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Effect of additives on fermentation characteristics, nutrient values and *in vitro* fermentation profile of *Neolamarckia cadamba* leaf silage

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Abstract

This study was designed to investigate the effects of additives on the ensiling characteristics of *Neolamarckia cadamba* leaf (NCL) silage. The NCL were allocated to six ensiling treatments, namely control (C-S), uncompacted (UC-S) (negative control), with the addition of sodium sulphite (S-S), with propionate (P-S), with citric acid plus sodium acetate (CA-S), and with glucose (G-S). Fermentation parameters, chemical compositions and *in vitro* fermentation profile of the silages were investigated after 60 days' storage. Compared with the control, the application of additives improved dry matter recovery (DMR), while UC-S had a poorer DMR. All the silages increased in pH value and acetate content, except for P-S. Lactate concentration was higher in S-S and G-S, and lower in UC-S, P-S, and CA-S. P-S, CA-S, and G-S had increased water soluble carbohydrate (WSC) concentrations. Soluble crude protein was higher in UC-S and CA-S. All the treated silage had greater gas production at slower rates, except for S-S. The fermentation of S-S, P-S and CA-S resulted in higher content of short-chain fatty acids and microbial crude protein relative to UC-S. S-S (38.82%) and CA-S (37.89%) had higher organic matter digestibility relative to the control (36.43%). S-S (42.80%) and P-S (43.99%) had higher crude protein digestibility, which was lower in UC-S (30.73%) when compared with the control (37.74%). There was no difference in the predicted available energy of these silages. The low ammonium nitrogen ratio and the high proportion of true protein inferred that protein was well preserved in these silages, even the uncompacted silage. These results suggest that sulphite and organic acids, such as propionate and citrate, could be an option to improve the feed quality of NCL silage, and its great protein conservation deserves more attention.

Keywords: feed quality, *in vitro* technique, *Neolamarckia cadamba,* ruminant nutrition, silage processing
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Introduction

Neolamarckia cadamba, an evergreen tropical tree that is native to South and South East Asia, has been exploited as woody forage in the past few years, because of its rapid growth, biomass yield and high protein content, and abundant components such as plant polyphenols (He *et al*., 2018). A previous study showed that harvesting *N. cadamba* at intervals of two months for silage processing would obtain the largest biomass yield over the year (Zhang, 2018). Moreover, *N. cadamba* could be high-quality forage that is comparable with the traditional *Leucaena leucocephala* and *Medicago sativa* for ruminant animals (Zayed *et al.*, 2014). Partially replacing corn silage with *N. cadamba* silage in a fattening diet could improve the growth performance and meat quality of Lezhi black goats (Wang *et al*., 2017). Making full use of this unconventional feed resource could be an important measure to confront the challenge of feed supply.

Ensiling seems to be the most practical way of keeping forage supplied all year round. It is based on lactic acid bacteria (LAB), which convert WSC anaerobically into organic acids to decrease the pH and inhibit the activity of spoilage organisms (McDonald *et al.*, 1991). However, the water soluble carbohydrate (WSC) content of *N. cadamba* leaf (NCL) is typically lower than 5% (He *et al.*, 2018; Wang *et al.*, 2018), which is thought to be insufficient to support the pH decrease (Ni *et al.*, 2017). Generally, there are several potential

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strategies to improve the ensiling process, that is by increasing the LAB number by inoculating, lowering pH by acid addition, clearing oxygen residue by antioxidant application, and supplying a fermentable substrate such as sugar (Kung *et al*., 2003). An LAB inoculant that is prepared by incubating the extract anaerobically is usually applied to improve silage quality because of a lack of epiphytic LAB, but the inoculant seems to show inconsistent effects on different materials (Ellis *et al.*, 2016). However, the authors' previous research showed that the LAB number of inoculated NCL silage was even lower than the control silage, and improved effects were rarely found in the silage fermentation (He *et al.*, 2018). Therefore, to exploit suitable additives for NCL silage, several additives were selected in this study rather than LAB inoculant.

It was commonly supposed that chemical additives would improve the ensiling process of NCL silage, but few studies have been reported in the literature. Therefore, this study was conducted to investigate the effects of silage additives, namely sodium sulphite, propionic acid, citrate plus sodium acetate and glucose, on the fermentation characteristics, carbohydrate constituents, protein fractions, *in vitro* gas production and digestibility of NCL silage.

