



Effects of Forestry Waste *Neolamarckia cadamba* Leaf Meal as an Additive on Fermentation Quality, Antioxidant Activity, and Bacterial Community of High-Moisture Stylo Silage

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Specialty section:

This article was submitted to
Environmental Economics and
Management,
a section of the journal
Frontiers in Environmental Science

Received: 21 April 2022

Accepted: 17 June 2022

Published: 22 July 2022

Citation:

Wu S, Gao L, Chen D, Xue Y, Kholif AE,
Zhou W, Chen X and Zhang Q (2022)
Effects of Forestry Waste
Neolamarckia cadamba Leaf Meal as
an Additive on Fermentation Quality,
Antioxidant Activity, and Bacterial
Community of High-Moisture
Stylo Silage.
Front. Environ. Sci. 10:925400.
doi: 10.3389/fenvs.2022.925400

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N. cadamba leaves, a byproduct of wood production, are always discarded in the field. *N. cadamba* leaves have strong antibacterial property, which might be recycled to inhibit undesirable bacteria and enhance the fermentation quality of silage. Ensiling, a traditional conservation method for animal feed, is commonly used all over the world. It is known that high-moisture forages, especially legumes, are difficult to ensile directly as much ammonia-N and butyric acid produced by undesirable bacteria will reduce the feeding value. To investigate the effects of *N. cadamba* leaf meal on the fermentation quality of stylo silage, 5% and 10% *N. cadamba* leaf meal were mixed with stylo for 30 days of ensiling in two independent experiments. Results showed that the silage pH and butyric acid content of stylo silage were decreased ($p < 0.01$) by 10% *N. cadamba* leaf meal. In experiment 2, contents of nonprotein-N and ammonia-N were significantly decreased ($p < 0.05$), while the true protein content was significantly increased ($p < 0.05$). The same results on the changing tendency were also obtained in experiment 1. In addition, *N. cadamba* leaf meal addition also decreased the bacterial diversity. The relative abundance of *Clostridium* and *Lelliottia* decreased, whereas that of *Lactobacillus* increased when *N. cadamba* leaf meal was added. It is worth noting that the addition of *N. cadamba* leaf meal also improved the antioxidant activity of stylo silage. The aforementioned results suggested that mixing *N. cadamba* leaf meal to high moisture forages could be an effective strategy to enhance silage fermentation quality, and it is also a feasible way to recycle *N. cadamba* leaves.

Keywords: recycle, mixed ensiling, microorganism, legume silage, tannin

INTRODUCTION

N. cadamba is an evergreen, fast-growing, and semi-deciduous tree, and it is widely planted throughout the tropical or subtropical regions. It is traditionally used as an herbal medicine to cure various ailments in India. In recent years, it has been largely planted in countries like China, Malaysia, and Egypt for plywood and paper production (Zhao et al., 2017). The leaves with vast amount of biomass are discarded or burnt in the field, which produces greenhouse gases and air pollutants. Actually, it is a good resource for ruminants due to its high crude protein content (>22% DM, Wang et al., 2017) and digestibility (He et al., 2018). Better growth and slaughter performance and meat characteristics of goats were observed when *N. cadamba* leaves were added in their diet (Wang et al., 2017). Moreover, it contains many active metabolites, such as flavonoids and phenolic acids, which result in its strong antibacterial property. It is reported that the extract of *N. cadamba* leaves showed a strong inhibitory effect on undesirable bacteria like *Bacillus*, *Escherichia*, and *Staphylococcus* (Khandelwal et al., 2016; Pandey and Negi, 2016), which are also abundant in silage (Bai et al., 2020; Ni et al., 2020). Adding *N. cadamba* leaf meal to silage may not only decrease the moisture content but also inhibit the activities of undesirable microorganisms.

Ensiling is a traditional conservation method for fresh forages to avoid nutritional losses caused by weather. It is widely and commonly used due to the simple process, low-cost, and better preservation of nutrients (McDonald et al., 1991). But the activities of undesirable microorganisms often lead to protein degradation during ensiling. Moreover, most forages, especially legumes, are difficult to obtain a high quality of silage, owing to their high moisture and low soluble carbohydrate contents. As a result, the silage pH cannot decline rapidly, and undesirable microorganisms like *Clostridia* and *Enterobacter* can dominate, which lead to butyric acid production and proteolysis during ensiling (Wang et al., 2019a; Ni et al., 2020). Both butyric acid and proteolysis are undesirable in silage production. Butyric acid not only promotes the growth of spoilage microorganisms, resulting in the loss of dry matter, but also reduces animal intake directly (Wang et al., 2019a). Depending on the research of Tabacco et al. (2006), the degradation of proteins may lead to the inefficient rumen microbial nitrogen synthesis. More urinary nitrogen emission also has a negative impact on the environment (Adegbeye et al., 2019). As reported by Ni et al. (2020) and Wang et al. (2019a), the ammonia-N content could be higher than 20% total nitrogen, and the butyric acid content could be higher than 0.5% dry matter in legume silages like alfalfa and stylo. Thus, it is essential to find a suitable method to improve the conservation of high-moisture legume silages to minimize the economic loss and potential environmental pollution.

Therefore, stylo (*Stylosanthes guianensis*), an important legume in the tropical or subtropical regions, was used in the present study. In two independent experiments, 5% and 10% *N. cadamba* leaf meal were added to fresh stylo to investigate their effects on the fermentation quality, nitrogen fractions, antioxidant activities, and bacterial communities. In addition,

to obviate the influence of the moisture content, 5% and 10% stylo meal were added separately to the fresh stylo.

MATERIALS AND METHODS

Silage Preparation

Stylo (CIAT 184) and *N. cadamba* were grown in an experimental field at the South China Agricultural University (Guangdong, China). No herbicides and fertilizers were used during the growth stage. Stylo (at bloom stage) from two plots and leaves of *N. cadamba* (with a height of 1.5–2 m) from one plot (40 trees in total) were collected on 4 December 2018 (experiment 1) and 12 October 2019 (experiment 2), respectively. All the collected materials were chopped into 1–2 cm lengths. Prior to ensiling, stylo and *N. cadamba* leaves were dried at 65°C for 2 days and then ground immediately to obtain stylo meal (S) and *N. cadamba* leaf meal (N). In both experiments, fresh stylo was added with 0% (the control), 5% S, 10% S, 5% N, and 10% N (on a fresh matter basis). About 110 g of evenly mixed silage material was sealed in polyethylene plastic bags with a vacuum sealer. Three bags for each treatment were made, and these bags were kept in the shade (around 25°C). After 30 days of fermentation, silages were sampled to analyze their fermentation parameters, protein fractions, antioxidant activities, and bacterial communities.

