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Effects of mixing *Neolamarckia cadamba* leaves on fermentation quality, microbial community of high moisture alfalfa and stylo silage

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Summary

***Neolamarckia cadamba* is not only a fodder of high nutritional value, but also a source of natural antimicrobial agent. The silage quality of high moisture alfalfa and stylo with or without *N. cadamba* leaves (NCL) was investigated, and microbial community after ensiling was analysed. Results showed that the silage samples with NCL have lower pH (4.32 versus 4.88, 4.26 versus 4.71 in alfalfa and stylo silage, respectively), ammonia-N content (67.5 versus 146, 42.2 versus 95.1 g kg^{-1} total N) and higher lactic acid (13.3 versus 10.4, 17.3 versus 13.6 g kg^{-1} dry matter), true protein N (592 versus 287, 815 versus 589 g kg^{-1} total N). The addition of NCL also influenced the bacterial community distribution. The relative abundance of *Clostridium* and *Enterobacter* decreased, whereas *Lactobacillus* abundance increased when NCL was added. In conclusion, NCL could inhibit undesirable microorganisms in high moisture alfalfa and stylo silage. Mixing with NCL could be a feasible way to improve the quality of silage.**

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Introduction

Conservation of legumes as silage is an alternative to avoiding damage by weather and loss of leaf by shattering during the process of haymaking. However, legumes are difficult to ensile successfully without an additive due to their high buffering capacity, low water soluble carbohydrate (WSC) and dry matter (DM) content (Denek *et al*., 2011). High moisture legumes silage is more susceptible to the spoilage of *Clostridia*, *Bacilli* or *Enterobacter*. Consequently, high levels of butyric acid accumulation and proteolysis are occurred. Clostridial endospores may lead to clostridial contamination in milk and feeding silages of high butyric acid content will reduce animal DM intake (Pahlow *et al*., 2003). Furthermore, extensive proteolysis in ensiled legume forages during fermentation results in extensive degradation of proteins to ammonia-N, free amino acid N and peptide-N. These forms of nonprotein-N always result in inefficient N rumen microbial N synthesis (Tabacco *et al*., 2006). The economic loss and potential environmental pollution call for a better approach to minimize proteolysis in legumes silage.

Neolamarckia cadamba is a large, deciduous and fast-growing tropical tree species distributed widely in South and South-East Asia. It could grow up to 17 m in height and 25 cm in breast height with straight cylindrical bole within 9 years (Zhao *et al*., 2014). The leaves are 15–50 cm long by 8–25 cm wide. It is also a common traditional herbal medicine that can be used for the treatment of various ailments (Pandey and Negi, 2016). Due to tremendous economic and ecological value, it has been recently introduced to many tropical and subtropical countries, like Costa Rica, Puerto Rico, South Africa, Surinam, Venezuela (Pandey and Negi, 2016; Li *et al*., 2019). It also can be used for woody forage production. Previous studies indicated that *N. cadamba* had high

forage quality and had positive effect on animal performance and meat quality (Wang *et al*., 2017). Moreover, *N. cadamba* leaves (NCL) contain high content polyphenols like tannins (hydrolysable tannins: 44.2 g kg^{-1} DM, condensed tannins: 69.6 g kg^{-1} DM, He *et al*., 2018), which could restrict proteolysis in ensiled forage (Guo *et al*., 2008). The extract of NCL had significant antibacterial activities on undesirable bacteria like *Staphylococcus*, *Bacillus*, *Escherichia* (Khandelwal *et al*., 2016; Pandey and Negi, 2016), which are also frequently detected in silage (Yang *et al*., 2019; Zhang *et al*., 2018).

Therefore, we hypothesized that NCL could be used to improve legumes silage quality based on their antimicrobial and polyphenols attributers. In the present study, we evaluated the effects of mixing NCL on nitrogen distribution, fermentation characteristics and microbial communities of alfalfa (*Medicago sativa* L.) and stylo (*Stylosanthes guianensis* Sw.) silages.

Results and discussion

Characteristics of fresh material before ensiling

The chemical composition and microbial population of the three materials before ensiling are shown in Table 1. The DM contents of *N. cadamba* leaves, alfalfa and stylo were 217, 265 and 277 g kg^{-1}, respectively. The CP content of NCL (107 g kg^{-1} DM) was comparable with the data reported by He and colleagues (2018) but far lower than Wang and colleagues (2018). The CP content of alfalfa (159 g kg^{-1} DM) was lower than the value reported by Yang and colleagues (2019), while the CP content of stylo (132 g kg^{-1} DM) was a slightly higher than that determined by Liu and colleagues (2012). These differences might be because the forage quality could be influenced by factors like climate, fertilization (Van Soest *et al*., 1978) and harvest time (Zhang *et al*., 2016). The chemical composition of the silage material, especially the WSC content, is an important factor involved in assessing fermentation quality. Typically, alfalfa and stylo had relatively low WSC content, 37.6 and 16.4 g kg^{-1} DM, respectively. It indicates high-quality silage is difficult to obtain when ensile alfalfa and stylo directly. The hydrolysable and condensed tannins in NCL were 43.3 and 58.6 g kg^{-1}, respectively. It could be helpful for undesirable microorganism inhibition and protein preservation in alfalfa and stylo silage when NCL is introduced (Peng *et al*., 2018).