Materials and Methods

The ensiling procedures of this study were in accordance with He *et al.* (2018). In brief, NCL were collected manually from Yuejin North Experimental Plot of South China Agricultural University, and then chopped into a particle length of 2 - 3 cm with a hand hay cutter. The prepared NCL was ensiled with the following treatments in six replicates: control silage (C-S), uncompacted silage (UC-S) (negative control), silage with the addition of sodium sulphite (S-S), silage with propionic acid (P-S), silage with citric acid plus sodium acetate (1:1, w/w) (CA-S) and silage with D-glucose (G-S). All the chemical agents (analytical reagents) were purchased from Guangzhou Chengshuo Reagent Company (Guangzhou, China), and their inclusion levels in the silages were 1% (w/w) of fresh matter. The additives were dissolved in 10 mL distilled water and sprayed with a mini sprayer onto 1.0 kg leaves, while the control was sprayed with 10 mL distilled water. All the pre-mixed forage was compacted in laboratory polyethylene bags (20 cm by 30 cm) and sealed with a food vacuum sealing machine, except that the UC-S was sealed without extraction of air. Following 60 days' fermentation at room temperature (around 28 °C), each bag was unsealed and sampled twice, so that one part was used to determine silage fermentation, and the other was oven-dried at 65 °C for 48 hours and ground in a hammer mill to pass a 1-mm sieve for chemical analysis and *in vitro* culture.

The extract fluid was prepared by stirring fresh silage (25 g) in distilled water (225 mL) with a domestic fruit juicer for three minutes, and then filtered with four layers of cheesecloth to attain the supernatant. With the fluid, pH value was measured using a pH meter (PHS-3C, INESA INSTRUMENT, Shanghai, China). Organic acids, including lactic acid, acetic acid, propionic acid and butyric acid, were analysed by high performance liquid chromatography (HPLC), which was equipped and set as follows: Shodex RS Pak KC-811 column (Showa Denko K.K., Kawasaki, Japan), SPD-20A detector (Shimadzu Co., Ltd, Kyoto, Japan); eluent 3 mmol/L $HClO_4$ with a running rate of 1.0 mL/min, temperature of column oven 50 °C. Ammonium-nitrogen (NH_3-N) was measured according to the method of Broderick & Kang (1980). Dry matter recovery (DMR) was measured as the difference in dry matter (DM) content before and after 60 days' silage fermentation.

Samples were analysed in duplicate for DM, crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and ash according to AOAC (2000) procedures. Specifically, CP was analysed by an automatic Kjeldahl apparatus (K-9860, Hanon, Jinan, China). NDF, ADF and ADL were analysed using an A220 fibre analyser (ANKOM Technology Corp., Macedon, NY, USA) without sodium sulphite and heat stable amylase, and the results were expressed inclusive of ash. Ash was measured by combustion in a muffle furnace at 550 °C for four hours following calcination. Protein fractions, including non-protein nitrogen (NPN), true protein (TP) and soluble crude protein (SCP), were analysed according to the method of Licitra *et al.* (1996). WSC was measured with anthrone-sulphuric colorimetry according to the instructions of the test kit (Comin Biotechnology Ltd, Suzhou, China). Total phenols and hydrolysable tannins were determined with Folin-Ciocalteu colorimetry (Min *et al*. (2015). Condensed tannins were measured according to Coblentz & Grabber (2013).

In vitro incubation was carried out with an ANKOM RFS gas production meter (ANKOM Technology Corp., Macedon, NY, USA) according to the instrument's specifications. Before the morning feeding, rumen fluid was collected from three Holstein cows (approximately 500 kg bodyweight) that were fitted with permanent rumen cannulae and fed twice a day a total mixed ration consisted of 50% corn silage and 50% commercial concentrate, and then strained through four layers of cheesecloth into a vacuum bottle and transported to the laboratory within 20 min. The rumen fluid was mixed with the buffer solution in a 1:2 (v/v) proportion under a continuous flow of CO_2. The buffer solution and incubation fluid were prepared according to Menke & Steingass (1988). Each sample was weighed (1.0 g) in triplicate into 250 mL glass bottles in advance. All the bottles (with three blank controls), which were injected with 150 mL incubation fluid, were

incubated at 39 °C for 48 hours, and gas production (GP) (mL) was recorded automatically at 0.5-hour intervals. At the end, the fermentation fluid was measured for pH value (PHS-3C, INESA Scientific Instrument Co, Shanghai, China) and then centrifuged to obtain the supernatant that was designated for NH_3-N analysis. The residue was collected and dried at 65 °C for 48 hours to calculate *in vitro* dry matter digestibility (DMD). The residue of the same sample was mixed to determine the digestibility of NDF and ADF (NDFD and ADFD).

