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Effect on the ensilage performance and microbial community of adding *Neolamarckia cadamba* leaves to corn stalks

Yi Wang,[1,†] Wei Zhou,[1,†] Cheng Wang,[1] Fuyu Yang,[2] Xiaoyang Chen[1]* and Qing Zhang[1]**

[1]*College of Forestry and Landscape Architecture, Guangdong Province Research Center of Woody Forage Engineering Technology, Guangdong Research and Development Centre of Modern Agriculture (Woody Forage) Industrial Technology, Guangdong Key Laboratory for Innovative Development and Utilization of Forest Plant Germplasm, State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Integrative Microbiology Research Centre, South China Agricultural University, Guangzhou, China.*
[2]*College of Animal Science and Technology, China Agricultural University, Beijing, China.*

Summary

To comprehensively evaluate the fermentation performance and microbial community of corn stalks (CS) silage mixed with *Neolamarckia cadamba* leaves (NCL), CS were ensiled with four levels (0%, 10%, 30% and 50% of fresh weight) of NCL for 1, 7, 14, 30, 60 days in two trials. The results showed that all silages were well preserved with low pH (3.60–3.88) and ammonia nitrogen content (0.08–0.19% DM). The silage samples with NCL displayed lower ($P < 0.05$) acetic acid, propionic acid and ammonia nitrogen contents and lactic acid bacteria population during ensiling than control silages (100% CS). The addition of NCL also influenced the distribution of bacterial and fungal communities. Fungal diversity (Shannon's indices were 5.15–5.48 and 2.85–4.27 in trial 1 and trial 2 respectively) increased while the relative abundances of *Lactobacillus*, *Leuconostocs*, *Acetobacter* and two moulds (*Aspergillus* and *Fusarium*) decreased after added NCL. In summary, mixing NCL is a promising effective approach to preserve protein of CS silage and inhibit the growth of undesirable bacteria and mould, thus to improve the forage quality to some extent.

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For correspondence. *E-mail xychen@scau.edu.cn; Tel. +86 13500002882; **E-mail zqing_scau@163.com; Tel. +86 18320064984.
[†]These authors contributed equally to this work.
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Introduction

Corn stalks (CS) are abundant, low cost and widely available agricultural by-product. In China, more than 600 million tons of CS are generated annually, and most of them have been used as high-fibre feed for ruminant animals (Yuan *et al*., 2011; Menardo *et al*., 2015). However, CS are harvested only once per year, which need safe and effective conservation ways for ruminants, especially in the cold season when forage availability is lower. Ensiling is a practical preservation method for CS forage, which could prolong the storage time and supply year-round availability of nutritious and palatable diets for animals. In this process, epiphytic lactic acid bacterial (LAB) ferments water-soluble carbohydrates (WSC) into lactic acid causing a rapid decline in pH, thus inhibiting the activity of deleterious microorganisms (Dunière *et al*., 2013; Ni *et al*., 2017). However, CS are normally deficient in certain nutrients, including nitrogen and phosphorous for optimal animal performance (Wang *et al*., 2017b). Moreover, a large number of researches have shown that CS ensiled alone will cause excessive dry matter loss and protein breakdown as well as high ammonia nitrogen (NH_3-N) production, thus reducing the feeding value (Windle *et al*., 2014; Ogunade *et al*., 2017). Currently, co-ensiling might be an effective way to improve fermentation performance and nutritive value of CS (Wang *et al*., 2017b).

Neolamarckia cadamba, which belongs to *Rubiaceae* family, is a semi-deciduous, evergreen and fast-growing tree species and mainly distributed in tropical or subtropical countries, such as India, China, Nepal and Myanmar (Rahman *et al*., 2015). Under normal conditions, it reaches a height of 17 m and a diameter of 25 cm at breast height within 9 years (Zhao *et al*., 2014). The

leaves are 15–50 cm long by 8–25 cm wide. The tree has various therapeutic properties and been widely used as a remedy in the treatment of cough, fever, skin diseases and dysentery (Pandey and Negi, 2016). It can be also used for woody forage production. Many studies indicated that *N. cadamba* leaves (NCL) were rich in crude protein, crude fat, gross energy and organic matter, which can be used as a new type of forage for animals (Zayed *et al*., 2014; He *et al*., 2018). Wang *et al*. (2017a) claimed that substituting whole plant corn silage with NCL silage significantly increased the average feed intake, average daily gain and shoulder breadth, thus can improve growth development and meat quality of *Lezhi Black* goat. Furthermore, the leaves were also discovered to contain tannins, alkaloids, saponin and steroids, and these secondary metabolites have been proved to possess significant antimicrobial and antioxidant activities (Chen *et al*., 2018). It is known that ensiling is a complex process involving a wide variety of microorganisms. The antibacterial and antifungal properties of NCL may directly affect microbial activity during anaerobic fermentation process, especially those undesirable microorganisms, mainly yeast and mould. He *et al*. (2018) found the hydrolysable and condensed tannins were as high as 4.42% and 6.96%, respectively, in the fresh material and NCL silage, which could inhibit the growth of some microorganisms, including LAB and spoilage organisms. Wang *et al*. (2019a) also noted that bacterial community distribution could be influenced by enhanced hydrolysable and condensed tannins in NCL mixed silages.

Therefore, the objective of present study was to evaluate the fermentation performance, bacterial and fungal communities of CS ensiled with NCL in different ratios, which might provide technical support for the preparation of high-quality CS silage and its application in livestock feeding programmes.

Results and discussion

Chemical and microbial characteristics of fresh materials before ensiling

The chemical composition and microbial population of materials before ensiling were showed in Table 1. The dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents of CS in our study were lower than those reported by Menardo *et al*. (2015) but were similar to those obtained by Wang *et al*. (2017b). Besides, the DM, NDF and ADF contents of NCL were comparable with the data reported by Zhang *et al*. (2019) but relatively higher than our previous report (Wang *et al*., 2019a). However, the DM contents of these two fresh materials except CS in trial 2 were higher than 25%, a minimum DM content for forage to minimize the risk of effluent (Mcdonald *et al*., 1991). The crude protein (CP) content of CS (around 9% DM) was higher than the value reported by Wang *et al*. (2017b), while the CP content of NCL (around 12% DM) was lower than that determined by Zayed *et al*. (2014). However, both CS and NCL presented lower CP contents than those of tropical grass and many legume herbages (Przemysław *et al*., 2015). These differences of chemical composition might be because the forage nutrition could be influenced by factors like climate, plant genotype, harvest season, irrigation and fertilization (Vasco-Correa and Li, 2015). The relatively high content of CP (compared with CS) and low content of fibre suggest that NCL could be potentially applied at ensiling to enhance the nutritive quality of CS. According to the recommendation of Cai *et al*. (1998), the WSC content greater than 5% DM is necessary for desirable silage quality. Higher residual of WSC indicates smaller DM loss during fermentation. In the present study, the WSC contents in CS (10.19–16.61% DM) and NCL (7.49–8.45% DM) were higher than other conventional forage such as alfalfa (Zhang *et al*., 2017), which was sufficient as substrate for propagation and growth of LAB in the successive stage.

Table 1. Chemical and microbial composition of fresh materials before ensiling.