Quality of alfalfa and stylo silage

Alfalfa and stylo are important leguminous forages with high yield and quality. However, it is known that high moisture legumes are difficult to ensile due to low WSC content and high buffering capacity (Mcdonald *et al*., 1991). As shown in Table 2, alfalfa and stylo ensiled alone showed relatively high pH (4.88 and 4.71, respectively), which were far higher than 4.2, a benchmark for well-fermented high moisture silage (Edwards and McDonald, 1978). pH is an important parameter to evaluate the extent of silage fermentation quality. A low pH ensures better aerobic stability and keeps the forage from further fermentation. In the present study, pH decreased ($P < 0.01$) significantly after mixing with NCL, though all values were above 4.2. DM loss occurred during ensiling always due to the metabolism of yeasts, which utilizes soluble carbohydrates and produce ethanol (Avila *et al*., 2014). Dry matter loss of stylo silage decreased after mixing NCL. It might be because NCL decreased activities of yeasts. Patel and colleagues (2011) reported NCL extract had moderate inhibitory activity against *Aspagillus fumigatous and Candila albicans*.

Neolamarckia cadamba leaves also decreased ($P < 0.01$) lactic acid bacteria count, while increased ($P < 0.01$) lactic acid content of the two silages. This might be attributed to the decrease of cocci (such as *Leuconostocs*, *Pediococcus*, *Lactococci* and *Enterococci*), which initiate lactic fermentation at the early of ensiling process, had lower tolerance to low pH and lower lactic acid production efficiency than rod-shaped LAB (*Lactobacillus*; Cai *et al*., 1998; Pang *et al*., 2011). It also could explain the reduction of DM loss and acetic acid content in NCL-treated silages. On the other hand, the reduction may also attribute to the decrease of enterobacteria, which are responsible for acetic acid production, DM and energy losses (Pahlow *et al*., 2003). All these results indicate mixing NCL could improve fermentation quality of alfalfa and stylo silage.

As shown in Table 3, nonprotein-N in alfalfa silage mixed with NCL decreased ($P < 0.001$), it indicates ensiling alfalfa with NCL might improve the utilization of silage-N. Because the efficiency of rumen microbial-N synthesis could be improved by supplementing silage with protein-N, rather than nonprotein-N (Pahlow *et al*., 2003). The ammonia-N in silage was an indicator of proteolysis during ensiling and the accumulation of ammonia-N in silage is typically caused by synthetic effect of plant protease activity and microbial activity (Ogunade *et al*., 2018). At pH 5.0 to 6.0, both *clostridia* and plant proteolytic enzymes are active. The relatively high ammonia-N contents in alfalfa and stylo ensiled alone might be explained by the relatively high pH values of these silages. Addition NCL decreased ($P < 0.001$) ammonia-N content of alfalfa or stylo silage (Table 3). It might be because NCL inhibited the growth and proteolytic activity of microorganisms such as *Clostridium*, *Enterobacter*. On the other hand, NCL contain relatively

Table 1. Chemical composition and microbial population of fresh *Neolamarckia cadamba* leaves, alfalfa and stylo prior to ensiling (±SD, $n = 3$).

Item	*N. cadamba* leaves	alfalfa	stylo
Dry matter (g kg^{-1} FM)	217 ± 6.0	265 ± 2.2	277 ± 0.9
Crude protein (g kg^{-1} DM)	107 ± 3.1	159 ± 0.4	132 ± 1.9
Neutral detergent fibre (g kg^{-1} DM)	237 ± 1.0	438 ± 13.9	512 ± 11.0
Acid detergent fibre (g kg^{-1} DM)	156 ± 3.9	303 ± 8.3	348 ± 15.9
Water soluble carbohydrate (g kg^{-1} DM)	46.9 ± 2.64	37.6 ± 0.83	16.4 ± 1.40
Lactic acid bacteria (Log_{10} cfu g^{-1} FM)	4.21 ± 0.79	5.94 ± 0.03	4.96 ± 0.44
Yeast (Log_{10} cfu g^{-1} FM)	3.93 ± 0.08	4.17 ± 0.15	4.15 ± 0.22
Coliform bacteria (Log_{10} cfu g^{-1} FM)	4.44 ± 0.33	6.44 ± 0.06	5.67 ± 0.28
Hydrolysable tannins (g kg^{-1} DM)	43.3 ± 5.60	5.0 ± 0.20	5.4 ± 0.90
Condensed tannins (g kg^{-1} DM)	58.6 ± 5.50	7.8 ± 0.71	22.6 ± 0.36

DM, dry matter; FM, fresh matter.

