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

Cheng Wang,† Liwen He,† Yaqi Xing,‡ Wei Zhou,§ Fuyu Yang,∥ Xiaoyang Chen and Qing Zhang**

†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.
‡College of Animal Science and Technology, China Agricultural University, Beijing, China.

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.

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 attributes. 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 Asp agillus fumigatus and Candida 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 Clostridium 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 (Log₁₀ cfu g⁻¹ FM)  | 4.21 ± 0.79       | 5.94 ± 0.03 | 4.96 ± 0.44 |
| Yeast (Log₁₀ cfu g⁻¹ FM)                 | 3.93 ± 0.08       | 4.17 ± 0.15 | 4.15 ± 0.22 |
| Coliform bacteria (Log₁₀ 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

| Item                                      | Trial 1 M-CK | Trial 1 M-25 | Trial 1 M-50 | SEM | P value | Trial 2 S-CK | Trial 2 S-25 | Trial 2 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  | < 0.001 | 23.7         | 18.0         | ND           | 3.64  | < 0.001 |
| Butyric acid (g kg⁻¹ DM)                  | ND           | ND           | ND           | –    | –       | ND           | ND           | ND           | –    | –       |
| Lactic acid bacteria (Log₁₀ 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 (Log₁₀ cfu g⁻¹ FM)                  | < 2.00       | < 2.00       | < 2.00       | –    | –       | < 2.00       | < 2.00       | < 2.00       | –    | –       |
| Yeast (Log₁₀ cfu g⁻¹ FM)                  | < 2.00       | < 2.00       | < 2.00       | –    | –       | < 2.00       | < 2.00       | < 2.00       | –    | –       |
| Coliform bacteria (Log₁₀ 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.

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

| Item                                      | Trial 1 M-CK | Trial 1 M-25 | Trial 1 M-50 | SEM | P value | Trial 2 S-CK | Trial 2 S-25 | Trial 2 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  | < 0.001 | 18.5         | 22.5         | 31.3         | 2.26  | 0.029   |

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

© 2019 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology. Microbial Biotechnology, 12, 869–878
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)](image1)

![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)](image2)
Methylobacterium was also detected in alfalfa (9.5%) and stylo (3.0%) before ensiling.

Exiguobacterium was also an abundant genus in most silage samples (42.2–45.1% in stylo silage and 18.3–36.0% in alfalfa silage). Similar results have 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) © 2019 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology, Microbial Biotechnology, 12, 869–878]
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 Boja Packaging, Dongguan, China), which were vacuumed and sealed by vacuum sealer (Lyve D2Z80; 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 HClO₄; 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 (2019). Samples (10 g) were mixed with 90 ml of sterilized 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 Bio-tek, 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), and amplified tRNA genes 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 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 UCHIME (v 1.2.11; Magoc 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 CHIMERA (v 1.9.1; Caporaso et al., 2010). Clean tags were searched against the reference database (http://drive5.com/uchime/uchime_down load.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 analyzed using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools).

Acknowledgements

This work was financially supported by Science and Technology Program of Guangdong, China (2017B020201008), 2018 Big Pig-producing County Reward Funds (Research and promotion of key technologies for healthy feeding of pigs and resource utilization of manure pollution), National Key R&D Projects (Grant No. 2017YFD0502102-02), Guangzhou Science Forestry Technology and Innovation Commission (Grant No. 2018KJCX001).

Conflict of interest

None declared.

