713.33	34282.67	46180.67
Shannon	0.45±0.10 b	1.58±0.09 a	0.65±0.23 b	1.89±0.09 a
Chao	118.04±13.22 b	144.09±12.07 a	121.54±5.47 b	137.19±8.32 ab
Ace	118.10±10.49 b	165.49±28.37 a	119.13±5.76 b	149.30±15.14 ab
Simpson	0.82±0.19 a	0.31±0.01 b	0.75±0.11 a	0.19±0.04 b
Shannoneven	0.10±0.04 b	0.34±0.02 a	0.14±0.05 b	0.41±0.02 a
Coverage	0.999	0.999	0.999	0.999

Values are means ± standard deviation, means with different letters within columns differ from each other ($p < 0.05$). Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW).

***Alpha diversity of pigeon pea silage*:** The diversity and richness of microbial communities are indicated by the α diversity index. The α-diversity index of microorganisms showed that the number of sequences was large, with the coverage of each treatment group being > 0.99 in the pigeon pea silage, thereby indicating that the core composition of all microorganisms in the sample was basically covered (Table 5). As compared to the control group, the Chao index and the Ace index of each group of the added bacterial agents slightly increased, with both being significantly higher in the MFA group (Table 5). Upon compared to the control and HECBEP groups, not only did the Simpson index decrease in MFA and BFFA but also the Shannon and Shannoneven indexes increased in the MFA and BFFA groups (Table 5).

***Structure of the microbial community of pigeon pea silage at the phylum level*:** To determine how bacterial agents affect the dominant populations of the microbial community in silage, the phylum with higher abundances, i.e., > 1% at the taxonomic level, was further analyzed. As shown in Table 6, the relative abundance of Firmicutes significantly increased in the MFA- and BFFA-treated silage as compared to the control (0.38%), and reached up to 30.13% and 33.99%, respectively. However, the abundance of Proteobacteria was reduced compared to the control, due to treatment with MFA and BFFA. Furthermore, despite the number of Cyanobacteria and unclassified bacteria in the HECBEP- and BFFA-treated silage being significantly higher than those in the control and MFA-treated silage, their numbers were low (Table 6).

***The correlation of fermentation parameters with the dominant phylum*:** Spearman correlation analysis was performed to evaluate the correlation of fermentation parameters with the dominant phylum in the pigeon pea silage. In the MFA-treated silage, a significant positive correlation was found between Proteobacteria and pH, LA, LA/AA (Fig. 2). Conversely, Firmicutes was negatively related to the pH, LA, LA/AA (Fig. 2).

***Microbial community of pigeon pea silage at the level of bacteria genus*:** We further identified genera from the dominant phylum to better understand the effects of bacterial agents on the microbial community structure. As compared to the control, the *Lactobacillus* abundance in MFA and BFFA increased significantly. Furthermore, common genus, including *Pseudomonas* and *Pantoea*, and the Enterobacteriaceae family were also found in the MFA- and BFFA-treated silage with (Table 7).

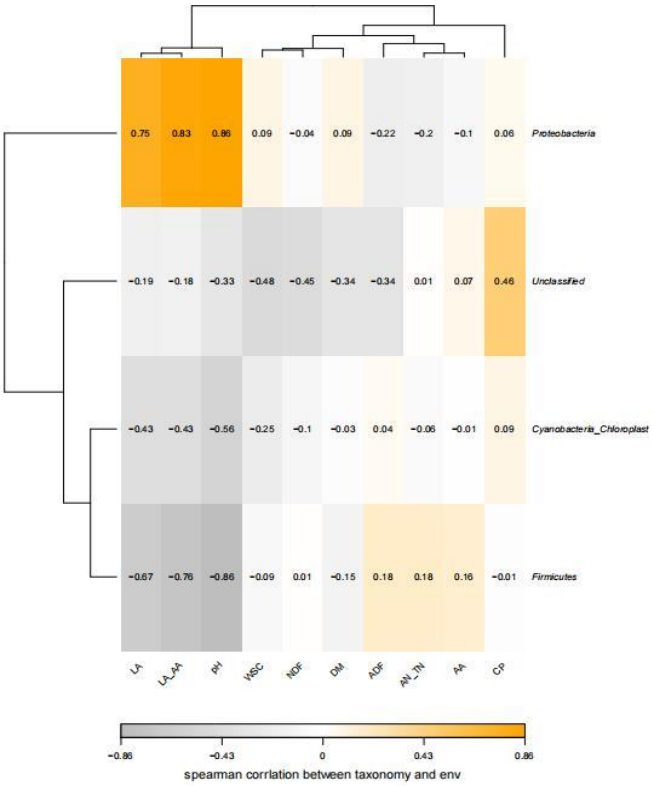

Figure 2. Heat map of correlation analysis between silage fermentation parameters and the dominant phylum.