Table 2. Organic acid contents, pH and microbial population of alfalfa or stylo silage with *Neolamarckia cadamba* leaves

	Trial 1					Trial 2				
Item	M-CK	M-25	M-50	SEM	*P* value	S-CK	S-25	S-50	SEM	*P* value
Dry matter (g kg^{-1} FM)	267	248	246	3.6	0.02	269	265	242	4.4	0.001
Dry matter loss (%)	4.31	5.27	2.54	0.482	0.031	5.86	0.17	4.67	0.992	0.014
pH	4.88	4.60	4.32	0.082	< 0.001	4.71	4.41	4.26	0.067	< 0.001
Lactic acid (g kg^{-1} DM)	10.4	12.9	13.3	0. 47	< 0.001	13.6	16.7	17.3	0.67	0.019
Acetic acid (g kg^{-1} DM)	3.82	2.60	1.55	0.332	< 0.001	1.92	1.23	0.81	0.17	< 0.001
Propionic acid (g kg^{-1} DM)	18. 1	20.2	22.4	1.11	0.307	23.7	18.0	ND	3.64	< 0.001
Butyric acid (g kg^{-1} DM)	ND	ND	ND	–	–	ND	ND	ND	–	–
Lactic acid bacteria(Log_{10} cfu g^{-1} FM)	7.73	7.37	6.81	0.139	< 0.001	7.41	6.15	6.59	0.212	0.013
Mould (Log_{10} cfu g^{-1} FM)	< 2.00	< 2.00	< 2.00	–	–	< 2.00	< 2.00	< 2.00	–	–
Yeast (Log_{10} cfu g^{-1} FM)	< 2.00	< 2.00	< 2.00	–	–	< 2.00	< 2.00	< 2.00	–	–
Coliform bacteria (Log_{10} cfu g^{-1} FM)	< 2.00	< 2.00	< 2.00	–	–	< 2.00	< 2.00	< 2.00	–	–

DM, dry matter; FM, fresh matter; ND, not detected; SEM, standard error of means.

high tannins, which could restrict proteolysis in ensiled forage (Guo *et al*., 2008). As expected, both hydrolysable and condensed tannins were increased ($P < 0.05$) in NCL mixed silages, which could partly explain the decrease of nonprotein-N and ammonia-N content. Similar results have been reported by Peng and colleagues (2018), who found condensed tannins reduced proteolysis in silage. Therefore, NCL could be used as alternative treatment of proteolysis in legume silages.

Microbial community of alfalfa and stylo silage

The result of principal component analysis, which clearly reflected the variance of the microbial community, was shown in Fig. 1. Alfalfa and stylo samples before ensiling were separated from the silage samples, which suggested that microbial community changed during ensiling process. The variation of microbial community might explain the difference in silage quality (Ni *et al*., 2018).

Table 3. Fibre, tannins content and protein fractions of alfalfa or stylo silage with *Neolamarckia cadamba* leaves

	Trial 1					Trial 2				
Item	M-CK	M-25	M-50	SEM	*P* value	S-CK	S-25	S-50	SEM	*P* value
Crude protein (g kg^{-1} DM)	163	148	145	3.1	0.007	126	118	112	1.61	0.082
True protein N (g kg^{-1} TN)	287	426	592	5.0	< 0.001	589	715	815	33.86	< 0.001
Nonprotein-N (g kg^{-1} TN)	714	574	408	5.0	< 0.001	411	285	185	33.86	< 0.001
Ammonia-N (g kg^{-1} TN)	146	110	67.5	11.69	< 0.001	95.1	60.5	42.2	8.10	< 0.001
Neutral detergent fibre (g kg^{-1} DM)	452	417	371	1.2	< 0.001	534	465	417	17.41	< 0.001
Acid detergent fibre (g kg^{-1} DM)	311	293	246	1.3	< 0.001	388	319	294	14.36	< 0.001
Hydrolysable tannins (g kg^{-1} DM)	7.38	14.3	22.1	2.18	< 0.001	4.06	13.6	15.6	1.86	< 0.001
Condensed tannins (g kg^{-1} DM)	9.20	19.9	30.3	3.41	0.008	18.5	22.5	31.3	2.26	0.029

DM, dry matter; SEM, standard error of means; TN, total N.

Distinctions among bacterial communities in silages mixed with two ratios of NCL were also very clear. Similar results have been reported by Ni and colleagues (2018), who found mixed ensiling had an impact on microbial community. It indicates mixing NCL had an impact on microbial community and fermentation quality of the two silages.