Fermentation Characteristics, Chemical Components, and Antioxidant Capacity Analyses

The methods used in this part were similar to our previously study (He et al., 2020) unless otherwise specified. The silage sample (20 g) was fully soaked in sterilized physiological saline (180 ml), and then, the leaching solution was diluted in gradient. Then, the plate counting method was used to determine the number of lactic acid bacteria (LAB), coliform bacteria, yeasts, and molds. Another 20 g silage sample was mixed with 180 ml distilled water and stored at 4°C for 18 h. Then, one aliquot of the filtrate was used to measure the pH value with a pH meter. Organic acids including lactic acid, acetic acid, propionic acid, and butyric acid were determined using an HPLC method, in accordance with the condition and procedure of He et al. (2020).

About 50 g silage were oven-dried for calculating the dry matter (DM) and ground for chemical analysis. The determination methods of chemical compositions, including protein, water-soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), and tannin, were the same as that of Wang et al. (2019b). The antioxidant activity including radical 2, 2-diphenyl-1-picrylhy-drazyl (DPPH) and radical 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), ammonium salt (ABTS)-scavenging activity, and ferric-reducing antioxidant power (FRAP) were measured with the method of He et al. (2020). The scavenging activity and reducing power were expressed as milligrams of trolox equivalents per gram of the dry matter (mg trolox g⁻¹ DM).

TABLE 1 | Characteristics of fresh stylo and *N. cadamba* leaf meal prior to ensiling (\pm SD, $n = 3$).

Item	Experiment 1		Experiment 2	
	Stylo	<i>N. cadamba</i> leaf meal	Stylo	<i>N. cadamba</i> leaf meal
Dry matter (g kg ⁻¹ FM)	266 \pm 2.60	—	343 \pm 6.62	—
Crude protein (g kg ⁻¹ DM)	116 \pm 0.65	127 \pm 4.85	132 \pm 9.96	84.7 \pm 1.13
True protein-N (g kg ⁻¹ TN)	820 \pm 31.2	901 \pm 28.2	580 \pm 37.7	966 \pm 10.3
Non-protein-N (g kg ⁻¹ TN)	180 \pm 31.2	99.0 \pm 28.2	420 \pm 37.7	34.0 \pm 10.3
Neutral detergent fiber (g kg ⁻¹ DM)	521 \pm 6.10	227 \pm 7.85	602 \pm 6.40	235 \pm 6.37
Acid detergent fiber (g kg ⁻¹ DM)	419 \pm 6.02	163 \pm 4.75	469 \pm 0.95	169 \pm 3.88
Water-soluble carbohydrate (g kg ⁻¹ DM)	15.1 \pm 1.81	33.9 \pm 5.75	16.2 \pm 1.14	34.7 \pm 4.58
Lactic acid bacteria (log ₁₀ CFU g ⁻¹ FM)	4.20 \pm 0.46	—	4.93 \pm 0.19	—
Yeasts (log ₁₀ CFU g ⁻¹ FM)	4.65 \pm 0.16	—	3.83 \pm 0.39	—
Molds (log ₁₀ CFU g ⁻¹ FM)	4.30 \pm 0.61	—	3.75 \pm 0.37	—
Coliform bacteria (log ₁₀ CFU g ⁻¹ FM)	4.94 \pm 0.55	—	5.68 \pm 0.18	—
Hydrolyzable tannins (g kg ⁻¹ DM)	4.68 \pm 0.17	37.1 \pm 5.15	3.8 \pm 0.21	25.9 \pm 4.52
Condensed tannins (g kg ⁻¹ DM)	4.02 \pm 0.33	56.6 \pm 2.52	4.1 \pm 0.19	75.3 \pm 11.2

FM, fresh matter; DM, dry matter; TN, total nitrogen; CFU, colony-forming unit.

Bacterial Community Sequencing Analysis

The stylo silage in the two experiments was sampled, and the total bacterial DNA was extracted with a DNA Kit (Omega Bio-Tek, Norcross, GA, United States), following the attached instructions. The specific steps were similar to Bai et al. (2020). The V3-V4 regions of 16S rDNA were amplified using the primers (341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATC TAAT). Polymerase chain reactions (PCRs) were conducted in a 50 μ l mixture (5 μ l of 10 \times KOD Buffer, 1.5 μ l of each primer (5 μ M), 1 μ l of KOD polymerase, 5 μ l of 2.5 mM dNTPs, and 100 ng of template DNA), and the same reaction procedures were reported by He et al. (2019b). After being purified and quantified, the purified PCR products were sequenced on an Illumina HiSeq 2500 Sequencing System (Illumina, Inc., San Diego, CA, United States), and the raw sequences were analyzed as according to the procedures of Wang et al. (2018). The bioinformatic data were analyzed using the free online platform (<http://www.omicshare.com/tools>). The α -diversity was calculated in the QIIME bioinformatic pipeline (<https://qiime.org>). The β -diversity was analyzed with principal component analysis (PCA). The relative abundances of different bacterial communities at the phylum and genus levels were also analyzed.

Statistical Analysis

The effects of mixing stylo meal and *N. cadamba* leaf meal on the stylo fermentation quality and chemical composition were analyzed with IBM SPSS 20.0 for Windows statistical software. The results were assessed using the one-way analysis of variance (ANOVA), with Duncan's multiple range test. The statistical significance was determined at the $p < 0.05$ level. All the figures were made by Adobe Illustrator CS 6.0 software.