Correlation coefficient *r* is between $^{-1}$ and 1, when *r*<-0.5, it is a significant negative correlation, and when *r*>0.5, it is a significant positive correlation. Color shades indicate the magnitude of the correlation coefficient. Dry matter=DM; crude protein=CP; water-soluble carbohydrates=WSC; neutral detergent fiber=NDF; acid detergent fiber=ADF; Lactic acid=LA; acetic acid=AA; propionic acid=PA; butyric acid=BA; Ammonia nitrogen=AN; Total nitrogen=TN.

Table 6. Structure of the microbial community of pigeon pea silage at the level of phylum.

Phylum	Treatment			
	Control	MFA	HECBEP	BFFA
Proteobacteria	94.05±6.85 a	61.33±3.80 b	93.02±3.74 a	42.15±11.7 c
Firmicutes	0.38±0.02 b	30.13±8.35 a	0.45±0.09 b	33.99±9.70 a
Cyanobacteria	1.46±0.71 c	4.88±1.62 bc	7.68±1.70 ab	9.85±1.81 a
Unclassified Bacteria	0.10±0.06 c	0.25±0.15 bc	0.76±0.12 a	0.62±0.21 ab
Others	0.14±0.11	0.11±0.04	0.10±0.01	0.07±0.01

Values are means ± standard deviation, means with different letters within columns differ from each other ($p < 0.05$). Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW). Classification of bacteria (Proteobacteria, Firmicutes, Cyanobacteria, Unclassified Bacteria and Others).

Table 7. Microbial community of pigeon pea silage at the level of bacteria genus.

Bacteria Genus	Bacterial agents			
	Control	MFA	HECBEP	BFFA
Lactobacillus	0.29±0.03 b	30.00±8.34 a	0.35±0.12 b	33.84±9.65 a
Streptophyta	1.46±0.71 c	4.88±1.62 bc	7.68±1.70 ab	12.60±3.15 a
Pseudomonas	2.58±4.24 b	10.93±0.18 a	2.00±0.34 b	12.15±4.11 a
Kosakonia	0.02±0.01	0.03±0.02	0.11±0.16	0.01±0.00
Pantoea	0.14±0.07 b	0.32±0.25b	0.99±0.42 a	0.28±0.07 b
Unclassified Enterobacteriaceae	90.58±11.50 a	51.18±6.43 b	87.32±6.81 a	21.66±7.53 c
Unclassified Bacteria	0.27±0.30	0.57±0.56	0.56±0.36	0.62±0.21
Others	0.97±0.45	0.99±0.25	1.11±0.16	0.93±0.08

Values are means ± standard deviation, means with different letters within columns differ from each other ($p < 0.05$). Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW). Bacteria Genus (*Lactobacillus*, *Streptophyta*, *Pseudomonas*, *Kosakonia*, *Pantoea*, Unclassified Enterobacteriaceae, Unclassified Bacteria and Others).

Table 8. The rank of different silage quality after application of bacterial agents calculated by function values.

Parameter	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Mean	Rank
Control	0.847	1.000	0.000	0.000	0.000	0.000	0.523	0.707	0.000	0.000	0.308	IV
MFA	0.000	0.184	0.569	0.763	0.640	0.614	0.000	0.000	0.724	0.761	0.425	III
HECBEP	0.369	0.000	1.000	1.000	1.000	0.080	1.000	0.960	0.023	0.002	0.543	II
BFFA	1.000	0.132	0.141	0.065	0.097	1.000	0.012	1.000	1.000	1.000	0.545	I

R1~R10 respectively represent the membership function values of DM, WSC, CP, NDF, ADF, pH, LA, AN/TN, Proteobacteria, and Firmicutes. Control (no additive); MFA (*Bacillus*, *Lactobacillus*, yeast; 5 g·kg^{-1} FW); HECBEP (*Bacillus*, *Lactobacillus*, yeast, enzymes; 1 g·kg^{-1} FW); BFFA (*Bacillus*, *Lactobacillus*, yeast; 4 g·kg^{-1} FW).