The relative abundance of bacterial communities in alfalfa and stylo before and after ensiling is shown in Figs 2 and 3. *Exiguobacterium* was dominant in alfalfa and stylo before ensiling. Lund and Schleifer (1983) found that *Exiguobacterium* is a Gram-positive facultative anaerobe and can convert glucose to lactic acid and acetic acid. Therefore, it may be helpful for alfalfa and stylo silage preservation. White and colleagues (1996) reported that *Sphingomonas*, Gram-negative aerobic bacteria, are animal pathogens and can readily degrade the copper pipes in drinking water distribution systems. The relative abundance of *Sphingomonas* genus decreased from 4.1% in alfalfa to 0.3% after ensiling, which means ensiling is an effective method to control this genus. It also indicates that feeding animals with alfalfa silage is better than fresh alfalfa. Similar trend in Italian ryegrass silage had been reported by Ni and colleagues (2017). Sy and colleagues (2005) reported species of *Methylobacterium* were facultative methylotrophic bacteria and were commonly found in association with plants. In the present study,

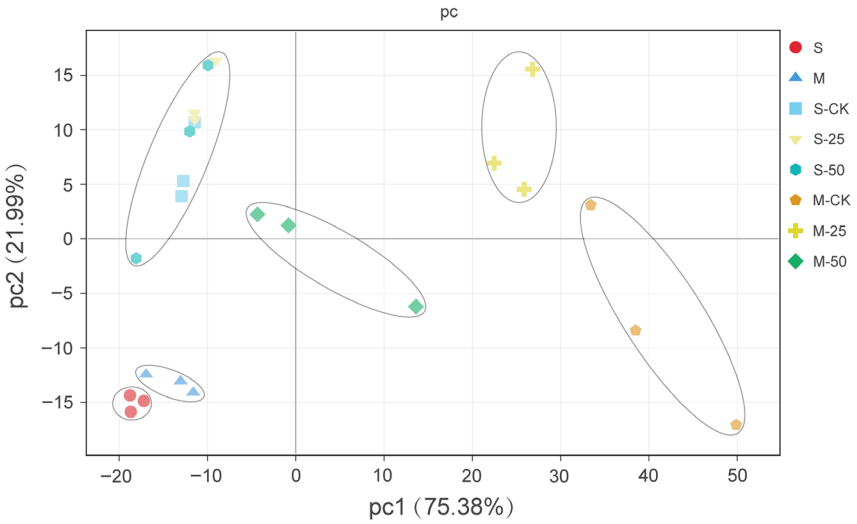

Fig. 1. Principal component analysis of bacterial communities for alfalfa or stylo silage with *Neolamarckia cadamba* leaves (M, alfalfa material; S, stylo material; CK; 0% *N. cadamba* leaves; 25, 25% *N. cadamba* leaves; 50, 50% *N. cadamba* leaves; 1, 2, 3, three mini-silos of each treatment)

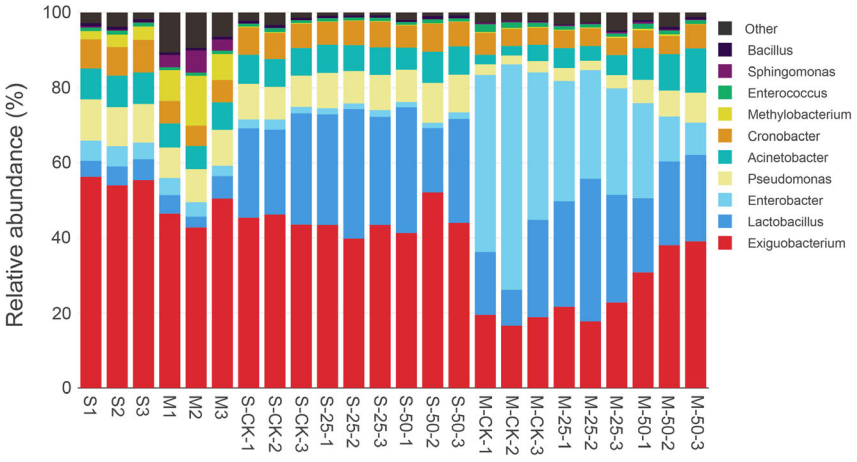

Fig. 2. Bacterial community and relative abundance by genus for alfalfa or stylo silage with *Neolamarckia cadamba* leaves (M, alfalfa material; S, stylo material; CK; 0% *N. cadamba* leaves; 25, 25% *N. cadamba* leaves; 50, 50% *N. cadamba* leaves; 1, 2, 3, three mini-silos of each treatment)

Methylobacterium was also detected in alfalfa (9.5%) and stylo (3.0%) before ensiling.