	Trial 1		Trial 2	
	CS	NCL	CS	NCL
Dry matter (%)	25.67 ± 0.46	28.13 ± 1.21	24.58 ± 0.52	28.50 ± 1.04
Crude protein (%DM)	9.47 ± 0.57	11.56 ± 0.19	9.88 ± 0.49	13.17 ± 0.20
Neutral detergent fibre (%DM)	59.87 ± 3.67	29.57 ± 1.92	48.50 ± 0.44	27.11 ± 1.54
Acid detergent fibre (%DM)	30.33 ± 1.02	23.48 ± 1.63	25.44 ± 0.15	20.88 ± 1.06
Acid detergent lignin (%DM)	3.22 ± 0.41	13.27 ± 0.90	2.34 ± 0.31	10.09 ± 1.34
Water-soluble carbohydrate (%DM)	10.19 ± 2.19	7.49 ± 0.14	16.61 ± 2.86	8.75 ± 0.36
Lactic acid bacteria (log cfu per gram FM)	7.08 ± 0.04	5.92 ± 0.63	5.22 ± 0.52	3.52 ± 0.07
Yeast (log cfu per gram FM)	5.93 ± 0.11	5.80 ± 0.19	5.96 ± 0.21	4.52 ± 0.07
Coliform (log cfu per gram FM)	> 7.0	5.98 ± 0.06	7.27 ± 0.23	3.89 ± 0.48

CS, corn stalks; DM, dry matter; FM, fresh material; NCL, *Neolamarckia cadamba* leaves. Trial 1 was conducted on September (2017) and trial 2 was conducted on November (2017).

Generally, the naturally epiphytic LAB population is considered as a crucial factor in determining the pH decline during the early stage of ensiling. The meta-analysis of Oliveira *et al*. (2017) indicated that LAB population reaches over 5 $\log_{10}$ cfu g^{-1} FM could ensure effective fermentation, whereas lower than 4 $\log_{10}$ cfu g^{-1} FM might decrease DM recovery and increase ammonia-N content. As shown in Table 1, the LAB populations in fresh CS were 5.2 and 7.1 $\log_{10}$ cfu g^{-1} FM in trial 1 and 2, respectively, which were enough to initiate the silage fermentation during the early stage of CS ensiling. However, high populations of the undesirable yeast and coliform were also detected in the CS, which was similar with the results reported by Yan *et al*. (2019). Their study showed higher amounts of undesirable microorganisms (4–8 $\log_{10}$ cfu g^{-1} FM) in Italian ryegrass and dry corn stover, which could impair silage preservation and affect animal performance and health (Dunière *et al*., 2013). Although the LAB population in fresh NCL was relatively low and the populations of undesirable microorganisms were relatively high, it will not affect the normal fermentation. Our previous study confirmed that NCL could inhibit undesirable microorganisms such as *Clostridium* and *Enterobacter* and improve the quality of high moisture alfalfa and stylo (Wang *et al*., 2019a). He *et al*. (2019) also proved that the protein of NCL can be well preserved during ensiling due to its low protease and bacterial activity. Therefore, mixing NCL might be helpful for undesirable microorganism inhibition and protein preservation in CS silage.

Fermentation performance of silage

The dynamics of fermentation performance during ensiling are shown in Tables 2 and 3. In total, the organic acids, pH, NH_3-N of trial 1 and trial 2 showed similar trends during whole ensiling process.

Silage pH is a traditional and good indicator for assessing the extent of fermentation quality, especially for high moisture silages. The goal of ensiling is to reduce the pH of the silage as rapidly as possible to ≤ 4.2, but preferably to ≤ 4.0, so that the forage is maintained in a stable form (Mcdonald *et al*., 1991). In the present study, the decline of pH values mainly occurred in the first 7 or 14 days of ensiling ($P < 0.01$) and then stabilized at a range of 4.0–3.5 (< 4.2) in all silages, which is a characteristic of well-preserved silages. These results were consistent with the study of Xu *et al*. (2018), who reported that pH of corn stalks rapidly dropped during the early stage of ensiling process (3 days of ensiling), and the pH value declined from 5.73 to 4.09. These phenomena possibly attributed to the high efficient conversion of fermentable WSC by epiphytic LAB in CS (Table 1) into the intensive production of organic acid. In addition, the relatively low buffering capacity of CS, as evidenced by Jatkauskas *et al*. (2013), making it less resistance to change in pH. Generally, organic acid in silage is conventionally produced by various microorganisms, which is normally detected to evaluate the fermentation quality. Lactic acid is the main organic acid responsible for pH reduction during the early stage of ensiling, while the butyric acid is usually caused by undesirable clostridial fermentation (Mcdonald *et al*., 1991). In this study, lactic acid was the dominant fermentation product and its content increased to 1.74–2.17% DM after 1 day of ensiling. The production rate and content were much higher than other material silages, such as *Manyflower silvergrass* and soybean (Li *et al*., 2015; Ni *et al*., 2017). Even though NCL had a very significantly effect on the lactic acid content over the entire ensilage time in trial 1 ($P < 0.01$), it did not influence the lactic acid content after 60 days of ensiling ($P > 0.05$). In addition, NCL showed no influence on lactic acid content in trial 2. The discrepancy between two trials was probably due to different fermentation conditions, especially the ambient temperature (Borreani *et al*., 2018). In the present study, acetic acid was detected in all silages and continuously increased until day 60 ($P < 0.01$). Moreover, NCL significantly decreased the content of acetic acid ($P < 0.05$). Similar results had been reported by Wang *et al*. (2019a), who found the significant reduction of acetic acid content in NCL-treated silages. This could be attributed to the inhibition of NCL by restriction the growth of some acetic acid bacteria and coliform (Muck, 2010). The content of propionic acid in mixed silages was also significantly ($P < 0.05$) lower than control silages, once again confirming that the antimicrobial property of NCL (Chen *et al*., 2018). However, the butyric acid was not detected in our study. It might be caused by the inhibition of harmful microorganisms such as *Clostridium* during ensiling, as a result of the rapid decrease of pH (Heinritz *et al*., 2012).

The primary goal of ensiling is to maximize the preservation of nutrients in economic way, especially the preservation of CP. NH_3-N level reflected the CP degradation in silage, which represents another important parameter for assessing silage quality. As shown in Tables 2 and 3, all mixed silages had lower NH_3-N content compared with the control in the whole ensilage time ($P < 0.05$), suggesting that NCL had a positive impact on the conservation of protein. Similarly, our previous study found high ratio of true protein (TP) to CP, the large proportion of free amino acid (FAA) in non-protein nitrogen (NPN) and low NH_3-N content of NCL sole silage, and protein was well preserved in the ensiling process (He *et al*., 2018). These results might be

Table 2. Dynamic of organic acid, pH and microbial population during ensiling process (trial 1).