RESULTS

Characteristics of Fresh Stylo and *N. cadamba* Leaves Prior to Ensiling

The chemical composition and microbial population of fresh stylo and *N. cadamba* leaf meal before ensiling in experiments 1 and 2 are listed in Table 1. The DM contents of stylo in the two experiments were

266 and 343 g kg⁻¹, respectively. The protein contents of stylo in experiment 2 were slightly higher than those in experiment 1 (132 vs. 116 g kg⁻¹ DM). But the NDF and ADF contents of stylo in experiment 1 were lower than those in experiment 2 (521 and 419 g kg⁻¹ vs. 602 and 469 g kg⁻¹ DM). The WSC contents of stylo in experiment 1 and 2 were very poor (15.1 and 16.2 g kg⁻¹ DM, respectively). The LAB numbers of fresh stylo in our two experiments were insufficient (4.20 and 4.93 log₁₀ CFU g⁻¹ FM, respectively). More unfortunately, the counts of undesirable microorganisms, fungi, and coliform bacteria (4.65 and 3.83, 4.30 and 3.75, and 4.94 and 5.68 log₁₀ CFU g⁻¹ FM for yeasts, molds, and coliform bacteria, respectively) were relatively high. Tannin contents of *N. cadamba* leaves in experiments 1 and 2 (37.1 and 25.9 g kg⁻¹, 56.5 and 75.3 g kg⁻¹ DM for hydrolyzable tannins and condensed tannins, respectively) were abundant and far higher than those of stylo. In addition, *N. cadamba* leaf meal contains relatively higher WSC (34 g kg⁻¹ DM), which were supposed to be helpful for fermentation.

Fermentation Quality and Microbial Population of Stylo Silage

As shown in Table 2, the DM content of stylo ensiled alone (249 and 330 g kg⁻¹ fresh matter) was relatively low, and the relatively high pH value (5.15 and 5.18, respectively) indicated the poor fermentation quality. The DM content and pH value were decreased significantly ($p < 0.01$) by *N. cadamba* leaf meal. Lactic acid, the dominant fermentation product in this study, increased by 10% *N. cadamba* leaf meal treatment in experiment 1 (8.70–10.6 g kg⁻¹ DM) and experiment 2 (5.78–7.15 g kg⁻¹ DM). The contents of acetic acid and butyric acid were significantly decreased ($p < 0.01$) when 10% *N. cadamba* leaf meal was added. For butyric acid, it decreased from 13.2 to 0.476 g kg⁻¹ DM and from 1.19 to not detected in experiments 1 and 2, respectively.

Nitrogen Fractions and Antioxidant Activities of Stylo Silage

As shown in Table 3, in experiment 2, the 5% and 10% *N. cadamba* leaf meal increased the true protein content and

TABLE 2 | Fermentation characteristics and microbial populations of stylo silage ($n = 3$).

Item	Experiment 1								Experiment 2							
	CK	S5	S10	N5	N10	SEM	p -value	CK	S5	S10	N5	N10	SEM	p -value		
Dry matter (g kg ⁻¹ FM)	249 ^c	293 ^{ab}	314 ^{ab}	283 ^{bc}	329 ^a	8.63	0.007	330 ^c	365 ^b	373 ^b	366 ^b	405 ^a	6.53	<0.01		
pH	5.15 ^a	5.20 ^a	5.15 ^a	4.70 ^b	4.65 ^b	0.066	<0.01	5.18 ^a	5.10 ^b	5.05 ^{bc}	5.01 ^c	4.90 ^d	0.026	<0.01		
Lactic acid (g kg ⁻¹ DM)	8.70 ^c	7.60 ^d	7.93 ^c	11.7 ^a	10.6 ^b	0.432	<0.01	5.78	6.65	7.15	6.29	7.15	0.316	0.667		
Acetic acid (g kg ⁻¹ DM)	10.4 ^a	9.30 ^{ab}	6.29 ^c	11.2 ^a	7.74 ^{bc}	0.546	0.004	6.99 ^a	5.84 ^b	4.75 ^c	5.87 ^b	4.11 ^c	0.287	<0.01		
Propionic acid (g kg ⁻¹ DM)	ND	ND	ND	ND	ND	—	—	ND	ND	ND	ND	ND	—	—		
Butyric acid (g kg ⁻¹ DM)	13.2 ^a	16.6 ^a	14.5 ^a	3.52 ^b	0.48 ^b	1.77	<0.01	1.19 ^{bc}	1.32 ^b	2.60 ^a	0.29 ^c	ND	0.269	<0.01		
Lactic acid bacteria (Log ₁₀ cfu g ⁻¹ FM)	6.45 ^b	6.37 ^b	6.80 ^b	7.40 ^a	7.61 ^a	0.142	<0.01	7.14	7.12	7.15	7.38	6.86	0.076	0.33		
Molds (Log ₁₀ cfu g ⁻¹ FM)	<2.00	<2.00	<2.00	<2.00	<2.00	—	—	<2.00	<2.00	<2.00	<2.00	<2.00	—	—		
Yeasts (Log ₁₀ cfu g ⁻¹ FM)	<2.00	<2.00	<2.00	<2.00	<2.00	—	—	<2.00	<2.00	<2.00	<2.00	<2.00	—	—		
Coliform bacteria (Log ₁₀ cfu g ⁻¹ FM)	3.55	3.26	2.49	3.73	2.54	0.323	0.709	<2.00	<2.00	<2.00	<2.00	<2.00	—	—		

CK, control; S5, stylo meal at 5%; S10, stylo meal at 10%; N5, N. cadamba leaf meal at 5%; N10, N. cadamba leaf meal at 10%; DM, dry matter; FM, fresh matter; ND, not detected; SEM, standard error of means; CFU, colony-forming unit. Means with different letters in the same row (a–d) indicate a significant difference ($p < 0.05$).

TABLE 3 | Protein fractions of stylo silage ($n = 3$).