Exiguobacterium was also a abundant genus in most silage samples (42.2–45.1% in stylo silage and 18.3–36.0% in alfalfa silage). Similar result has been reported by Wang and colleagues (2018), who reported *Exiguobacterium* was dominated in *Moringa oleifera* leaves silage. In our study, the relative abundance of *Enterobacter* decreased from 5.1% in stylo to 2.3%, and the *Lactobacillus* increased from 4.9% to 25.3% after ensiling. Parvin and colleagues (2010) also reported a similar shift of the bacterial communities from *Enterobacter* to *Lactobacillus* and *Lactococcus* after fermentation of whole corn silage. It is known that alfalfa is difficult to ensile and pH is uneasy to decrease due to its high buffer capacity. In the present study, the relative abundance of *Lactobacillus* (17.4%) in alfalfa ensiled alone was far below it of *Enterobacter* (48.8%). Interestingly, *Lactobacillus* increased to 21.7% and *Enterobacter* decreased to 15.3% in alfalfa silage when mixed with 50% NCL. It indicates mixing NCL might enhance fermentation quality of alfalfa and stylo silage by inhibiting undesirable microorganisms like *Enterobacter* and promoting profitable microorganisms like *Lactobacillus*.

During ensiling, the presence of *enterobacteria* is undesirable as they may compete with the LAB for nutrients and produce ammonia-N. The reduction of *enterobacteria* in silage reflects the combined presence of good ensiling conditions, the availability of nutrients and water, an efficient conversion of those nutrients to

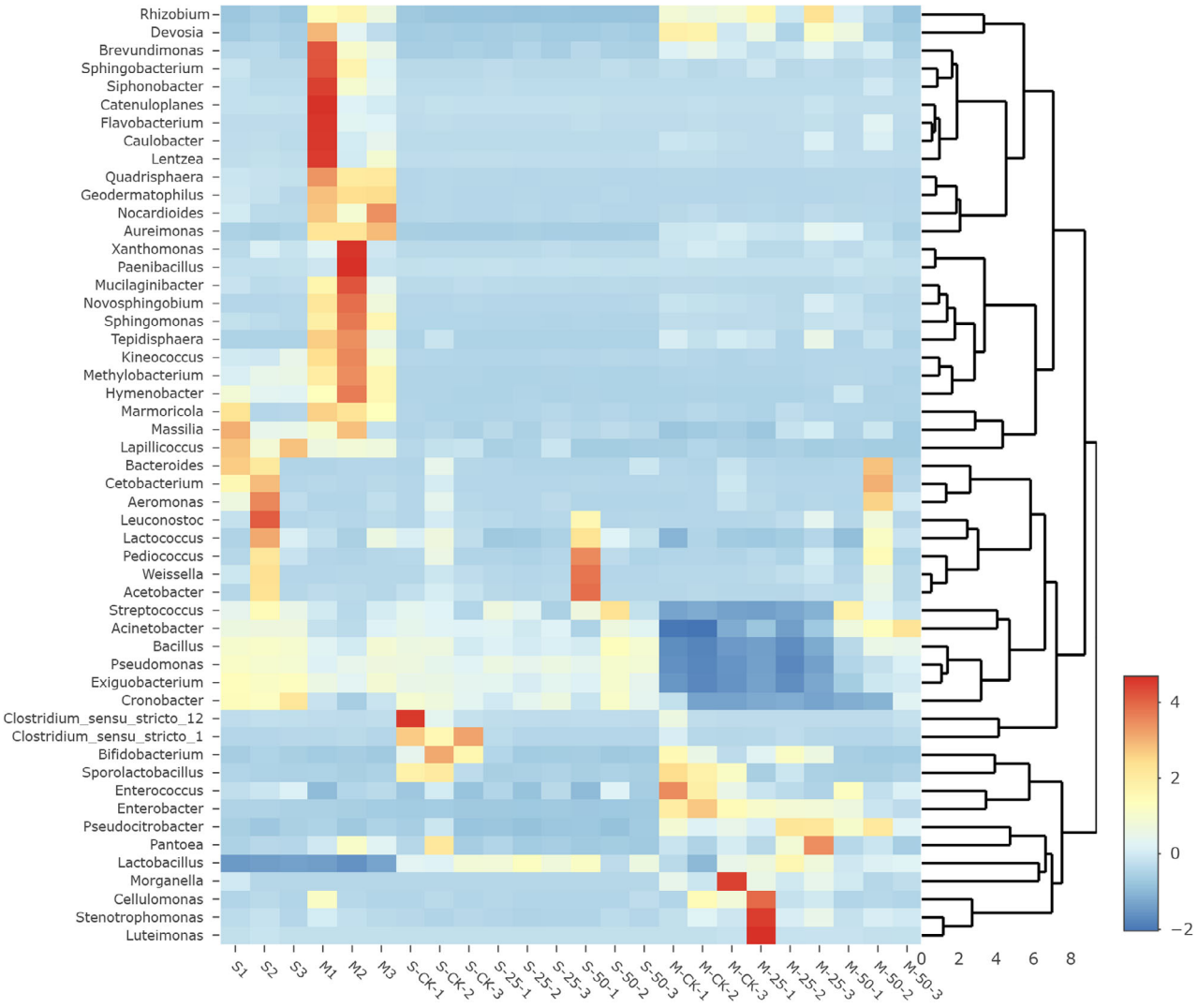

Fig. 3. Heatmap of prominent bacterial genera (35 most abundant genera) for alfalfa or stylo silage with *Neolamarckia cadamba* leaves (M, alfalfa material; S, stylo material; CK; 0% *N. cadamba* leaves; 25, 25% *N. cadamba* leaves; 50, 50% *N. cadamba* leaves; 1, 2, 3, three mini-silos of each treatment)

fermentation products and a low pH by LAB, and also moderate temperatures (Pahlow *et al*., 2003). In the present study, the relative abundance of *Enterobacter* in alfalfa and stylo silage mixed with NCL decreased from 48.8% to 15.3% and from 2.3% to 1.5%, respectively. It means better fermentation quality is obtained when ensile alfalfa and stylo by mixing with NCL.