		%DM						log_{10} cfu per gram FM		
Ensilage times	Sample ID	Lactic acid	Acetic acid	Propionic acid	Butyric acid	NH_3-N	pH	LAB	Yeast	Coliform
1	100% CS	2.17 ± 0.09^{a}	1.10 ± 0.26^{a}	ND	ND	0.07 ± 0.00^{a}	4.74 ± 0.06^{a}	8.00 ± 1.08^{a}	5.17 ± 0.15^{a}	6.66 ± 0.14^{a}
	10% NCL	2.59 ± 1.10^{a}	0.72 ± 0.38^{ab}	ND	ND	0.06 ± 0.01^{a}	4.86 ± 0.15^{a}	8.64 ± 0.20^{a}	5.16 ± 0.23^{a}	6.63 ± 0.41^{a}
	30% NCL	2.71 ± 0.57^{a}	0.42 ± 0.28^{bc}	ND	ND	0.04 ± 0.00^{b}	4.79 ± 0.11^{a}	8.40 ± 0.07^{a}	5.21 ± 0.29^{a}	6.91 ± 0.12^{a}
	50% NCL	3.18 ± 0.86^{a}	0.27 ± 0.15^{c}	ND	ND	0.03 ± 0.00^{b}	4.68 ± 0.03^{a}	8.11 ± 0.16^{a}	4.92 ± 0.15^{a}	6.63 ± 0.11^{a}
7	100% CS	5.36 ± 0.89^{a}	1.62 ± 0.15^{a}	ND	ND	0.15 ± 0.06^{a}	4.07 ± 0.05^{a}	8.56 ± 0.09^{a}	4.96 ± 0.44^{a}	3.50 ± 0.34^{a}
	10% NCL	4.04 ± 0.97^{a}	1.19 ± 0.21^{b}	ND	ND	0.12 ± 0.04^{ab}	4.10 ± 0.05^{a}	8.57 ± 0.07^{a}	3.91 ± 0.73^{a}	< 2.0^{b}
	30% NCL	5.37 ± 1.13^{a}	0.54 ± 0.12^{d}	ND	ND	0.05 ± 0.02^{b}	4.24 ± 0.27^{a}	8.40 ± 0.17^{ab}	3.95 ± 0.63^{a}	< 2.0^{b}
	50% NCL	5.44 ± 0.86^{a}	0.87 ± 0.16^{c}	ND	ND	0.10 ± 0.00^{ab}	4.24 ± 0.01^{a}	8.22 ± 0.11^{b}	4.12 ± 0.34^{a}	< 2.0^{b}
14	100% CS	6.07 ± 0.73^{a}	1.92 ± 0.42^{a}	ND	ND	0.22 ± 0.04^{a}	4.00 ± 0.20^{a}	7.90 ± 0.13^{a}	3.46 ± 0.15^{a}	< 2.0
	10% NCL	4.22 ± 0.88^{b}	1.08 ± 0.31^{b}	ND	ND	0.17 ± 0.00^{a}	3.93 ± 0.10^{a}	7.91 ± 0.14^{a}	3.52 ± 0.54^{a}	< 2.0
	30% NCL	5.33 ± 0.09^{ab}	1.02 ± 0.39^{b}	ND	ND	0.08 ± 0.03^{b}	3.91 ± 0.05^{a}	7.37 ± 0.11^{b}	3.58 ± 0.49^{a}	< 2.0
	50% NCL	5.22 ± 1.02^{ab}	0.82 ± 0.06^{b}	ND	ND	0.07 ± 0.03^{b}	3.97 ± 0.04^{a}	7.23 ± 0.09^{b}	3.26 ± 0.24^{a}	< 2.0
30	100% CS	5.94 ± 0.63^{a}	2.38 ± 0.02^{a}	0.44 ± 0.15^{a}	ND	0.12 ± 0.02^{a}	3.83 ± 0.08^{a}	7.10 ± 0.09^{a}	3.65 ± 0.37^{a}	< 2.0
	10% NCL	5.39 ± 0.30^{a}	1.33 ± 0.45^{b}	ND	ND	0.12 ± 0.02^{a}	3.83 ± 0.04^{a}	6.67 ± 0.31^{a}	3.60 ± 0.30^{a}	< 2.0
	30% NCL	5.37 ± 0.52^{a}	1.43 ± 0.25^{b}	ND	ND	0.11 ± 0.00^{a}	3.89 ± 0.02^{a}	6.01 ± 0.45^{b}	3.60 ± 0.00^{a}	< 2.0
	50% NCL	5.07 ± 0.43^{a}	0.94 ± 0.23^{b}	ND	ND	0.07 ± 0.02^{b}	3.92 ± 0.06^{a}	5.83 ± 0.27^{b}	4.08 ± 0.45^{a}	< 2.0
60	100% CS	6.58 ± 1.22^{a}	2.40 ± 0.44^{a}	0.41 ± 0.12^{a}	ND	0.19 ± 0.04^{a}	3.87 ± 0.02^{a}	7.30 ± 0.03^{a}	3.80 ± 0.44^{a}	< 2.0
	10% NCL	4.78 ± 0.07^{ab}	1.35 ± 0.60^{b}	ND	ND	0.14 ± 0.01^{ab}	3.86 ± 0.04^{a}	6.32 ± 0.52^{ab}	3.80 ± 0.62^{a}	< 2.0
	30% NCL	3.96 ± 1.28^{b}	0.95 ± 0.41^{b}	ND	ND	0.11 ± 0.02^{bc}	3.89 ± 0.04^{a}	5.76 ± 0.85^{bc}	3.88 ± 0.86^{a}	< 2.0
	50% NCL	5.46 ± 0.67^{ab}	0.79 ± 0.10^{b}	ND	ND	0.08 ± 0.01^{c}	3.98 ± 0.04^{a}	5.22 ± 0.43^{c}	3.81 ± 0.47^{a}	< 2.0
T		**	**	*	—	**	**	**	**	**
NCL		**	**	**	—	**	NS	**	NS	**
T*NCL		NS	NS	**	—	**	NS	*	NS	**

CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves; FM, fresh material; DM, dry matter; ammonia nitrogen, NH_3-N; LAB, lactic acid bacteria; T, ensilage times; T*NCL, the interaction between ensilage times and *N. cadamba* leaves; ND, not detected; —, not analysed; values within the same column under same ensiling days with different superscripts in lowercase letter differ significantly from each other at $P < 0.05$; * and **, significant at $P < 0.05$ and 0.01 respectively; NS, no significant.

Table 3. Dynamic of organic acid, pH and microbial population during ensiling process (trial 2).