Enzyme degradation of crude protein (CPD) was conducted with cellulase (400 U/mg, *Trichoderma viride*) and pepsin (3000 U/g, porcine mucosa) that were purchased from Beizhuo Biotech Company (Shanghai, China), according to the method of Faithfull (2002). First, Erlenmeyer flasks (150 mL) containing 0.5 g feed sample and 40 mL cellulase citrate-phosphate buffer (6 g/L, pH = 4.6) were incubated in the shaker setting at 150 rpm/min and 39 °C for 24 hours. After that, the enzymes were inactivated in boiling water for 10 min. Subsequently, the flasks were filled with 10 mL pepsin-0.1 M HCl solution (35 g/L) and incubated for another 48 hours. Finally, the residue was collected by filtering and analysed for nitrogen content.

To estimate the kinetic parameters of gas production, gas production records were fitted using the nonlinear option of SAS (version 9.0), with the model described by France *et al.* (2000):

$$GP_t = b \times (1-e^{-ct})$$

where: GP_t (mL/g DM) is the volume of GP at time t
b (mL/g DM) is the asymptotic GP
c (/h) is the rate of gas production

The partitioning factor at 48 hours of incubation (PF_{48}), a measure of fermentation efficiency, was calculated as the ratio of DMD (mg/g) to the volume (mL/g) of GP at 48 hours (i.e., DMD/GP_{48}) according to Blümmel *et al.* (1997). Gas yield (GY_{24}) was calculated as the volume of gas (mL/g DM) produced after 24 hours of incubation divided by the amount of DMD (g) (GP_{24} /DMD). Short-chain fatty acid (SCFA) concentration was calculated according to Getachew *et al.* (2002) as:

$$SCFA\ (mmol/0.2\ g\ DM) = 0.0222\ GP_{24}\ (mL/0.2\ g\ DM) - 0.00425$$

Microbial crude protein (MCP) biomass production was calculated according to Blümmel *et al.* (1997) as:

$$MCP\ (mg/g\ DM) = DMD\ (mg) - GP\ (mL) \times 2.2\ mg/mL$$

where: 2.2 mg/mL is a stoichiometric factor, which expresses mg of C, H and O required for the production of SCFA gas associated with the production of 1 mL of gas.

Organic matter digestibility (OMD) and metabolizable energy (ME) were calculated according to Menke & Steingass (1988), and the net energy for maintenance (NE_m) and for gain (NE_g) were calculated according to NRC (2000):

$$OMD\ (g/kg) = 145.1 + 8.490GP_{24}\ (mL/0.2\ g\ DM) + 0.653CP\ (g/kg\ DM) + 0.686Ash\ (g/kg\ DM)$$

$$ME\ (MJ/kg) = 2.20 + 0.1357GP_{24}\ (mL/0.2\ g\ DM) + 0.0057CP\ (g/kg) + 0.0002859CP^2\ (g/kg)$$

$$NE_m\ (MJ/kg) = [1.37ME\ (Mcal/kg) - 0.138ME^2\ (Mcal/kg) + 0.0105ME^3\ (Mcal/kg) - 1.12] \times 4.184$$

$$NE_g\ (MJ/kg) = [1.42ME\ (Mcal/kg) - 0.174ME^2\ (Mcal/kg) + 0.0122ME^3\ (Mcal/kg) - 1.65] \times 4.184$$

All data were subjected to the GLM procedure of SAS (version 9.0, SAS Institute Inc., Cary, NC, USA), considering additive treatments as fixed factors in the linear model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where: Y_{ij} is every observation of silage treatment i (T_i)
μ is the general mean; T_i is the effect of treatment i
e_{ij} is experimental random residual error

Duncan's test was used to do multiple comparisons with differences being declared significant at $P < 0.05$.