Clostridia are considered undesirable in silage, as they may result in excessive protein degradation, DM loss and butyric acid production, which can promote the growth of less acid-tolerant spoilage microorganisms and result in reduced silage intake. Their spores have ability to survive in the gastrointestinal tract in dairy cows and their contamination in milk can lead to off-flavours and excessive gas formation in cheeses. Some species even produce an extremely pathogenic toxin. Their occurrence and transmission through the dairy chain always causes death of animals and humans (Dunière *et al*., 2013). The relative abundance of *Clostridium* in stylo silage decreased in NCL-treated groups (Fig. 3). It might be attributed to the strong antimicrobial activities against undesirable microorganisms of NCL (Khandelwal *et al*., 2016; Pandey and Negi, 2016). It is consistent with the decrease of ammonia-N and nonprotein-N. Furthermore, Flythe and Russell (2004) found some *Clostridium* could produce large amounts of acetic acid apart from butyric acid. The decrease in acetic acid in alfalfa and stylo silage mixed with NCL might be explained by lower abundance of *Clostridium*.

Some genera like *Pseudomonas*, *Cronobacter* and *Acinetobacter*, whose roles in silage have not been extensively studied, were affected by NCL mixing in alfalfa and stylo silage. *Pseudomonas* might be undesirable in silage due to its possibility of biogenic amines production (Dunière *et al*., 2013). *Cronobacter*, formerly known as *Enterobacter sakazakii*, is a genus consisting of Gram-negative, facultatively anaerobic bacterial pathogens belonging to the *Enterobacteriaceae* family (Joseph