References

AOAC (1990) Official Methods of Analysis, 15th edn. Arlington, VA: Association of Official Analytical Chemists. Avila, C.L.S., Carvalho, B.F., Pinto, J.C., Duarte, W.F., and Schwan, R.F. (2014) The use of Lactobacillus species as starter cultures for enhancing the quality of sugar cane silage. J Dairy Sci 97: 940–951. Broderick, G.A., and Kang, J.H. (1980) Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J Dairy Sci 63: 64–75. Cai, Y., Benno, Y., Ogawa, M., Ohamomo, S., Kumai, S., and Nakase, T. (1998) Influence of Lactobacillus spp. from an inoculant and of Weissella and Leuconostoc spp. from forage crops on silage fermentation. Appl Environ Microb 64: 2982–2987. Coblentz, W.K., and Grabber, J.H. (2013) In situ protein degradation of alfalfa and birdsfoot trefoil hays and
silages as influenced by condensed tannin concentration. J Dairy Sci 96: 3120–3137.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., and Fierer, N. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335–336.
Denek, N., Can, A., Avci, M., Aksu, T., and Durmaz, H. (2011) The effect of molasses-based pre-fermented juice on the fermentation quality of first-cut lucerne silage. Grass Forage Sci 66: 243–250.
Dunière, L., Sindou, J., Chaucheyras-Durand, F., Chevallier, I., and Thévenot-Sergentet, D. (2013) Silage processing and strategies to prevent persistence of undesirable microorganisms. Anim Feed Sci Technol 182: 1–15.
Edwards, R.A., and McDonald, P. (1978) The Chemistry of Silage. In Fermentation of silage-a review. McCullough, M.E. (ed). NFIA, West Des Moines, IA, pp. 27–60.
Flythe, M.D., and Russell, J.B. (2004) The effect of pH and a bacteriocin (bovicin HCS) on Clostridium sporogenes MD1, a bacterium that has the ability to degrade amino acids in ensiled plant materials. FEBS Microbiol Ecol 47: 215–222.
Fuhs, G.W., and Chen, M. (1975) Microbiological basis of phosphate removal in the activated sludge process for treatment wastewater. Microb Ecol 2: 119–138.
Guo, X.S., Ding, W.R., Han, J.G., and Zhou, H. (2008) Characterization of protein fractions and amino acids in ensiled alfalfa treated with different chemical additives. Anim Feed Sci Tech 142: 89–98.
Han, L.Y., Li, J., Na, R.S., Yu, Z., and Zhou, H. (2013) Effect of two additives on the fermentation, in vitro digestibility and aerobic security of Sorghum–sudangrass hybrid silages. Grass Forage Sci 70: 185–194.
He, L.W., Zhou, W., Wang, Y., Wang, C., Chen, X.Y., and Zhang, Q. (2018) Effect of applying lactic acid bacteria and cellulase on the fermentation quality, nutritive value, tannins profile and in vitro digestibility of Neolamarckia cadamba leaves silage. J Anim Physiol Anim Nutr 102: 1429–1436.
Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., and Forsythea, S.J. (2012) Diversity of the Cronobacter genus as revealed by multilocus sequence typing. J Clin Microbiol 50: 3031–3039.
Khandelwal, V., Bhatia, A.K., and Goel, A. (2016) Antimicrobial and antioxidant efficacy of aqueous extract of Anthocephalus cadamba leaves. J Pure Appl Microbiol 10: 209–216.
Li, J.J., Zhang, D., Que, Q.M., Chen, X.Y., and Ouyang, K.X. (2019) Plant regeneration and Agrobacterium-mediated transformation of the miracle tree Neolamarckia cadamba. Ind Crop Prod 130: 443–449.
Licitra, G., Hernandez, T.M., and Van Soest, P.J. (1996) Standardization of procedures for nitrogen. Anim Feed Sci Tech 57: 347–358.
Liu, Q.H., Chen, M.X., Zhang, J.G., Shi, S.L., and Cai, Y.M. (2012) Characteristics of isolated lactic acid bacteria and their effectiveness to improve stylo (Stylosanthes guianensis Sw.) silage quality at various temperatures. Anim Sci J 83: 128–135.
Liu, B.Y., Huan, H.L., Gu, H.R., Xu, N.X., Shen, Q., and Ding, C.L. (2019) Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. Bioresource Technol 273: 212–219.
Lund, B.M., and Schleifer, K.H. (1983) Chemotaxonomic study of an alkaliophilic bacterium, Exiguobacterium aurantiacum gen. nov., sp. nov. Microbiology 129: 2037–2042.
Magoc, T., and Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 27: 2957–2963.
Mcdonald, P., Henderson, A.R., and Heron, S.J.E. (1991) The Biochemistry of Silage, 2nd edn. Marlow, UK: Chalcombe Publications.
Ni, K., Minh, T.T., Tu, T.T.M., Tsuruta, T., Pang, H., and Nishino, N. (2017) Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. Appl Microbiol Biot 101: 1385–1394.
Ni, K.K., Zhao, J.Y., Zhu, B.G., Su, R.N., Pan, Y., Ma, J.K., et al. (2018) Assessing the fermentation quality and microbial community of the mixed silage of forage soybean with crop corn or sorghum. Bioresource Technol 265: 563–567.