Item	Experiment 1								Experiment 2							
	CK	S5	S10	N5	N10	SEM	p value	CK	S5	S10	N5	N10	SEM	p value		
Crude protein (g kg ⁻¹ DM)	120	122	120	125	126	1.33	0.605	106	97	104	101	104	1.22	0.211		
True protein-N (g kg ⁻¹ TN)	601	640	679	670	736	21.6	0.092	547 ^b	561 ^b	556 ^b	585 ^{ab}	643 ^a	11.7	0.029		
Non-protein-N (g kg ⁻¹ TN)	399	360	321	330	264	21.6	0.092	453 ^a	439 ^a	444 ^a	415 ^{ab}	357 ^b	11.7	0.029		
Ammonia-N (g kg ⁻¹ TN)	110 ^a	94.9 ^{ab}	84.9 ^{bc}	67.8 ^c	46.2 ^d	6.45	<0.01	169 ^a	126 ^b	99.0 ^c	87.0 ^c	69.0 ^d	9.52	<0.01		

CK, control; S5, stylo meal at 5%; S10, stylo meal at 10%; N5, N. cadamba leaf meal at 5%; N10, N. cadamba leaf meal at 10%; DM, dry matter; N, nitrogen; TN, total nitrogen; SEM, standard error of means. Means with different letters in the same row (a–d) indicate a significant difference ($p < 0.05$).

TABLE 4 | Tannin contents and antioxidant activities of stylo silage ($n = 3$).

Item	Experiment 1								Experiment 2							
	CK	S5	S10	N5	N10	SEM	p -value	CK	S5	S10	N5	N10	SEM	p -value		
Hydrolyzable tannins (g kg ⁻¹ DM)	3.48 ^c	4.60 ^c	3.78 ^c	8.18 ^a	11.0 ^a	0.85	<0.01	2.56 ^c	3.18 ^c	3.55 ^c	6.42 ^b	9.66 ^a	0.748	<0.01		
Condensed tannins (g kg ⁻¹ DM)	2.72 ^c	3.51 ^c	3.09 ^c	5.85 ^b	8.73 ^a	0.632	<0.01	2.36 ^d	2.59 ^d	4.46 ^c	6.54 ^b	11.0 ^a	0.926	<0.01		
DPPH (mg of trolox equivalent g ⁻¹ of DM)	1.41 ^c	1.87 ^c	2.17 ^c	11.7 ^b	13.9 ^a	1.45	<0.01	1.14 ^c	1.87 ^c	2.17 ^c	11.7 ^b	13.9 ^a	1.45	<0.01		
ABTS (mg of trolox equivalent g ⁻¹ of DM)	28.2 ^b	26.5 ^b	30.2 ^{ab}	28.6 ^b	34.0 ^a	0.87	0.038	23.2	23.9	23.5	20.9	21.4	0.54	0.32		
FRAP (mg of trolox equivalent g ⁻¹ of DM)	6.59 ^c	6.15 ^c	6.80 ^c	7.84 ^{ab}	9.25 ^a	0.345	0.005	4.95 ^b	4.85 ^b	4.97 ^b	4.96 ^b	6.67 ^a	0.206	0.001		

CK, control; S5, stylo meal at 5%; S10, stylo meal at 10%; N5, N. cadamba leaf meal at 5%; N10, N. cadamba leaf meal at 10%; DM, dry matter; DPPH, free radical DPPH, scavenging activity; ABTS, radical ABTS, scavenging activity; FRAP, ferric-reducing antioxidant power; SEM, standard error of means. Means with different letters in the same row (a–d) indicate a significant difference ($p < 0.05$).

decreased the nonprotein-N ($p < 0.05$) and ammonia-N ($p < 0.01$) contents, although the crude protein content showed no change. The same results on the changing tendency were also obtained in experiment 1. The ammonia-N content decreased from 110 to 46.2 g kg⁻¹ total nitrogen and from 169 to 69.0 g kg⁻¹ total nitrogen by 10% *N. cadamba* leaf meal in experiments 1 and 2. On the other hand, as shown in **Table 4**, the contents of hydrolyzable tannins and condensed tannin content increased in *N. cadamba* leaf meal-treated silage. Also, the DPPH scavenging activity and FRAP of *N. cadamba* leaf meal-treated stylo silage were significantly increased ($p < 0.01$). However, the addition of *N. cadamba* leaf meal had no significant effects on the ABTS scavenging activity of stylo silage.

Bacterial Diversity of Stylo Silage

Bacterial diversity of stylo silage mixed with stylo meal and *N. cadamba* leaf meal is shown in **Table 5**. The good coverage values for all the treatments in the two experiments were all above 0.99. The change in different indexes in the two experiments showed a similar trend. The Sobs, Shannon, Simpson, and Ace indices in experiments 1 and 2 decreased in 10% *N. cadamba* leaf meal treatment. The results of unweighted principal coordinate analysis (PCoA) could reflect the distinction of the bacterial community among the treatments. As shown in **Figure 1**, PCoA 1 and PCoA 2 were 66.1% and 12.1% of the total variance in experiment 1 and 38.0% and 15.3% of the total variance in experiment 2, respectively. The relative abundances of bacterial

TABLE 5 | Alpha diversity of the bacterial community of stylo silage mixed with stylo meal and *N. cadamba* leaf meal ($n = 3$).

	Treatment	Sobs	Shannon	Simpson	Chao	Ace	Coverage
Experiment 1	CK	803	3.28	0.76	1,325	1,301	0.998
	S5	727	2.94	0.73	1,243	1,283	0.998
	S10	859	3.26	0.76	1,326	1,357	0.998
	N5	664	3.28	0.77	1,132	1,179	0.998
	N10	781	4.07	0.83	1,206	1,282	0.998
Experiment 2	CK	1,129	5.09	0.91	1,474	1,455	0.996
	S5	1,159	5.07	0.91	1,597	1,559	0.996
	S10	1,226	5.55	0.94	1,538	1,554	0.996
	N5	1,119	5.16	0.91	1,468	1,479	0.996
	N10	1,225	5.85	0.94	1,529	1,516	0.997

CK, control; S5, stylo meal at 5%; S10, stylo meal at 10%; N5, *N. cadamba* leaf meal at 5%; N10, *N. cadamba* leaf meal at 10%.