Results and Discussion

The chemical composition of raw NCL is presented in Table 1. The results showed that NCL contained about 14% CP and 30% NDF, in which the protein level was high enough for beef cattle and the fibre content fell into the dietary range (NRC, 2000), thus promoting its high inclusion in the diet. These data were in line with the results of Wang *et al*. (2018) and Zhang (2018). WSC is critical for silage fermentation, acting as the fermentation substrate, in which 5% is thought to be the threshold for acceptable fermentation (Ni *et al.*, 2017), inferring that ensiling NCL (4.42% WSC) without the application of additives may not work well. Moreover, NCL contains high contents of phenols and tannins, which might affect palatability and feed utilization because of their protein binding and precipitating properties (Estell, 2010; Huang *et al.*, 2016). It has also been reported that forage conserved as silage greatly diminished the extractability of protein-protecting polyphenols (Terrill *et al*., 1990; Minnee *et al*., 2002), most of which were transformed into compounds during ensiling (unpublished data), suggesting that NCL silage may be superior in palatability relative to its raw material.

Table 1 Chemical composition of *Neolamarckia cadamba* leaves used for silage

Item	***Neolamarckia cadamba* leaves**
Dry matter (%)	32.66
Crude protein (% DM)	13.97
Neutral detergent fibre (% DM)	30.43
Acid detergent fibre (% DM)	22.59
Acid detergent lignin (% DM)	9.26
Ash (% DM)	8.03
Water soluble carbohydrate (% DM)	4.19
Total phenols (% DM)	5.56
Hydrolysable tannins (% DM)	4.42
Condensed tannins (% DM)	6.96

The data that reflect the fermentation quality of NCL ensiled with various additives (C-S, UC-S, S-S, P-S, CA-S and G-S) are summarized in Table 2. There were significant ($P < 0.01$) differences in DMR (88.31% - 94.93%), pH value (4.16 - 4.84), and the contents of lactate (3.03% - 6.19%), acetate (0.48% - 1.84%), and propionate (0.08% - 3.22%) of NCL ensiled with various additives. Dry matter loss occurred inevitably during ensiling owing to the respiration of plant cell and aerobic fermentation in the initial phase (McDonald *et al.*, 1991; Dos Santos *et al.*, 2015), but it could be minimized. Any strategies that could inhibit these activities may benefit nutrient preservation. In the present study, additives improved ($P < 0.05$) DMR, as expected, and the uncompacted treatment resulted in a poorer ($P < 0.05$) DMR. The additives would alter the ensiling process in alternative pathways by promoting pH decrease, inhibiting microbial activity and accelerating the dominance establishment of LAB. The study of Zhao *et al.* (2018), which investigated dynamic variations in pH and DM loss of rice straw silage, showed that additives (hemicellulase and *Lactobacillus plantarum*) resulted in a rapid pH reduction at the early stage of ensiling and significantly decreased DM loss. pH is a simple parameter to evaluate the extent of silage fermentation and silage quality (Wang *et al*., 2009). A lower pH generally inferred further fermentation and better aerobic stability. Compared with the control, all the silages had higher ($P < 0.01$) pH values and acetate contents, except for P-S, in which lactate concentration was higher ($P < 0.01$) in S-S and G-S, and lower ($P < 0.01$) in UC-S, P-S and CA-S. Not compacting resulted in decreased lactate content and thus increased the pH value ($P < 0.01$) because more fermentable substrate was consumed in aerobic activity. In view of the changes of lactate content, the addition of sodium sulphite and glucose promoted the activity of acid-producing bacteria, while the addition of acids inhibited LAB fermentation. The chemical nature of the additives would also make a difference. The addition of sodium sulphite led to a higher pH value (4.84) because of its alkalinity, which might impair the aerobic stability of the silage. It is reported that moulds are usually inhibited at pH < 4.5 (Kung *et al.*, 2003). Adding acetate and

propionate led to a greatly increased concentration of the corresponding acids, but did not decrease the pH of the silages, which may be attributed partly to the differences in their acidity and buffering capacity. Similarly, Li *et al.* (2016) showed that citrate inhibited the production of lactic acid and acetic acid of alfalfa silage, resulting in a relatively higher final pH value. Additionally, all these silages had low NH_3-N proportions, with butyric acid being undetected, indicating good preservation of protein. Given that the silage pH values did not seem low enough to achieve such high true protein recovery, which was not expected for high-protein silage, it is speculated that this is correlated with their high tannin content and other functional components. The naturally occurring condensed tannins were verified for reducing proteolysis of alfalfa during conservation (Grabber & Coblentz, 2009; Grabber *et al.*, 2011).