Ogunade, I.M., Jiang, Y., Pech Cervantes, A.A., Kim, D.H., Oliveira, A.S., Vyas, D., et al. (2018) Bacterial diversity and composition of alfalfa silage as analyzed by Illumina MiSeq sequencing: Effects of Escherichia coli O157:H7 and silage additives. J Dairy Sci 101: 2048–2059.
Pahlow, G., Muck, R., Driehuis, F., Oude Elferink, S., and Spoelstra, S. (2003) Microbiology of ensiling. In Silage Science and Technology Agronomy. Buxton, D. R., Muck, R., and Harrison, J. H. (eds). Madison, WI, USA: ASA, CSSA, SSSA, pp. 31–93.
Pandy, A., and Negi, P.S. (2016) Traditional uses, phytochemistry and pharmacological properties of Neolamarckia cadamba: a review. J Ethnopharmacol 181: 118–135.
Pang, H.L., Qin, G.Y., Tan, Z.F., Li, Z.W., Wang, Y.P., and Cai, Y.M. (2011) Natural populations of lactic acid bacteria associated with silage fermentation as determined by phenotype, 16S ribosomal RNA and recA gene analyses. Syst Appl Microbiol 34: 235–241.
Parvin, S., Wang, C., Li, Y., and Nishino, N. (2010) Effects of inoculation with lactic acid bacteria on the bacterial communities of Italian ryegrass, whole crop maize, guinea grass and Rhodes grass silages. Anim Feed Sci Technol 160: 160–166.
Patel, D., Darji, V.C., Bariya, A.H., Patel, K.R., and Sonpal, R.N. (2011) Evaluation of anti-fungal activity of Neolamarckia cadamba (Roxb.) Bosser leaf and bark extract. Int Res J Pharm 2: 192–193.
Peng, K., Jin, L., Niu, Y.D., Huang, Q.Q., McAllister, T.A., Yang, H.E., et al. (2018) Condensed tannins affect bacterial and fungal microbiomes and mycotoxin production during ensiling and upon aerobic exposure. Appl Environ Microbiol 84: e00274–17.
Van Soest, P.J., Mertens, D.R., and Deinum, B. (1978) Pre-harvest factors influencing quality of conserved forage. J Anim Sci 47: 712–720.
Van Soest, P.J., Robertsom, J.B., and Lewis, B.A. (1991) Methods for dietary fiber, neutral detergent fiber and
nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583–3597.
Sy, A., Timmers, A.C.J., Knief, C., and Vorholt, J.A. (2005) Methylo trophic metabolism is advantageous for Methylobacterium extorquens during colonization of Medicago truncatula under competitive conditions. Appl Environ Microbiol 71: 7245–7252.
Sy, A., Timmers, A.C.J., Knief, C., and Vorholt, J.A. (2005) Methylotrophic metabolism is advantageous for Methylobacterium extorquens during colonization of Medicago truncatula under competitive conditions. Appl Environ Microbiol 71: 7245–7252.
Tabacco, E., Borreani, G., Crovetto, G.M., Galassi, G., Colombo, D., and Cavallarin, L. (2006) Effect of chestnut tannin on fermentation quality, proteolysis, and protein rumen degradability of alfalfa silage. J Dairy Sci 89: 4736–4746.
Wang, S., Liu, G., Li, Y., Cui, Z., Zhou, D., Liu, D., and Sun, B. (2017) Effect of different proportion silage Anthoccephalus chinensis substitute silage whole plant corn on growth performance, slaughter performance and meat quality of Lezhi Black goat in fattening period. Feed Industry 21: 37–44.
Wang, Y., Wang, X.K., Zhou, W., Yang, F.Y., Zhang, Q., and Chen, X.Y. (2018) Effects of moisture content and additive on silage quality of Neolamarckia cadamba leaves. Journal of South China Agricultural University 39: 80–86.
White, D.C., Sutton, S.D., and Ringelberg, D.B. (1996) The genus Sphingomonas: physiology and ecology. Curr Opin Biotech 7: 301–306.
Xie, X., Yang, C.L., Guan, L.L., Wang, J.K., Xue, M.Y., and Liu, J.X. (2018) Persistence of cellulosolytic bacteria Fibrobacter and Treponema after short-term corn stover-based dietary intervention reveals the potential to improve rumen fibrolytic function. Front Microbiol 9: 1363.
Yang, L.L., Yuan, X.J., Li, J.F., Dong, Z.H., and Shao, T. (2019) Dynamics of microbial community and fermentation quality during ensiling of sterile and nonsterile alfalfa with or without Lactobacillus plantarum inoculants. Bioresource Technol 275: 280–287.
Zhang, Q., Yu, Z., Yang, H., and Na, R.S. (2016) The effects of stage of growth and additives with or without cellulase on fermentation and in vitro degradation characteristics of Leymus chinensis silage. Grass Forage Sci 71: 595–606.
Zhang, Q., Zhao, M.M., Wang, X.G., Yu, Z., and Na, R.S. (2017) Ensiling alfalfa with whole crop corn improves the silage quality and in vitro digestibility of the silage mixtures. Grassl Sci 63: 211–217.
Zhang, Q., Yu, Z., Wang, X.G., and Tian, J.P. (2018) Effects of inoculants and environmental temperature on fermentation quality and bacterial diversity of alfalfa silage. Anim Sci J 89: 1085–1092.
Zhao, X.H., Ouyang, K.X., Gan, S.M., Zeng, W., Song, L.L., Zhao, S., et al. (2014) Biochemical and molecular changes associated with heteroxylan biosynthesis in Neolamarckia cadamba (Rubiaceae) during xylogenesis. Front Plant Sci 5: 602.