***The correlation of fermentation parameters with the dominant genus*:** *Lactobacillus* and *Pseudomonas* had a significant negative correlation with LA, pH, and LA/AA (Fig. 3).

***Silage quality comprehensive score*:** After combining all parameters regarding silage quality and microbial community structure to rank the bacterial agents performance-wise, I and IV represented the highest and lowest scores, respectively. The overall score of the control silage and bacterial agent-treated silage showed that the BFFA had the best performance (score of 0.545), followed by the HECBEP (0.543) and MFA (0.425) groups (Table 8).

DISCUSSION

Silage is a commonly used form of forage preservation for livestock feed. The dry matter content of silage is an important factor affecting the quality of silage production. The BFFA and control groups had higher DM content than the MFA group. This could be due to the reduction in harmful microorganisms, consequently, reduction in the degradation of DM during ensiling. The MFA, HECBEP, and BFFA groups showed a greater CP concentration than the control group, possibly due to the restriction of clostridial growth and proteolysis by LAB in the additives (Arriola *et al*., 2011). Among the three treatments *viz.*, MFA, HECBEP, and BFFA, the MFA and HECBEP showed the best inhibitory effect, mainly due to their significantly higher CP content.

The decrease in NDF and ADF levels indicates an increased nutritional value through the ensiling process. Consistent with previous studies (Ligoski *et al*., 2020), as mentioned above, although the NDF and ADF were positively correlated, a negative correlation existed between the CP and NDF contents in the MFA group, with lower NDF and ADF contents being found in the MFA and HECBEP groups. These results could be explained by the presence and degradation of cellulose enzyme (Wang *et al*., 2023). Since a low pH is also essential for the well-preserved silage (Jones, 1991), the pH of bacterial agent-treated silage was measured and compared against the control. The application of MFA and BFFA significantly reduced the LA/AA ratio, which resulted in pH values of silage being much lower than those in the control. This is probably because bacterial agents can promote LA accumulation for better forage preservation (Li and Nishino, 2010). Furthermore, BA content < 2 g·kg^{-1} of DM and AN/TN percentage < 11% are defined as standards of good fermentation (Catchpoole and Henzell, 1971; Buxton *et al*., 2003). Although the BA content and AN/TN showed no significant difference as compared to the control, they were all within these above-mentioned specific ranges of the treated silage (Table 4), thereby suggesting that the bacterial agent-treated pigeon pea silage met the required standard of good quality silage.

From the results of the basic analysis of bacterial OTUs, although the addition of bacteria agents had little effect on the difference in species distribution in the pigeon pea silage, 175

higher OTUs were found in it. The microbial community altered during the silage process and the resulting microbial diversity is highly related to the silage quality (Huang *et al*., 2021).

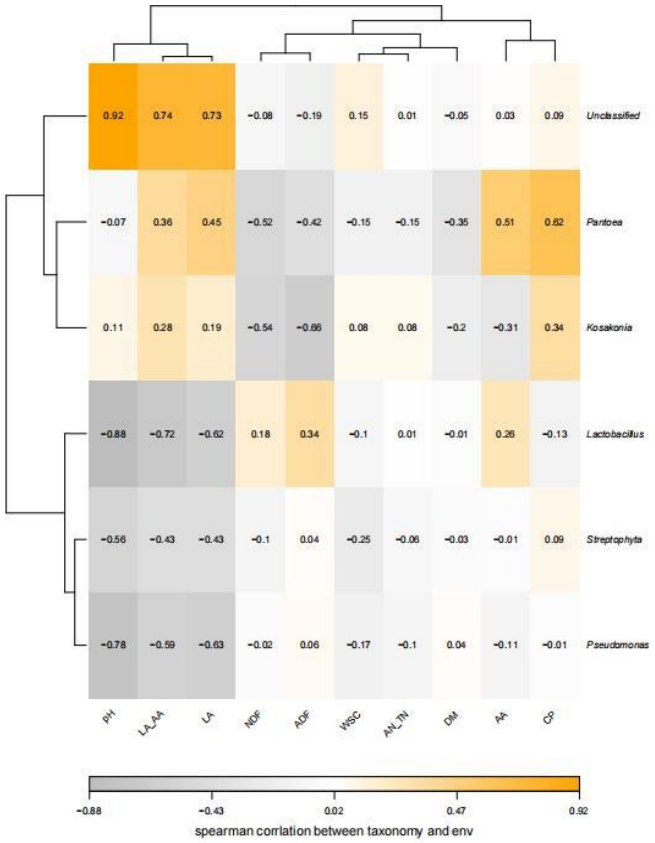

Figure 3. Heat map of correlation analysis between silage quality indicators and bacterial flora according to the genus level.