		%DM						log_{10} cfu per gram FM		
Ensilage times	Sample ID	Lactic acid	Acetic acid	Propionic acid	Butyric acid	NH_3-N	pH	LAB	Yeast	Coliform
1	100% CS	2.07 ± 0.04^{a}	1.26 ± 0.32^{a}	0.37 ± 0.16^{a}	ND	0.07 ± 0.01^{a}	4.71 ± 0.43^{a}	8.92 ± 0.11^{a}	5.10 ± 0.54^{a}	7.47 ± 0.36^{a}
	10% NCL	1.74 ± 0.63^{a}	1.23 ± 0.28^{a}	ND	ND	0.05 ± 0.00^{b}	4.54 ± 0.20^{a}	8.86 ± 0.23^{a}	4.80 ± 0.32^{a}	7.04 ± 0.60^{a}
	30% NCL	2.01 ± 0.52^{a}	0.83 ± 0.04^{a}	ND	ND	0.04 ± 0.00^{b}	4.61 ± 0.14^{a}	8.61 ± 0.12^{a}	4.93 ± 0.56^{a}	7.49 ± 0.29^{a}
	50% NCL	1.78 ± 0.39^{a}	0.10 ± 0.15^{b}	ND	ND	0.04 ± 0.00^{b}	4.71 ± 0.01^{a}	8.05 ± 0.36^{b}	4.45 ± 0.34^{a}	7.48 ± 0.14^{a}
7	100% CS	3.43 ± 0.27^{a}	1.21 ± 0.01^{a}	0.38 ± 0.14^{a}	ND	0.11 ± 0.02^{a}	3.67 ± 0.07^{c}	9.09 ± 0.02^{a}	5.10 ± 0.77^{a}	< 3.0
	10% NCL	3.90 ± 0.66^{a}	1.02 ± 0.29^{a}	0.30 ± 0.17^{ab}	ND	0.11 ± 0.01^{a}	3.74 ± 0.07^{bc}	8.86 ± 0.11^{a}	4.23 ± 0.21^{ab}	< 3.0
	30% NCL	4.56 ± 0.70^{a}	0.42 ± 0.19^{b}	0.10 ± 0.07^{bc}	ND	0.08 ± 0.02^{ab}	3.82 ± 0.05^{ab}	8.80 ± 0.27^{a}	3.83 ± 0.22^{b}	< 3.0
	50% NCL	3.87 ± 0.69^{a}	0.20 ± 0.08^{b}	ND	ND	0.06 ± 0.01^{b}	3.91 ± 0.03^{a}	8.81 ± 0.08^{a}	3.46 ± 0.15^{b}	< 3.0
14	100% CS	5.73 ± 0.58^{a}	1.49 ± 0.08^{a}	1.25 ± 0.19^{a}	ND	0.19 ± 0.05^{a}	3.55 ± 0.03^{c}	8.42 ± 0.32^{a}	5.74 ± 0.02^{a}	< 2.0
	10% NCL	4.28 ± 1.05^{b}	0.86 ± 0.35^{ab}	0.77 ± 0.32^{ab}	ND	0.09 ± 0.02^{b}	3.58 ± 0.01^{c}	8.35 ± 0.25^{a}	5.66 ± 0.20^{a}	< 2.0
	30% NCL	5.81 ± 0.58^{a}	0.79 ± 0.19^{ab}	0.58 ± 0.14^{ab}	ND	0.07 ± 0.01^{b}	3.69 ± 0.05^{b}	8.05 ± 0.14^{a}	4.04 ± 0.04^{b}	< 2.0
	50% NCL	3.90 ± 0.44^{b}	0.37 ± 0.09^{b}	0.17 ± 0.15^{b}	ND	0.06 ± 0.00^{b}	3.83 ± 0.04^{a}	8.07 ± 0.16^{a}	3.65 ± 0.49^{b}	< 2.0
30	100% CS	5.66 ± 1.39^{a}	0.83 ± 0.59^{a}	1.22 ± 0.41^{a}	ND	0.14 ± 0.03^{a}	3.59 ± 0.00^{d}	6.84 ± 0.15^{a}	5.65 ± 0.19^{a}	< 2.0
	10% NCL	5.49 ± 0.62^{a}	0.64 ± 0.22^{a}	0.97 ± 0.06^{a}	ND	0.13 ± 0.06^{a}	3.66 ± 0.02^{c}	6.82 ± 0.17^{a}	5.38 ± 0.31^{a}	< 2.0
	30% NCL	5.65 ± 0.94^{a}	0.49 ± 0.01^{a}	0.72 ± 0.33^{a}	ND	0.08 ± 0.03^{a}	3.76 ± 0.03^{b}	6.28 ± 0.32^{b}	5.27 ± 0.11^{a}	< 2.0
	50% NCL	5.84 ± 1.03^{a}	0.43 ± 0.14^{a}	0.81 ± 0.28^{a}	ND	0.09 ± 0.04^{a}	3.81 ± 0.03^{a}	6.19 ± 0.33^{b}	4.61 ± 0.17^{b}	< 2.0
60	100% CS	4.94 ± 0.81^{a}	0.95 ± 0.26^{a}	1.68 ± 0.41^{a}	ND	0.18 ± 0.03^{a}	3.60 ± 0.05^{c}	6.45 ± 0.05^{a}	5.28 ± 0.92^{a}	< 2.0
	10% NCL	5.39 ± 0.39^{a}	0.62 ± 0.17^{a}	1.11 ± 0.37^{ab}	ND	0.16 ± 0.05^{ab}	3.62 ± 0.04^{c}	5.46 ± 0.19^{b}	5.28 ± 0.34^{a}	< 2.0
	30% NCL	5.43 ± 0.56^{a}	0.58 ± 0.25^{a}	0.83 ± 0.24^{b}	ND	0.11 ± 0.01^{bc}	3.70 ± 0.02^{b}	5.47 ± 0.10^{b}	5.26 ± 0.27^{a}	< 2.0
	50% NCL	5.10 ± 0.59^{a}	0.15 ± 0.13^{b}	0.21 ± 0.18^{c}	ND	0.08 ± 0.00^{c}	3.81 ± 0.02^{a}	5.28 ± 0.13^{b}	4.85 ± 0.12^{a}	< 2.0
T		**	NS	**	—	**	**	**	**	**
NCL		NS	**	**	—	**	**	**	**	NS
T*NCL		NS	NS	NS	—	*	NS	**	**	NS

CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves; FM, fresh material; DM, dry matter; ammonia nitrogen, NH_3-N; LAB, lactic acid bacteria; T, ensilage times; T*NCL, the interaction between ensilage times and *N. cadamba* leaves; ND, not detected; —, not analysed; Values within the same column under same ensiling days with different superscripts in lowercase letter differ significantly from each other at $P < 0.05$; * and **, significant at $P < 0.05$ and 0.01 respectively; NS, no significant.

explained by the high contents of condensed tannin (Wang *et al*., 2019a) and low activity of protease in NCL (He *et al*, 2019). With regard to tannin, many studies have reported that tannin can bind to protein by forming insoluble complexes resistance to rumen fermentation for better nitrogen utilization in ruminants (Huang *et al*., 2010; Jayanegara *et al*., 2015). Therefore, the role of NCL in forage protein conservation should deserve more attentions and require further studies.

LAB number increased at the initial fermentation period of ensiling ($P < 0.01$), and then decreased with storage period, which was in accordance with the results reported by Xu *et al*. (2017). It might be because the lactic acid-producing *cocci* (e.g. heterofermentative *Weissella*, *Lactococci*, *Leuconostocs*, *Pediococcus* and *Enterococci*) grew vigorously and reduced pH at the early stage of ensiling process, and then decreased due to low WSC content and their low tolerance to low pH (Cai *et al*., 1998; Xu *et al*., 2017). The three mixed silages had significantly lower ($P < 0.05$) LAB population than the control after 60 days of ensiling, and 50% NCL-treated silages had the lowest LAB number. However, NCL did not decrease the population of yeast after 60 days of ensiling as expected, indicating that the growth of yeast could not be inhibited by NCL. The population of yeast was different between trial 1 and trial 2. Ensiling decreased the yeast number in trial 1 while enhanced it in trial 2. It might be due to different microorganisms of fresh materials (Table 1) and different ambient temperature of two trials. Trial 1 was conducted on September (average temperature > 35°C (http://data.cma.cn/en)), while trial 2 was conducted at a lower ambient temperature (in November, average temperature is 25–28°C (http://data.cma.cn/en)), which could indirectly favour the yeast survival by allowing the slower metabolism and reducing the permeability of cell membrane to organic acids (Borreani *et al*., 2018). Coliform is Gram-negative facultative anaerobic bacteria, which could deaminate and decarboxylate amino acids in silages and reduce NO_3, thereby enhancing ammonia and biogenic amine production. Queiroz *et al*. (2018) reported that some species of coliform can produce endotoxins, which may cause severe diseases in animals. However, their growth and viability decrease as the pH decline. In the current study, coliform was detected in all treatments, but decreased to 2 $\log_{10}$ cfu g^{-1} FM after ensiling 7 days. Similarly, Ni *et al*. (2017) reported that coliform was detected in the control and LAB-treated soybean silages, but decreased to below the detectable level after 7 or 14 days of ensiling. In a word, the addition of NCL reduced acetic acid, propionic acid, NH_3-N contents and LAB population. All these results indicate that mixing NCL could improve fermentation quality of CS silage.