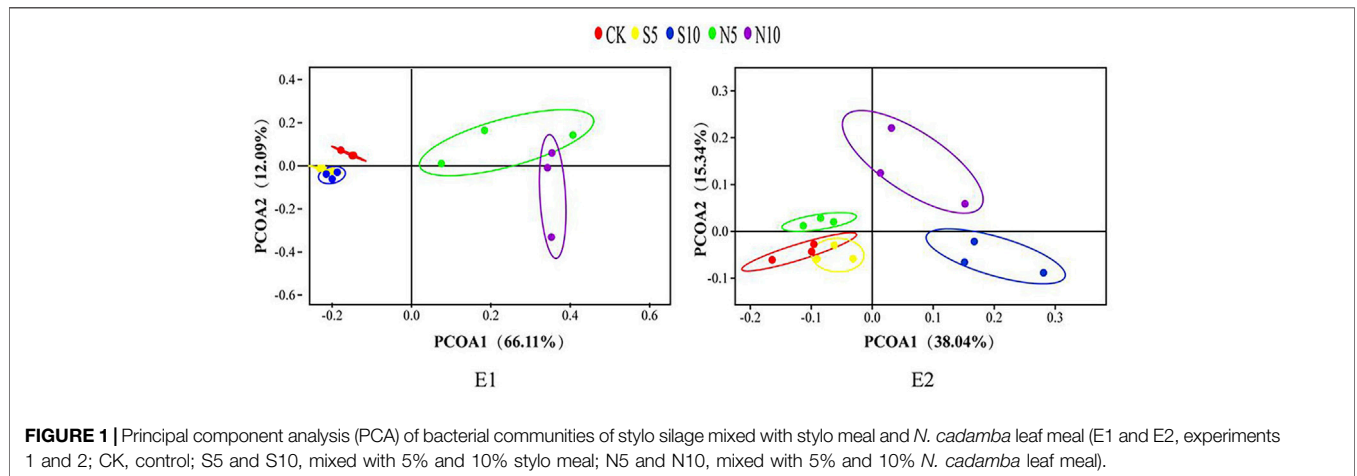


FIGURE 1 | Principal component analysis (PCA) of bacterial communities of stylo silage mixed with stylo meal and *N. cadamba* leaf meal (E1 and E2, experiments 1 and 2; CK, control; S5 and S10, mixed with 5% and 10% stylo meal; N5 and N10, mixed with 5% and 10% *N. cadamba* leaf meal).

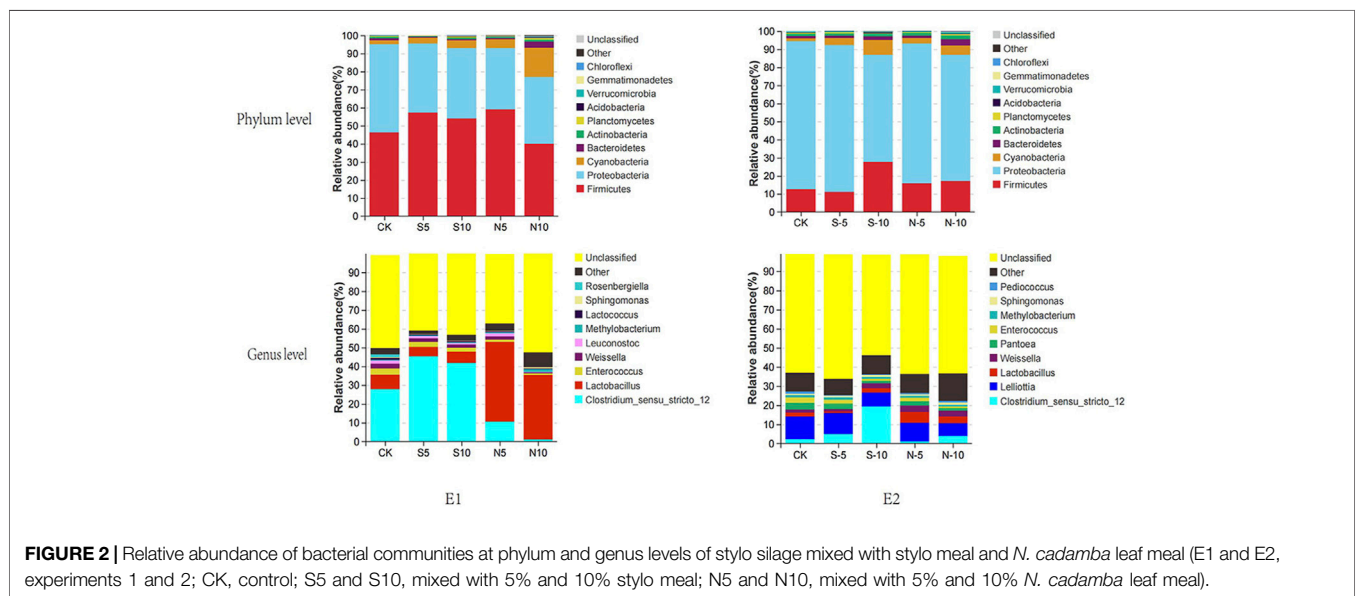
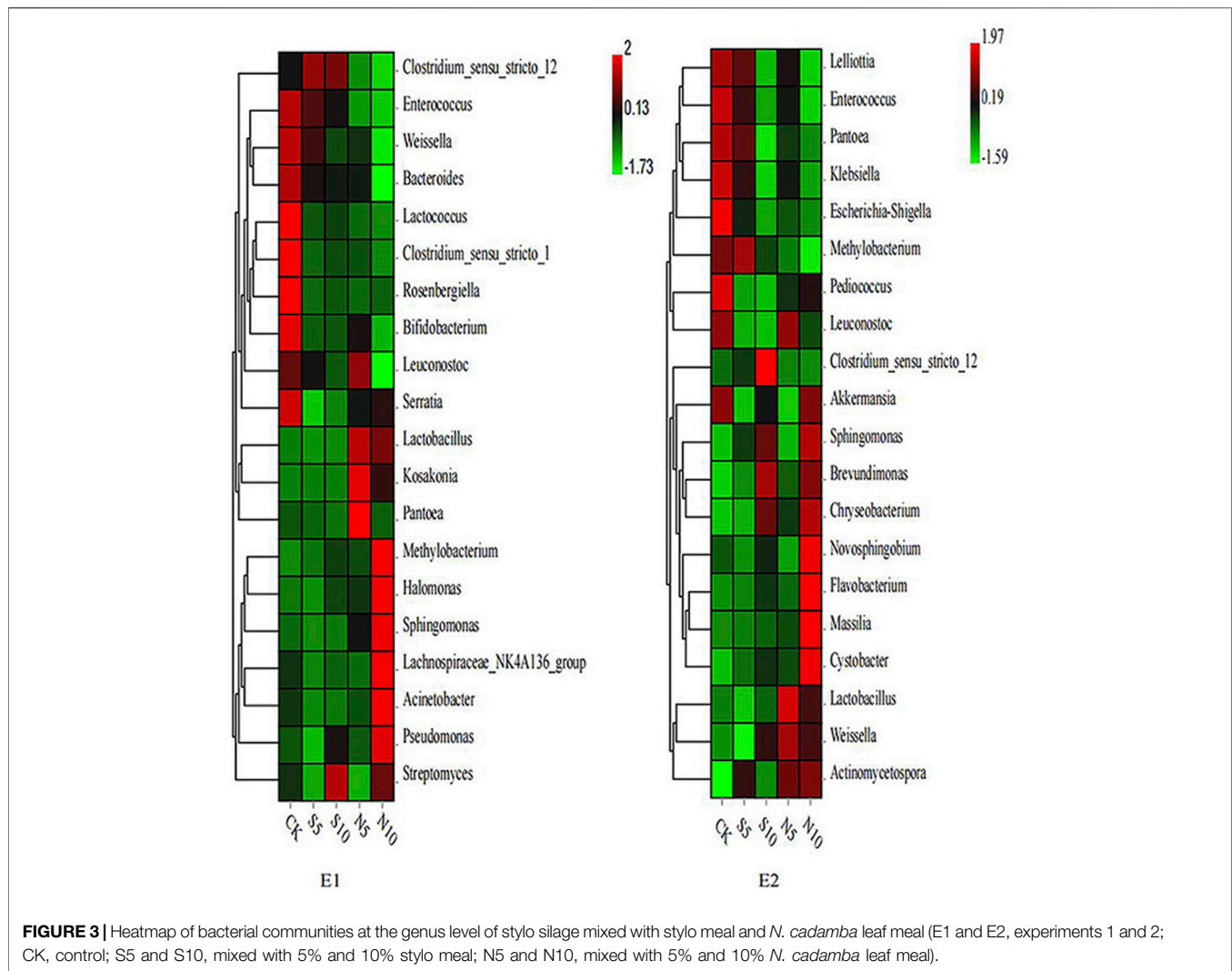