Table 2 Effect of additives on the dry matter recovery and fermentation parameters of *Neolamarckia cadamba* leaf silage

Item	Treatment						SEM	*P*-value
	C-S	UC-S	S-S	P-S	CA-S	G-S		
Dry matter recovery (%)	88.6^{e}	88.3^{f}	92.4^{b}	89.2^{d}	94.9^{a}	90.9^{c}	0.08	<0.01
Fermentation parameters (on a DM basis)								
pH	4.22^{c}	4.36^{b}	4.84^{a}	4.16^{c}	4.31^{b}	4.31^{b}	0.03	<0.01
NH_3-N/TN (%)	0.65	0.77	0.67	0.54	0.65	0.73	0.06	0.11
Lactate (%)	5.30^{b}	4.06^{c}	6.12^{a}	4.47^{c}	3.03^{d}	6.19^{a}	0.16	<0.01
Acetate (%)	0.48^{c}	0.71^{b}	0.74^{b}	0.49^{c}	1.84^{a}	0.80^{b}	0.04	<0.01
Propionate (%)	0.13^{bc}	0.14^{bc}	0.11^{bc}	3.22^{a}	0.08^{c}	0.18^{b}	0.02	<0.01
Butyrate (%)	ND	ND	ND	ND	ND	ND	-	-

C-S: compacted silage (control); UC-S: uncompacted silage (negative control); S-S: silage with sodium sulphite (1% of fresh matter); P-S: silage with propionate acid (1% of fresh matter); CA-S: silage with citrate plus sodium acetate (1:1) (1% of fresh matter); G-S: silage with D-glucose (1% of fresh matter); SEM: standard error of means.
pH: pH value of silage extract fluid; NH_3-N/TN: ratio of ammonium nitrogen to total nitrogen; ND: undetected; -: default.
[a-f] Means followed by different superscripts in the same row are significantly different (P <0.05)

The nutrient values, including DM, ash, carbohydrate constituents and protein fractions, of various NCL silages are shown in Table 3. In the present study, uncompacted silage showed a similar chemical profile to the control, mainly because the residual air was not sufficient to make a difference, suggesting that there should be an optimum compacting density for practical silage production. CA-S increased (P <0.05) in the contents of DM and ash, and S-S presented a higher (P <0.01) ash content, which should be attributed to the addition of sodium sulphite and sodium acetate. P-S, CA-S and G-S had increased (P <0.01) WSC concentrations. that is, the application of propionate, citrate plus sodium acetate and glucose resulted in higher concentrations of WSC. WSC in the silage generally derived from the residual WSC of feedstuff and the outcome of polysaccharide hydrolysis. The higher WSC content in the silages with the addition of acid should be attributed to their fermentation inhibition and the acidolysis of cellulose and hemicellulose (McDonald *et al.*, 1991; Zhang *et al*., 2016). Owing to the effect of plant protease and microbial activity during ensiling, part of the true protein is degraded to non-protein nitrogen (NPN), including mainly peptides, free amino acids, and NH_3-N (McDonald *et al*., 1991). To evaluate the extent of protein conservation, there is a need to analyse their protein fractions, which would determine the bioavailability of CP (Licitra *et al.*, 1996). Protein solubility is an important indicator for protein feed as it largely determines the ratio of rumen by-pass protein (Hvelplund & Madsen, 1990). In the present study, all the silages had relative low protein solubility in the borate-phosphate buffer, where UC-S and CA-S had increased (P <0.05) soluble crude protein, inferring that uncompacted silage suffered more severe proteolysis, given its high NPN concentration, and the addition of citrate caused more protein acidolysis. The low protein solubility may be because of the high tannin content (condensed tannin>7%), because tannin extracts are able to bind with proteins to form complexes and prevent protein hydrolysis in silage and in the rumen (Piluzza *et al*., 2014; Herremans *et al*., 2019). It was reported that naturally occurring condensed tannins contributed to the reduced proteolysis of alfalfa during conservation (Grabber & Coblentz, 2009). NPN often accounts for more than half of the total N in alfalfa silage and can result in inefficient N utilization by ruminants (Li *et al.*, 2016). The present results

inferred that the protein was well preserved in the silages in view of the low NH_3-N concentration and the high ratio of true protein, which should be taken advantage of in practical production to modify dietary by-pass protein, improving the efficiency of nitrogen utilization in ruminants and decreasing nitrogen excretion.