Bacterial and fungal diversities after 60 days of ensiling

The diversity of bacterial and fungal communities in each sample based on α-diversity was listed in Table 4. The coverage values of all samples were around 0.99, suggesting that most bacteria and fungi were adequately captured. The OTUs, Chao1 index, Shannon index showed the low bacterial biodiversity once material was ensiled ($P < 0.05$). This result was likely due to the relatively low pH values in all silages inhibiting the growth of bacteria that had lower adaptability to the acid condition (Ni *et al*., 2017). Interestingly, although the addition of NCL did not affect the bacterial biodiversity, it had an effect on fungal biodiversity ($P < 0.05$). The silages containing NCL had higher fungal diversity (Shannon's index) than those of the control in two trials, and 50% NCL silages had the highest diversity ($P < 0.05$). The probable reason for this result was the relatively higher acetic and propionic acids production in control silages. Mcdonald *et al*. (1991) concluded that acetic and propionic acids are two fermentation products with strong antifungal and antimycotic properties, which play an important role in aerobic deterioration. Besides, the effect of acetic acid on fungal activity is related to the undissociated concentration in silage; thus, a given concentration of acetic acid becomes more inhibitory to yeasts as silage pH decrease. It is possible that low pH values (3.87 and 3.60 in trail 1 and trial 2 respectively) and higher contents of acetic and propionic acid in control silages tend to suppress the growth of some fungus such as lactate-assimilating yeast and subsequently reduce the fugal diversity (Kung *et al*., 2018).

Then, the unweighted PCoA revealed the existence of microbial structural difference (Fig. 1), the principal coordinate 1 (PCoA 1) and 2 (PCoA 2) explained 14.29% and 7.18% of total variance in Figure 1A, and the PCoA 1 and PCoA 2, respectively, explained 17.95% and 12.91% of total variance in Figure 1B. In both bacterial and fungal communities, the silages mixed with NCL were clearly separated from the control silages, which suggested that NCL not only affected bacterial community but also influence fungal community structures. This might be a vital factor leading to difference in silage quality (Yang and Wang, 2018). However, there was less variation in three mixed silages. This result agreed with the finding reported by Ni *et al*. (2018), who found the forage soybean mixed with crop corn or sorghum had a similar microbial community and believed that the microbial community of mixed silage was relatively stable.

Bacterial composition after 60 days of ensiling

In order to obtain the further knowledge associated with the potential nature of bacterial and fungal that was

Table 4. Alpha diversity of bacterial and fungal diversity at the day 0 and 60 of ensiling.

	Bacterial diversity					Fungal diversity				
Sample ID	Reads	OTUs	Chao1	Good's coverage	Shannon	Reads	OTUs	Chao1	Good's coverage	Shannon
Trial 1										
FM	103 813 ± 9288^{a}	1000 ± 42^{a}	1266 ± 46^{a}	0.99 ± 0.00	5.97 ± 0.09^{a}	150 190 ± 20 439^{a}	552 ± 55^{a}	666 ± 110^{a}	0.99 ± 0.00	4.87 ± 0.01^{c}
100% CS	84 821 ± 7237^{b}	536 ± 72^{b}	780 ± 92^{b}	0.99 ± 0.00	3.52 ± 0.17^{b}	98 544 ± 2507^{c}	349 ± 34^{b}	565 ± 55^{ab}	0.99 ± 0.00	3.84 ± 0.45^{b}
10% NCL	81 639 ± 3370^{b}	505 ± 25^{b}	783 ± 83^{b}	0.99 ± 0.00	3.27 ± 0.13^{b}	132 605 ± 30211^{ab}	301 ± 15^{bc}	493 ± 34^{bc}	0.99 ± 0.00	5.19 ± 0.19^{ab}
30% NCL	78 228 ± 4727^{b}	562 ± 119^{b}	853 ± 101^{b}	0.99 ± 0.00	3.36 ± 0.38^{b}	106 577 ± 3061^{bc}	299 ± 4^{bc}	430 ± 31^{c}	0.99 ± 0.00	5.15 ± 0.45^{ab}
50% NCL	85 086 ± 7203^{b}	589 ± 51^{b}	859 ± 53^{b}	0.99 ± 0.00	3.38 ± 0.27^{b}	106 541 ± 6835^{bc}	280 ± 23^{c}	384 ± 21^{c}	0.99 ± 0.00	5.48 ± 0.09^{a}
Trial 2										
FM	89 554 ± 8221^{a}	713 ± 127^{a}	966 ± 139^{a}	0.99 ± 0.00	4.05 ± 0.38^{a}	106 452 ± 15724^{a}	464 ± 27^{a}	582 ± 12^{a}	0.99 ± 0.00	3.96 ± 0.44^{ab}
100% CS	89 224 ± 5229^{a}	603 ± 47^{ab}	852 ± 4^{ab}	0.99 ± 0.00	3.65 ± 0.18^{ab}	126 799 ± 32946^{a}	306 ± 9^{b}	437 ± 49^{b}	0.99 ± 0.00	2.64 ± 1.21^{b}
10% NCL	85 868 ± 5419^{a}	504 ± 25^{b}	724 ± 67^{b}	0.99 ± 0.00	3.41 ± 0.04^{b}	121 808 ± 50379^{a}	308 ± 37^{b}	543 ± 51^{a}	0.99 ± 0.00	3.68 ± 0.09^{ab}
30% NCL	74 960 ± 15 874^{a}	483 ± 61^{b}	786 ± 91^{b}	0.99 ± 0.00	3.26 ± 0.12^{b}	89 445 ± 23162^{a}	232 ± 22^{c}	385 ± 56^{b}	0.99 ± 0.00	4.27 ± 0.24^{a}
50% NCL	87 015 ± 3568^{a}	500 ± 66^{b}	719 ± 75^{b}	0.99 ± 0.00	3.57 ± 0.26^{b}	91 842 ± 3294^{a}	248 ± 17^{c}	392 ± 50^{b}	0.99 ± 0.00	4.10 ± 0.56^{a}

CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves; FM, fresh material (pre-ensiled material). Trial 1 was conducted on September (2017) and Trial 2 was conducted on November (2017).