FIGURE 2 | Relative abundance of bacterial communities at phylum and genus levels of stylo silage mixed with stylo meal and *N. cadamba* leaf meal (E1 and E2, experiments 1 and 2; CK, control; S5 and S10, mixed with 5% and 10% stylo meal; N5 and N10, mixed with 5% and 10% *N. cadamba* leaf meal).

communities at the phylum and genus levels in the two experiments are presented in **Figure 2** and **Figure 3**. Overall, Proteobacteria and Firmicutes were the main phyla

in stylo silage. The bacterial composition of the two experiments is a little different, and the bacterial community was dramatically altered by *N. cadamba* leaf



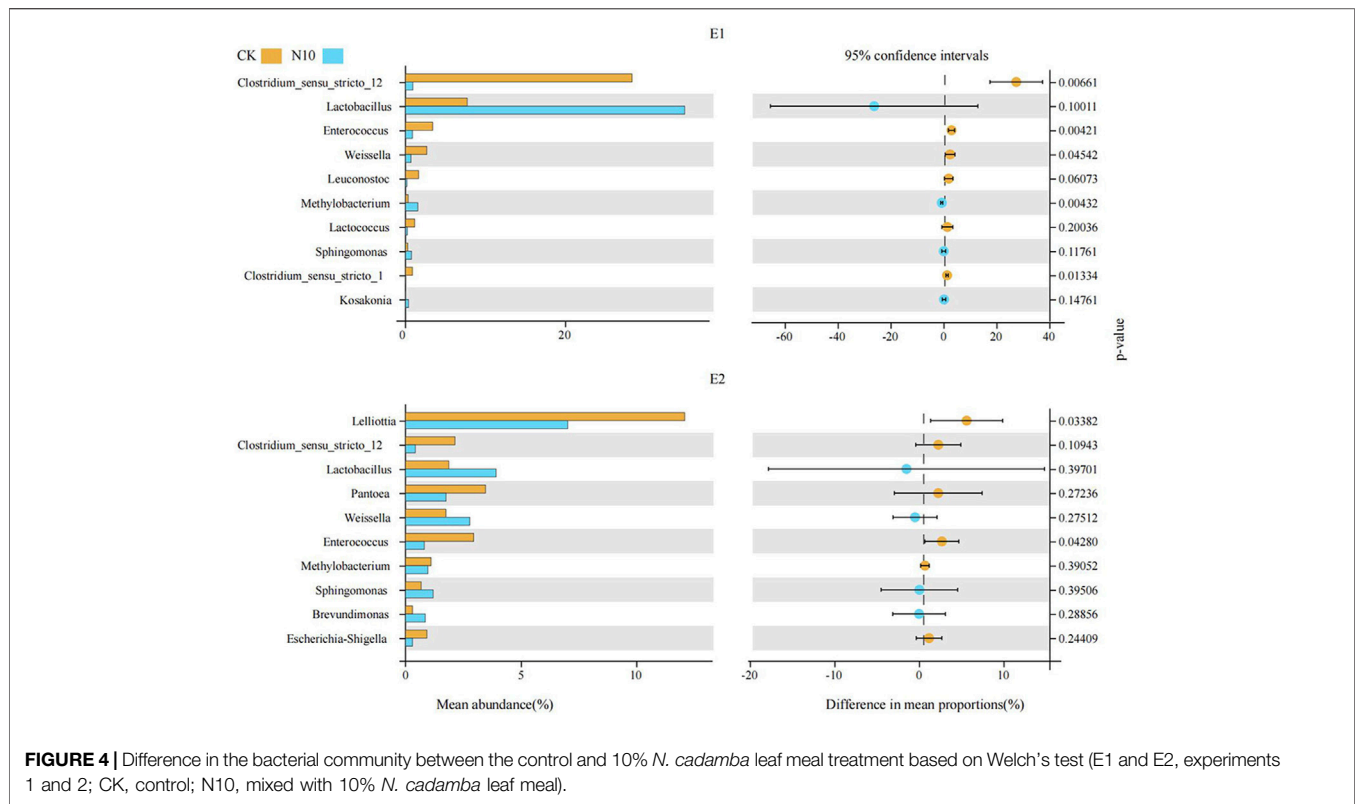
meal. In experiment 1, *Clostridium* (27.9%) was the most dominant genera in the control. The relative abundance of *Clostridium* increased to 45.3% and 41.7% after 5% and 10% stylo meals were added. In contrast, *Clostridium* decreased to 10.4% and 0.91% in *N. cadamba* leaf meal-treated silage ($p < 0.01$, **Figure 4**). In experiment 2, the relative abundance of *Clostridium* increased from 2.14% to 19.5% in 10% stylo meal-treated silage. The relative abundance of *Clostridium* decreased from 2.14% to 0.42% in *N. cadamba* leaf meal-treated silage. *Lactobacillus* was the most dominant species in experiment 1 (42.6% and 34.3%) when *N. cadamba* leaf meal was added. In experiment 2, the relative abundance of *Lactobacillus* increased from 1.88% to 5.81% after the addition of *N. cadamba* leaf meal. Moreover, *Lelliottia* was detected in stylo silage in experiment 2, and the relative abundance was relatively high (6.87%–12.0%), although *Enterobacter* was not detected in the present study. The good news is that the relative abundance of *Lelliottia* decreased by the addition of 10% *N. cadamba* leaf meal ($p < 0.05$, **Figure 4**).