Correlation coefficient *r* is between -1 and 1, when *r*<-0.5, it is a significant negative correlation, and when *r*>0.5, it is a significant positive correlation. Color shades indicate the magnitude of the correlation coefficient. Dry matter=DM; crude protein=CP; water-soluble carbohydrates=WSC; neutral detergent fiber=NDF; acid detergent fiber=ADF; Lactic acid=LA; acetic acid=AA; propionic acid=PA; butyric acid=BA; Ammonia nitrogen=AN; Total nitrogen=TN.

Exogenous additive treatments promote the bacterial community diversity with differing trends observed between species (Li *et al*., 2018). As compared to the control group, the Chao and the Ace indexes of each bacterial agent-treated group slightly increased, and both indexes were significantly elevated in the MFA group (Table 5), thus indicating that the microbial flora abundance had increased. The increase of the Shannon index and the decrease of Simpson index in MFA and BFFA suggested their positive effects on the microbiota diversity. Also, the increased Shannoneven index was observed in the MFA and BFFA groups, which highlighted that the MFA and BFFA addition promoted the uniformity of the flora (Table 5). Therefore, adding MFA and BFFA were attributed to the improvement of the uniformity and abundance of the microbial community in the pigeon pea silage.

Since the relative abundance of Firmicutes increased significantly in the MFA- and BFFA-treated silage as compared to the control, it suggested that Firmicutes was the most dominant phylum in the MFA- and BFFA-treated silage. Furthermore, the abundance of phylum Proteobacteria was reduced due to the treatment with MFA and BFFA as compared to the control. According to previous data from the alfalfa silage, Firmicutes and Proteobacteria were found to be dominant phyla that played important roles in altering the microbial community structure of the leaf silage (Mahanna, 1993). Therefore, this suggested that Firmicutes and Proteobacteria were the dominant phyla in MFA and BFFA-treated silage.

As compared to the control, the abundance of *Lactobacillus* in MFA and BFFA increased significantly, which suggested that the *Lactobacillus* was the dominant genera in the MFA and BFFA-treated silage. This genus has been widely reported previously in maize, alfalfa, guinea grass, and other forage grasses grown in anaerobic conditions to protect themselves against unfavorable conditions (Pereira *et al*. 2007; Parvin and Nishino, 2010; Silva *et al*., 2016; Santos *et al*., 2016).

The percentage of LAB had gradually decreased, probably due to the microbiota evaluation started at the beginning of fermentation rather than the whole process (Li *et al*., 2019). Our results agreed with the assumption that two species were correlated with LA, pH, and LA/AA in naturally fermented silage (Ni *et al*., 2017). However, *Pantoea* and *Kosakonia* showed a significant negative correlation with NDF and ADF, which might be due to the decomposition effect on the acidic detergent fiber.

***Conclusions*:** This study showed that all three bacterial agents could potentially improve the pigeon pea silage quality. The ability of bacterial agent BFFA to improve the pigeon pea silage quality was stronger than those of the bacterial agents, MFA and HECBEP. BFFA significantly improved the pigeon pea silage quality, mainly in terms of higher DM content along with lower pH and AN/TN contents. The microbial analysis and Spearman correlation analysis demonstrated that the application of bacterial agent BFFA increased the bacterial community richness, *Lactobacillus* abundance, Firmicutes abundance, while decreasing the Proteobacteria abundance. Therefore, the bacterial agent BFFA was recommended to be applied in pigeon pea for high-quality silage production.

Conflict of Interest: The Authors declare that there is no conflict of interest.

Authors' Contribution Statements: PJH, WY and WYY executed the research, WJ, CJ and YS laboratory analyses, whereas LXD, WZY, OWJ and LL conceived the idea and supervised the work.

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