concerned with the ensiling process, the phylogenetic analysis was also performed at genus level. The relative abundance of bacterial community on the genus level was exhibited in Figure 2A. Similarly, the changes in microbial composition of trial 1 and trial 2 were basically the same. The most abundant genera in the pre-ensiled samples were *Exiguobacterium* (6.01–33.08%), *Pseudomonas* (4.60–10.68%) and *Acinedomonas* (2.58–5.13%), while the portion of *Exiguobacterium* increased greatly and became dominant genus after ensiling. Similar result had been reported in our previous study in *Moringa oleifera* leaves silage and NCL silage (Wang *et al*., 2018; He *et al*., 2019). However, many studies indicated that *Lactobacillus* could dominate the fermentation (Li *et al*., 2015; Ni *et al*., 2017). This might be because the bacterial community would vary depending on the silage material, growing season and climate (Dunière *et al*., 2013). *Exiguobacterium* is Gram-positive facultative anaerobe, non-spore, non-acid, and can ferment glucose to lactic acid, acetic acid and formic acid during anaerobic fermentation (Lund and Schleifer, 1983). Vijayalaxmi *et al*. (2013) also reported that *Exiguobacterium* could effectively hydrolyse lignocellulolytic materials, with a high substrate conversion yield, high productivity and high optical purity. Therefore, it is reasonable to suspect that partial lactic acid and acetic acid produced on the day 1 of ensiling might derive from *Exiguobacterium*, and the rapid acidification might inhibit their activities. At present, *Exiguobacterium* is more widely used in decomposition of organic pollutants (azo dyes, pesticides and petroleum), transformation of heavy metals, rhizosphere promotion, industrial waste water treatment and other fields (Zhang *et al*., 2013). However, more information needed to be uncovered to illuminate its roles during ensiling in the further studies. *Acinedomonas* is considered to be undesirable microorganism which can survive in an anaerobic condition by utilizing acetate as a substrate. It has also been found previously in corn silage, *Moringa oleifera* leaves silage and barely silage (Ogunade *et al*., 2017; Liu *et al*., 2019; Wang *et al*., 2019b). Ogunade *et al*. (2017) reported that the increased abundance of *Acinedomonas* may result from the increased acetate content in corn silage inoculated with *Escherichia coli* O157:H7 and *Lactobacillus buchneri*. In the present study, the abundance of *Acinedomonas* increased slightly after 60 days of ensiling, but no difference in all silages. The results may be due to the relatively low content of acetic acid after fermentation.

In general, LAB was typically associated with silage and belonged to the genera *Leuconostocs*, *Lactobacillus*, *Weissella*, *Pediococcus* and *Lactococcus* while lactic acid-rod (*Lactobacillus*) plays a critical role in enhancing lactic acid content and reducing pH values (Cai *et al*., 1998). In the present study, *Lactobacillus* was the main LAB in all silages, and undesirable *Enterobacter* was not

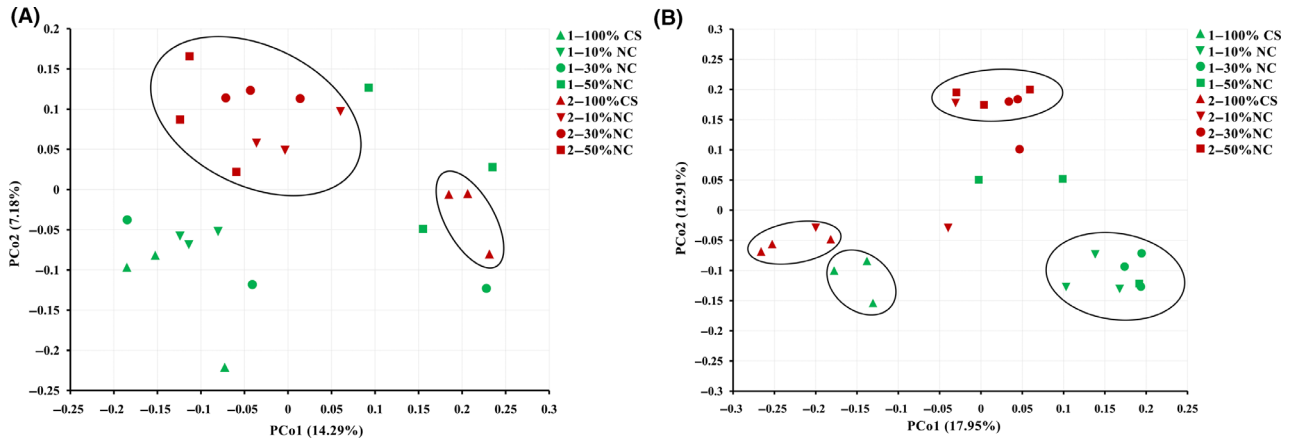

Fig. 1. The unweighted principal coordinate analyses (PCoA) of silages. The bacterial community structure (A) and the fungal community structure (B). PCoA1, principle coordinate 1; PCoA2, principle coordinate 2; red colour represents trial 1; green colour represents trial 2; different shapes represent different treatments; 1, trial 1; 2, trial 2; CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves.

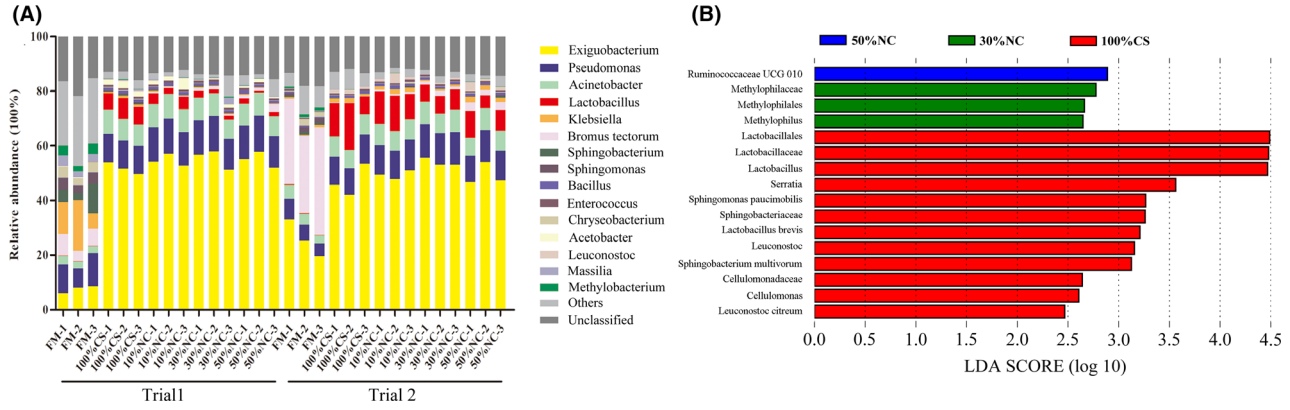

Fig. 2. The bacterial community of silages. The relative abundance of bacterial community at genus level (A) and LEfSe analysis of bacterial variations between control silages and mixed silages (B). FM, fresh material (pre-ensiled material); CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves.

detected, which could explain the relatively well fermentation quality of all silages. Compared with the control silages, lower abundances of *Lactobacillus* (especially *Lactobacillus brevis*) and *Leuconostocs* (especially *Leuconostocs citreum*) were found in the mixed silages (Fig. 2A and B). *Lactobacillus brevis* and *Leuconostocs* are heterofermentative LAB species, metabolizing WSC to produce lactic acid, acetic acid and ethanol (Pang *et al*., 2011). The above results indicated that the growth of LAB could be inhibited by NCL and also explained the relatively low acetic acid concentration in all mixed silages. *Acetobacter* is detrimental acetic bacteria as it may result in aerobic spoilage of corn silage by oxidizing lactate and acetate to carbon dioxide and water, which can impair the nutritive value of the silage (Dolci *et al*., 2011). As expected, the abundance of *Acetobacter* in control silages was 1.84-2.59%, and it was decreased after mixed NCL in trial 1. The presences of *Klebsiella* and *Bacillus* are usually associated with the production of biogenic amines and the growth of acid-tolerant spoilage microorganisms, resulting in significant economic loss (Dunière *et al*., 2013). Although the abundance of *Klebsiella* and *Bacillus* in this study increased after 60 days of ensiling in trial 2, they were detected at a low level. Other genera such as *Sphingobacterium*, *Chryseobacterium* and *Methylobacterium* also existed in silages, but their role had not been extensively studied.