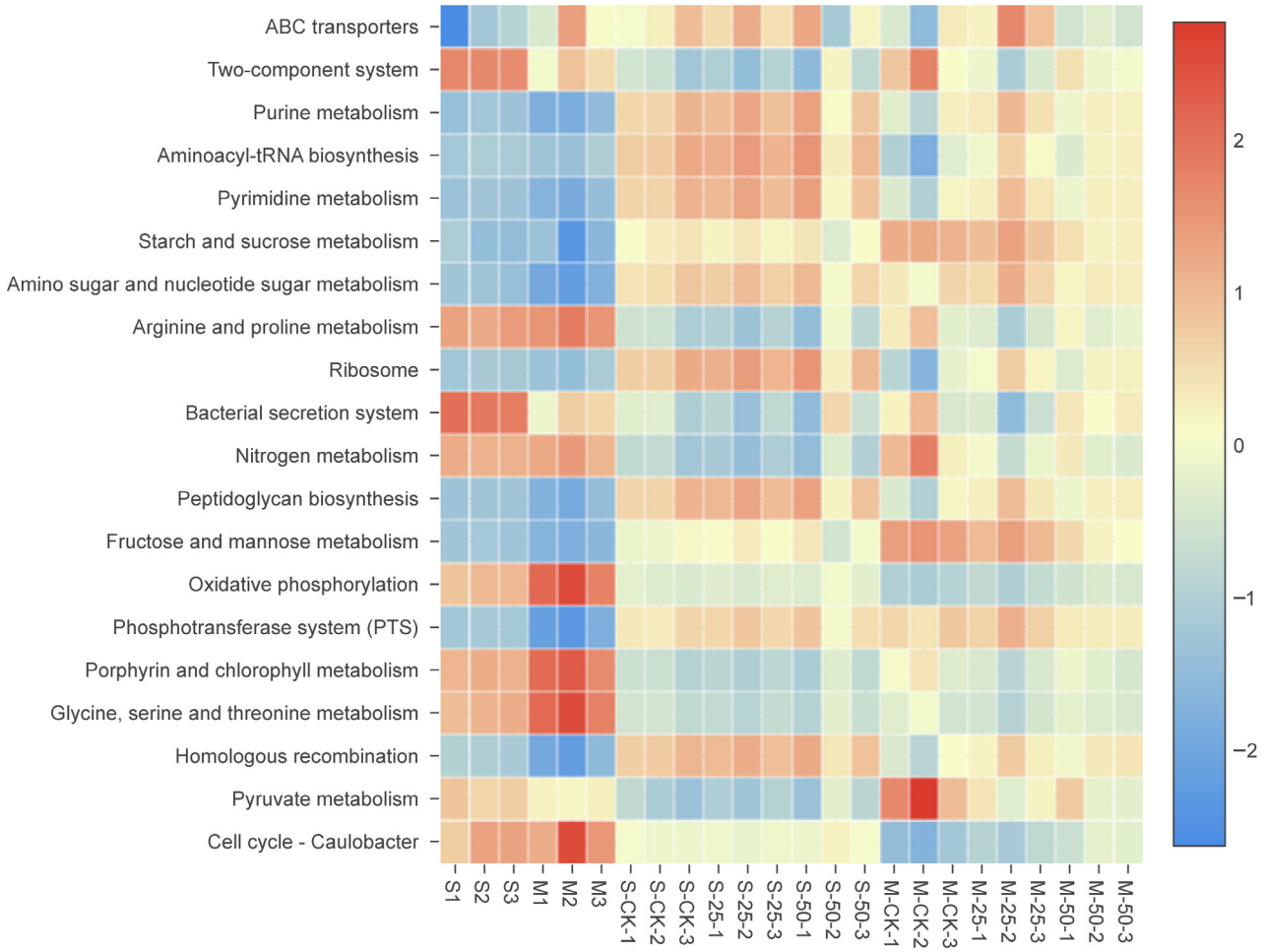

Fig. 4. Heatmap of 16S rRNA gene-predicted functional profiles obtained with Tax4Fun (M, alfalfa material; S, stylo material; CK; 0% *N. cadamba* leaves; 25, 25% *N. cadamba* leaves; 50, 50% *N. cadamba* leaves; 1, 2, 3, three mini-silos of each treatment)

et al., 2012). The abundance of *Cronobacter* in alfalfa and stylo silage (4.7–6.9%) was relative high, though it was decreased by mixing NCL. Perhaps more measures should be taken to control this genus. *Acinetobacter* species are aerobic bacteria and can be found in different environments. Fuhs and Chen (1975) found some *Acinetobacter* species can utilize acetate as a substrate and survive in an anaerobic environment. The utilization of acetic acid by *Acinetobacter* in anaerobic environment requires energy from carbohydrate degradation, thus silage DM loss increases during ensiling. The good news is that the two genera are not abundant in *M. oleifera* leaves silage (9.8%, 10.0% in maximum, respectively, Fig. 2). On the other hand, *Acinetobacter* might be concerned with aerobic stability of silage. Liu and colleagues (2019) investigated the bacterial community in barley silage during the fermentation process and aerobic exposure phase and found *Acinetobacter* proliferated rapidly and became the dominant genus after 7 days of exposure to air. In the present study, *Acinetobacter* was more commonly observed in alfalfa silages mixed with NCL. Therefore, studies on the aerobic stability of the silage and its relationship with *Acinetobacter* might be conducted in the future.

16S rRNA gene-predicted functional profiles are shown in Fig. 4. Metabolism of nitrogen, arginine, proline, glycine, serine and threonine was reduced in alfalfa and stylo silage mixed with NCL. *Clostridia* could produce ammonia by utilizing amino acids (Flythe and Russell, 2004). Therefore, the decrease of ammonia-N in alfalfa and stylo silage mixed with NCL might because NCL reduced the abundance and amino acid metabolism of *Clostridium* and *Enterobacter*. Apart from the decrease in the protein content and nutritional value of the silage, the decarboxylation of tryptophane, histidine and arginine will cause biogenic amines accumulation, which has negative effects on animal health (Dunière *et al*., 2013). These above phenomena suggest that NCL could be used as potential sources of natural antimicrobial agent in silage.

Conclusions

This study revealed that mixed ensiling of alfalfa and stylo with NCL is useful to improve the fermentation quality and nutrition. Nonprotein-N, ammonia-N content and pH of alfalfa or stylo silage decreased after mixing with NCL. The abundance of *Clostridium* and *Enterobacter* decreased, whereas *Lactobacillus* abundance increased when NCL was added. These results indicated that mixing with NCL could be an alternative approach to improve the quality of high moisture alfalfa and stylo silage.

Experimental procedures

Raw materials and silage preparation

Neolamarckia cadamba leaves, alfalfa (GEA) and stylo (CIAT 184) without use of herbicides and fertilizers were harvested from the experimental farm of South China Agricultural University (Guangzhou, China) in August, 2018. Legumes were mowed at full bloom in third cutting, using a sickle by hand and leaving a 5 cm stubble. In trial 1, alfalfa and NCL were mixed at ratios of 100: 0 (M-CK), 75: 25 (M-25), 50: 50 (M-50) after chopping to 1–2 cm by hand with a paper cutter. In trial 2, stylo and NCL were mixed at ratios of 100: 0 (S-CK), 75: 25 (S-25), 50: 50 (S-50), respectively. After that, the materials (about 180 g) were immediately packed into plastic silo bags (20 × 30 cm; Dongguan Bojia Packaging, Dongguan, China), which were vacuumed and sealed by vacuum sealer (Lvye DZ280; Dongguan Yijian Packaging Machinery, Dongguan, China). These 18 silage bags (2 forages × 3 treatments × 3 repeats) were opened to determine fermentation quality, chemical composition, bacteria communities after 60 days of storage at room temperature.