DISCUSSION

Characteristics of Fresh Stylo and *N. cadamba* Leaves Prior to Ensiling

The DM, NDF, and ADF contents of stylo in this study were comparable with the value reported by Wang et al. (2019a) but were lower than those showed by Liu et al. (2011). The crude protein content of *N. cadamba* leaves in experiment 1 was similar to that in our previous report (He et al., 2019a) and was much higher than those in experiment 2. Such variations might be due to the different factors like climate and season of harvest, which have influence on the chemical composition of the forage (Zhang et al., 2016). Trees like *N. cadamba* are widely planted for timber wood, and its leaf is the main byproduct. For a cleaner production of wood, using the residue as animal feed might be a good choice.

As the fermentation substrate, the WSC content of forage is an important factor for good silage fermentation quality. In our study, the WSC contents of stylo in experiment 1 and 2 were very poor and far lower than $60\text{--}70\text{ g kg}^{-1}$ DM, the theoretical requirement for obtaining well-preserved silage (Smith, 1962).



The epiphytic LAB of fresh material is also a crucial factor, and at least five \log_{10} CFU g^{-1} epiphytic LAB during ensiling are necessary to produce well-preserved silage (Cai et al., 1998). But the LAB numbers of stylo in our two experiments were below the value. Furthermore, the counts of undesirable microorganisms, like fungi and coliform bacteria, were relatively high. It indicates that measures should be taken to inhibit these undesirable microorganisms during ensiling. Jayanegara et al. (2018) reported that tannins had the ability to limit extensive proteolysis, which indicated *N. cadamba* leaf meal could be potentially used to enhance nitrogen conservation during the ensiling of legumes. Tannins (both hydrolyzable and condensed tannins) or tannin-containing forages are proved to be effective in reduction of greenhouse gases by ruminants (Kiggundu et al., 2019; Ugbogu et al., 2019). Therefore, mixing *N. cadamba* leaf meal might be helpful to reduce protein degradation, improve the fermentation quality of silage, and decrease the greenhouse gas emission from ruminants.

Fermentation Quality and Microbial Population of Stylo Silage

In this study, addition of *N. cadamba* leaf meal can significantly increase the DM contents of stylo silage. Excess moisture in silage could influence the silage pH and microbiota and increase the nitrogen level that is associated with the emission of nitrogen oxide. Higher DM contents could reduce the losses caused by effluent production and silo gas production during ensiling (Araújo et al., 2020). Silage mainly using lower pH inhibits the

activities of undesirable microorganisms so as to preserve nutrients (McDonald et al., 1991). Therefore, the accumulation of organic acids in silage is crucial. Lactic acid, the dominant fermentation product in this study, increased in 10% *N. cadamba* leaf meal treatment in both experiment 1 and experiment 2. The production of butyric acid is unfavorable during ensiling. However, the content of butyric acid was not reduced by the addition of 10% stylo meal. It indicates that *N. cadamba* leaf meal enhances the fermentation quality, which is not merely because of the improvement of DM content. It might be because some chemicals in *N. cadamba* leaf meal changed the bacterial community and fermentation process during ensiling.

Nitrogen Fractions and Antioxidant Activities of Stylo Silage

As we all know, the ultimate goal of silage is to preserve nutrients. From Table 3, *N. cadamba* leaf meal increased the true protein content and decreased the nonprotein-N and ammonia-N contents. It indicates that *N. cadamba* leaf meal has a positive effect on the conservation of protein during ensiling. The ammonia-N content in silage is a crucial indicator of protein breakdown (Pahlow et al., 2003). The decrease of the ammonia-N content and nonprotein-N content by *N. cadamba* leaf meal addition indicates the benefit of *N. cadamba* leaves further. Tannins can protect proteins by forming insoluble complexes with forage proteins or inhibiting aminopeptidase, carboxypeptidase, and acid proteinase during the ensiling process (Li et al., 2018). Ding et al. (2013) reported that the

addition of tannin acid could reduce ammonia-N and nonprotein-N contents in alfalfa silage. In our study, the contents of hydrolyzable tannins and condensed tannins increased in *N. cadamba* leaf meal-treated silage as expected. The higher content of tannins in *N. cadamba* leaf meal treatments might also explain the reduction of proteolysis. The DPPH scavenging activity of stylo silage was significantly increased in *N. cadamba* leaf meal treatment, which might also be because of higher tannin contents. A similar result has been reported by He et al. (2020), who also found that the antioxidant capacity increased by adding tannin acid in mulberry leaf and stylo silage.