Fungal composition after 60 days of ensiling

Generally, Fungi are considered to be detrimental group as they can reduce nutritional value and produce many potentially toxic secondary metabolites (Duniere *et al*., 2017). As observed by Spadaro *et al*. (2015), species

belonging to the genera *Cladosporium*, *Epicoccum*, *Alternaria*, *Penicillium* and *Ramularia* were usually associated with fresh corn sample. As shown in Figure 3, the dominant genera in the pre-ensiled samples were *Gibberella*, covering 4.09–32.28% of the sequences and followed by *Cladosporium* (11.94–28.57%), *Saitozyma* (5.98–14.88%), *Curvularia* (0.80–14.02%), *Aspergillus* (1.2–3.70%), *Fusarium* (0.34–3.27%) and others. Nevertheless, their proportions shifted dramatically after ensiling. The abundance of *Aspergillus* increased and became *the* dominant genus in all groups due to initial high oxygen and WSC content in CS silage. This result was in accordance with the report of El-Shanawany *et al*. (2005), who collected forty silage samples and found that the most prevalent genera were *Aspergillus* and *Penicillium* respectively. However, Keshri *et al*. (2018) reported *Candida* would become the most dominant genus in both untreated and treated corn silage. This discrepancy was probably related to difference in the original epiphytic population of fungi, silage ages, ensiling conditions or even differences in the soil fungal community (Carvalho *et al*., 2016). It has been well documented that *Aspergillus* and *Fusarium* sp. are the most frequent mycotoxigenic moulds isolated from corn silage (Dunière *et al*., 2013). Niderkorn *et al*. (2006) observed that more than 20 mycotoxins were produced by *Fusarium* sp., which could have adverse effects on the productivity and health of animals. As expected, the abundance of *Aspergillus* (especially *Aspergillus occultus* and *Aspergillus fumigatus*) and *Fusarium* in mixed silages was lower than the control silages in this study (Fig. 3A and B), indicating that NCL could suppress the growth of these two kinds of undesirable genera, thus reducing the potential risk of liver toxicity and improving the forage quality to some extent. In the present study, mixing NCL increased the abundance of *Gibberella*, *Cladosporium*, *Curvularia*, *Pseudocercospora*, *Kazachstania* and *Aureobasidium*. It may partly explain the higher fungal diversity in mixed silages. *Kazachstania,* belongs to family *Saccharomycetaceae*, is an ascomycetous yeast which is proposed by Zubkova with the description of *Kazachstania viticola* (Zubkova, 1971). *Aureobasidium* is an important biotechnological yeast as its ability to produce many extracellular enzymes such as cellulase and xylanase (Chi *et al*., 2009). When CS mixed with NCL, *Kazachstania* population increased from 0.05–17.14% to 1.07–53.80% in trial 2, and *Aureobasidium* population increased from 0.06–0.77% to 0.52–11.93%, which might be attributed to the relatively lower acetic acid and propionic acid contents in the mixed silages. These results further confirmed that NCL could not inhibit the growth of some species of yeast. In this study, the growth of genus *Saitozyma* was inhibited by NCL. *Saitozyma* is a basidiomycete yeast, which is often isolated from the soil as well as both below-ground parts of plants (Prakash *et al*., 2018). However, its exact function has not been reported yet. The further study could focus on revealing the underlying reason about the inhibition of *Saitozyma* after silages mixed with NCL.

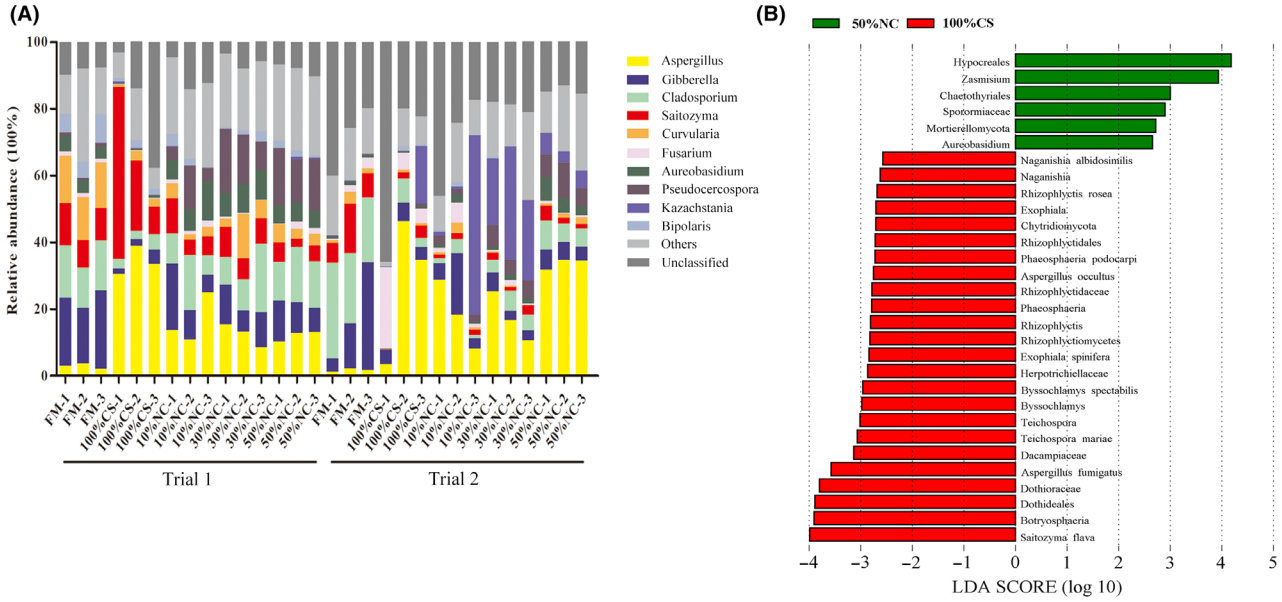

Fig. 3. The fungal community of silages. The relative abundance of fungal community at genus level (A) and LEfSe analysis of fungal variations between control silages and mixed silages (B). FM, fresh material (pre-ensiled material); CS, corn stalks; NCL, *Neolamarckia cadamba* leaves; 10% NCL, 90% corn stalks with 10% *N. cadamba* leaves; 30% NCL, 70% corn stalks with 30% *N. cadamba* leaves; 50% NCL, 50% corn stalks with 50% *N. cadamba* leaves.