Analysis of microbial population, organic acid and chemical composition

According to Wang and colleagues (2018), 20 g of each sample was immediately blended with 180 ml of sterilized saline water (8.5 g l^{-1} NaCl), and serially diluted from 10^{-1} to 10^{-6}. Lactic acid bacteria and coliform bacteria counts were estimated using Man, Rogosa, Sharpe (MRS) agar and Violet Red Bile agar after incubation at 30°C for 2 days. Yeast and mould counts were enumerated on Rose Bengal agar after incubation at 28°C for 3–5 days. All media were obtained from Guangdong Huankai Bio-tech (Guangzhou, China).

According to Han and colleagues (2013), 20 g of each silage sample was homogenized with 180 ml of distilled water in a blender for 1 min and then filtered through four layers of cheesecloth and filter paper. The pH of this filtrate was measured by a glass electrode pH meter (PHS-3C, INESA Scientific Instrument, Shanghai, China) immediately. The concentration of organic acids (lactic acid, acetic acid, propionic acid and butyric acid) was measured using high-performance liquid chromatography (HPLC) (column, Shodex RSpak KC-811S-DVB gel C (8.0 mm × 30 cm; Shimadzu, Tokyo, Japan); oven temperature, 50°C; mobile phase, 3 mmol l^{-1} $HClO_4$; flow rate, 1.0 ml min^{-1}; injection volume, 5 μL; and detector, SPD-M10AVP) (Zhang *et al*., 2017).

About 100 g of silage sample was dried at 65°C for 48 h to determine DM content. The dried samples were

ground to pass a 1-mm screen by a laboratory knife mill (FW100, Taisite Instrument, Tianjin, China). Crude protein (CP) was determined using the Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto-Analyzer, FOSS Analytical AB, Hoganas, Sweden) according to the methods of Association of Official Analytical Chemists (AOAC, 1990). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were measured without use of heat-stable amylase and sodium sulphite by an A220 Fiber Analyzer (ANKOM Technology Corp., Macedon, NY, USA) according to the method of Van Soest and colleagues (1991). The concentration of ammonia-N was determined by the method of Broderick and Kang (1980). Nonprotein-N and true protein were determined according to the method of Licitra and colleagues (1996). Hydrolysable tannins were determined using the Folin–Ciocalteu colorimetry according to He and colleagues (2018), and condensed tannins were determined by the method of Coblentz and Grabber (2013).

Microbial diversity analysis

DNA extraction was performed according to Liu and colleagues (2019). Samples (10 g) were mixed with 90 ml of sterile 0.85% NaCl solution with vigorous shaking at 120 r m^{-1} for 2 h. The mixture was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10 000 r m^{-1} for 10 min at 4°C. The deposit was resuspended in 1 ml of sterile 0.85% NaCl solution, and the microbial pellets were obtained by centrifugation at 12 000 r m^{-1} for 10 min at 4°C. The E.Z.N.A. stool DNA Kit (Omega Biotek, Norcross, GA, USA) was used to extract total DNA according to the manufacturer's protocols. The PCRs were performed in a 50 μL mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase and 100 ng of template DNA. The 16S rDNA V3-V4 regions were amplified using primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) according to Wang and colleagues (2018).

After purification and quantification, the PCR products were sequenced using Illumina platform (Guangzhou Gene Denovo, Guangzhou, China). The raw sequences were selected according to Wang and colleagues (2018). Paired-end clean reads were merged as raw tags using FLSAH (v 1.2.11; Magoč and Salzberg, 2011) with a minimum overlap of 10 bp and mismatch error rates of 2%. Noisy sequences filtering and data processing were performed using the QIIME (v 1.9.1; Caporaso *et al*., 2010). Clean tags were searched against the reference database (http://drive5.com/uchime/uchime_download.html) to perform Reference-based chimera checking using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). Chimeric sequences were removed and the effective tags with 0.97 identities were clustered into operational taxonomic units (OTU) using UPARSE pipeline. Taxonomy assignment of representative sequences was performed using Ribosome Database Project (RDP) classifier (Version 2.2). Finally, functional genes of the bacterial communities were predicted using Tax4Fun (Xie *et al*., 2018). The sequences data reported in this study were archived in the Sequence Read Archive (SRA) with the accession number SRP181994.

Statistical analyses

The effects of mixing *N. cadamba* leaves were evaluated using one-way analysis of variance, with Duncan's multiple range tests. All statistical analyses were conducted using SAS 9.3 software (SAS Institute, Cary, NC, USA). The data of high throughput sequencing were analysed using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools).

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Conflict of interest

None declared.

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