Bacterial Diversity of Stylo Silage

Next-generation sequencing has been widely applied to monitor the dynamic changes of bacterial communities in the diverse ensiling period (He et al., 2019b; Wang et al., 2019b). The good coverage values for all treatments in the two experiments were all above 0.99 in the present study, which indicates that the data were representative. Moreover, OTUs in experiment 2 (1,450–1,580) were higher than those in experiment 1 (1,100–1,360). It might be because that the bacterial community of silage is influenced by many factors, such as forage varieties, collected period, silage treatments, and storage conditions (Yang et al., 2016; He et al., 2019b).

As shown in **Figure 1**, the bacterial community of stylo ensiled alone showed a clear separation from the samples treated with *N. cadamba* leaf meal. It suggests that *N. cadamba* leaf meal had a significant influence on the bacterial community of stylo silage. Ensiling is a process in which microorganisms influence and compete with each other. Thus, the better fermentation quality of stylo silage treated with *N. cadamba* leaf meal might be due to the change in the microbial community.

Clostridium, a kind of obligate anaerobic bacteria, is undesirable during ensiling (Driehuis et al., 2018; He et al., 2019b). It is reported that *Clostridium* can degrade protein, promote the growth of spoilage microorganisms, and prevent a rapid fall of silage pH (He et al., 2019a; Zheng et al., 2017). Furthermore, *Clostridium* in silage might be harmful to animal health because some species of it could colonize in the gastro and intestinal tract and then produce pathogenic toxins (Dunière et al., 2013), but *Clostridium* has low tolerance to a low moisture content. Reducing moisture by wilting is commonly and widely used in silage production to inhibit the activities of *Clostridium* (Pahlow et al., 2003). In this study, the relative abundance of *Clostridium* did not decrease as expected, although stylo meal addition decreased the moisture content. On the contrary, it increased in stylo meal-treated silage (**Figures 2, 3**). It indicates that well-fermented stylo silage is difficult to obtain by reducing the moisture content alone. The relative abundance of *Clostridium* was decreased by *N. cadamba* leaf meal, which was consistent with the decrease in ammonia-N and butyric acid contents. It suggests mixing *N. cadamba* leaf meal might be an effective method to inhibit *Clostridia* and improve the silage quality. Daglia (2012) considered that tannins had an extensive range of antimicrobial activity. It may partially explain the inhibiting effects of *N. cadamba* leaf meal on *Clostridia* in stylo silage.

Generally, *Lactobacillus*, *Weissella*, *Enterococcus*, and *Lactococcus* are common lactate-producing bacteria in silage (Pahlow et al., 2003; Yang et al., 2016; Ni et al., 2018). *Weissella* is considered an initial colonizer microorganism, and then, it is inhibited ultimately by the low pH value (<4.2) caused by organic acid accumulation (Graf et al., 2016). It might be because of the relatively high pH value (>4.5 in all samples) of stylo silage; *Weissella* was detected in the two experiments. *Enterococcus* and *Lactococcus* are always used as LAB inoculants during the silage production. It could reduce the ammonia-N content of silage and affect ruminal fermentation by improving ruminal microbial biomass production (Weinberg et al., 2003). In the present study, *Enterococcus* was decreased by *N. cadamba* leaf meal addition (**Figure 4**). It might be because of the increase in other genera like *Lactobacillus*.

Lelliottia, a gram-negative and non-spore-forming bacterium belonging to the family Enterobacteriaceae, was first separated from the genus *Enterobacter* in 2013, and it is reclassified subsequently as a novel genus, according to genotypic and phenotypic characteristics (Yuk et al., 2018). It is reported that *Lelliottia* has been isolated from drinking water (Kampfer et al., 2018), long-standing arsenic contaminated environment (Tian et al., 2019), and even fecal samples from hospitalized patients (Kmpfer et al., 2014). It is seldom reported in silage. However, *Lelliottia* was detected in stylo silage in experiment 2, and the relative abundance was relatively high although *Enterobacter* was not detected in the present study. Based on the similarity with *Enterobacter*, it might also play the same function in silage. The relative abundance of *Lelliottia* was decreased by 10% *N. cadamba* leaf meal addition. It could explain the reduction of ammonia-N in 10% *N. cadamba* leaf meal treatment. Ogunade et al. (2018) supposed that *Pantoea* had the ability to reduce the ammonia-N content and pH value in alfalfa silage. Confusingly, the relative abundance of *Pantoea* increased in 5% *N. cadamba* leaf meal treatment in experiment 1 but decreased in experiment 2. The relative abundance of *Pantoea* in both experiments was very low. So the role of *Pantoea* in stylo silage needs further study.

CONCLUSION

Compared with discarding in the field, using *N. cadamba* leaf meal as a silage additive might be a great choice to recycle this resource. Moreover, the present study revealed that mixing *N. cadamba* leaf meal is an effective way to improve the fermentation quality of legume silage. In *N. cadamba* leaves meal treated silage, silage pH, butyric acid, nonprotein-N and ammonia-N content decreases, and antioxidant activities, lactic acid, and true protein content increases. The relative abundance of *Clostridium* and *Lelliottia* decreased, while the relative abundance of *Lactobacillus* increased in stylo silage when *N. cadamba* leaf meal was added. It might be because of the abundant active compounds like tannins in *N. cadamba* leaves. The aforementioned results suggested that mixing *N. cadamba* leaf meal to high-moisture silage could be an effective strategy to improve the silage conservation and a feasible way to use this kind of forestry waste.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: BioProject, accession number PRJNA846587.

AUTHOR CONTRIBUTIONS

SW: investigation and writing the original draft. LG: investigation and data curation. DC: investigation and software. YX: interpretation. AK: analysis. WZ: project administration and

resources. XC: supervision and validation. QZ: methodology and project administration.

FUNDING

This work was financially supported by the Guangzhou Forestry Science and Technology Innovation Commission (Grant Nos. 2018KJCX001 and 2019KJCX001), the Guangdong Natural Science Foundation (2020A1515011253), and the National Natural Science Foundation of China Project (Grant No. 31771345).

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