Conclusions

This study revealed that the addition of NCL apparently changed the microbial community and influenced fermentation performance of the CS silage. Acetic acid, propionic acid and NH_3-N contents of CS silage decreased after mixing with NCL. The fungal diversity increased while the abundance of *Lactobacillus*, *Leuconostocs*, *Acetobacter*, *Aspergillus* and *Fusarium* decreased when NCL were added. These results confirmed that mixing with NCL before ensiling appears to be a feasible approach to improve CS silage, which will aid in the livestock feed industry at a later date.

Experimental procedures

Raw materials and silage preparation

Corn stalks (CS) and *Neolamarckia cadamba* leaves (NCL) were cultivated at the experimental field of South China Agricultural University (Guangzhou, China) and applied with no herbicides and fertilizers. The CS for the ensiling trials were obtained from two varieties with different harvest times. Trail 1: CS (variety: Yuetian No.1) and NCL (2-year-old tree, with a height of approximately 2–2.5 m) were harvested on 7 September 2017. Trial 2: CS (variety: Suitian No.16) and NCL (2-year-old tree, with a height of approximately 2–2.5 m) were harvested on 1 November 2017. Both CS and NCL were cut into 20 cm lengths by hand with a sickle and immediately transported to the laboratory. Before ensiling, all of the materials were cut with a crop chopper into 2 cm theoretical lengths.

The treatments for making silage were combination of 100% CS with 0% NCL, 90% CS with 10% NCL, 70% CS with 30% NCL and 50% CS with 50% NCL respectively (on a fresh matter basis). After a thorough mixing, approximately 180 g of the mixture for each treatment was packed into plastic film bags (20 × 30 cm; Dongguan Bojia Packaging, Dongguan, China), vacuumed and sealed by a vacuum sealer (Lvye DZ280; Dongguan Yijian Packaging Machinery, Dongguan, China). A total of 120 samples (2 trials × 4 treatments × 5 ensiling times × 3 replicates) were made and stored at room temperature. Three bags for each treatment were randomly opened for analysing fermentation performance (pH, organic acid, NH_3-H and microbial population) after 1, 7, 14, 30 and 60 days of ensiling respectively. Fresh materials and silage samples (60 ensiling days) were collected for analysing the microbial community composition.

Analysis of microbial population, organic acid and chemical composition

For fermentation indices, the silage samples (20 g) were blended with 180 ml sterilized saline water (8.5 g l^{-1} NaCl) and serially diluted from 10^{-1} to 10^{-7}. The number of lactic acid bacteria (LAB) was measured by plate count on de Man, Rogosa and Sharpe (MRS) agar incubated at 37°C for 2 days under anaerobic conditions (LRH-250, Shanghai, China). Yeast was counted on Rose Bengal Agar, incubated at 28°C for 2 days under aerobic conditions. Coliform was counted on Violet Red Bile Agar incubated at 30°C for 2 days under aerobic conditions. Colonies were counted as viable numbers of microorganisms in colony forming unit (cfu) per gram of fresh material (FM).

For pH, NH_3-N and organic acid determination, 20 g of samples with 180 ml sterilized water was homogenized in a juicer for 1 min and then filtered through four layers of cheesecloth and Whatman filter paper. The pH of this filtrate was immediately measured by a glass electrode pH meter (PHS-3C, INESA Scientific Instrument, Shanghai, China). The NH_3-N content was determined by the method of Broderick and Kang (1980). The organic acid (including lactic acid, acetic acid, propionic acid and butyric acid) content was measured in 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} $HClO_4$; flowrate, 1.0 ml min^{-1}; injection volume, 5 μl; and detector, SPD-M10AVP) (Zhang *et al*., 2017).

Dry matter (DM) content was determined by oven drying at 65°C for 2 days. Water-soluble carbohydrate (WSC) content was analysed using the anthrone method (Murphy, 1958). Crude protein (CP) was measured by the method of Association of Official Analytical Chemists (AOAC, 2012). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were measured by the method of Van Soest *et al*. (1991).

Microbial diversity analysis

Fresh materials and silage samples (60 ensiling days) were collected for investigating the microbial community composition. A total of 30 samples (2 trials × 4 treatments × 1 ensiling times × 3 replicates + 6 fresh materials) were collected and stored at –20 before DNA extraction. The E.Z.N.A. stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) was used to extract microbial DNA. For bacteria, the 16S rDNA V3-V4 variable region was targeted using specific primers with barcode: 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT). For fungi, the ITS region was targeted using primers with barcode: ITS3_KYO2F (GATGAAGAACGYAGYRAA) and ITS4R (TCCTCC GCTTATTGATATGC) (Guo *et al*., 2018). Polymerase chain reactions (PCR) were carried out under the following conditions: hotstart 95°C for 2 min, followed by 27

cycles of denaturation at 98°C for 10 s, annealing at 62°C for 30 s, elongation at 68°C for 30 s and a final extension at 68°C for 10 min. The reactions were performed in a 50 μl mixture containing 5 μl of 10× KOD Buffer, 5 μl of 2.5 μl mM^{-1} dNTPs, 1.5 μl of each primer (5 μM), 1 μl of KOD Polymerase and 100 ng of template DNA. All of the PCR reactions for each sample were performed in triplicate.

The DNA samples were sequenced at Guangzhou Gene Denovo (Guangzhou, China) using Illumina Hiseq™ 2500 PE250 platform according to the standard protocols. To get high-quality clean reads, raw reads that contained > 10% of unknown nucleotides (N) and < 80% of bases with quality (*Q*-value) > 20 were removed. Paired-end clean reads were merged as raw tags using FLSAH (v 1.2.11) with a minimum overlap of 10 bp and mismatch error rates of 2% (Magoc and Salzberg, 2011). Noisy sequences of raw tags were filtered using the QIIME (v 1.9.1) pipeline under specific filtering conditions to obtain high-quality clean tags (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). All chimeric tags were removed and finally obtained effective tags for further analysis. The effective tags were clustered into operational taxonomic units (OTUs) of 97% similarity level using UPARSE pipeline (Edgar, 2013). The alpha diversity index, mainly of the Shannon index, Chao1 richness estimator and the Good's coverage were calculation in QIIME. Taxonomic classification at the genus level was performed using Ribosomal Database Project (RDP) classifier (version 2.2) (Wang *et al*., 2007). Biomarker features in each group was screened by LEfSe software. The unweighted principal coordinate analyses (PCoA) based on UniFrac metrics was calculated and plotted in R software.

Data accessibility

The sequences were archived in the Sequence Read Archive (SRA) with the accession number PRJNA490426.

Statistical analyses

The statistical analysis was performed using the general linear model procedure (GLM) of Statistical Analysis System (version 9.0, SAS Institute, Cary, NC, USA). Data were analysed using a two-way analysis of variance, with NCL inclusion and ensilage time as the main variables. The mathematical model is as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} was every observation; μ was the general mean; α_i represented the effect of NCL inclusion; β_j denoted the effect of ensilage times; $\alpha\beta_{ij}$ accounted for the interaction of NCL inclusion and ensilage times; and ε_{ijk} was random residual error. Additionally, Duncan's multiple comparison was used to compare the differences between the average value of each treatment and the significance and very significance were set to $P < 0.05$ and $P < 0.01$ respectively. All values in tables were presented as mean ± standard deviation ($n = 3$). 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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Conflicts of interest

None declared.

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