Table 3 Effect of additives on the chemical composition of *Neolamarckia cadamba* leaf silages

Item	Treatment						SEM	*P*-value
	C-S	UC-S	S-S	P-S	CA-S	G-S		
Dry matter	30.94^{b}	31.02^{b}	32.70^{ab}	31.87^{b}	33.92^{a}	32.38^{ab}	0.57	0.02
Ash	8.30^{cd}	8.65^{bcd}	11.06^{a}	8.16^{d}	9.05^{b}	8.83^{bc}	0.20	<0.01
Carbohydrate fractions (%, on a DM basis)								
Neutral detergent fibre	30.68	32.41	27.94	28.36	29.31	28.27	1.40	0.13
Acid detergent fibre	22.38	24.17	20.57	21.42	22.79	20.91	0.81	0.07
Acid detergent lignin	9.32	9.73	8.27	8.93	9.66	9.45	0.49	0.19
Water soluble carbohydrate	3.01^{de}	2.83^{e}	3.33^{cd}	4.30^{a}	3.91^{ab}	3.70^{bc}	0.13	<0.01
Protein fractions (%, on a DM basis)								
Crude protein	13.60	13.78	13.59	13.65	13.95	14.15	0.19	0.32
Ammonium nitrogen	0.09	0.11	0.09	0.07	0.09	0.10	0.01	0.07
Non-protein nitrogen	2.66	4.69	2.98	3.68	3.76	3.42	0.61	0.36
True protein	10.95	9.10	10.62	9.97	10.19	10.73	0.61	0.40
Soluble crude protein	3.48^{c}	5.52^{a}	4.02^{bc}	3.66^{bc}	4.51^{b}	3.95^{bc}	0.25	0.02

C-S: compacted silage (control); UC-S: uncompacted silage (negative control); S-S: silage with sodium sulfite (1% of fresh matter); P-S: silage with propionate acid (1% of fresh matter); CA-S: silage with citrate plus sodium acetate (1:1) (1% of fresh matter); G-S: silage with D-glucose (1% of fresh matter); SEM: standard error of means
$^{a-e}$ Means followed by different superscripts in the same row are significantly different (*P* <0.05)

Because *in vivo* measurement is time consuming and varies widely, *in vitro* techniques such as *in vitro* rumen culture and enzymatic digestion have been developed as common methods to evaluate the nutritional value of ruminant feeds (Wang, 2016). As one of the most widespread techniques, *in vitro* gas production is a simple way of evaluating feed quality that reflects the extent of feed fermentation and digestibility (He *et al.*, 2015). In the present study, the gas production curves of various NCL silages showed apparent gaps after 30 hours' incubation (Figure 1). Compared with the control, cumulative gas production was higher (*P* <0.01) in S-S and CA-S at 12 hours' fermentation and higher (*P* <0.05) in CA-S at 24 hours. All the silages had higher (*P* < 0.01) gas production (GP_{48} and b) with slower (*P* <0.01) rates of gas production (c), except for S-S (Table 4), suggesting that additive treatments resulted in more fermentable carbohydrates in the silages, which was in line with their increased WSC contents. Thus, SCFA, the primary fermentation product of carbohydrates, was expected to increase, probably contributing to the improved energy supply of the silage. Silage S-S, P-S, and CA-S resulted in higher (*P* <0.05) contents of SCFA and MCP relative to UC-S (Table 4), verifying that uncompacted silage had an inferior supply of energy and protein. It is inferred that poor energy supply would decrease the efficiency of microbial protein synthesis (Fijałkowska *et al.*, 2015). No differences (*P* >0.05) were found in the other fermentation parameters such as pH, NH_3-N, GY_{24} and PF_{48}.

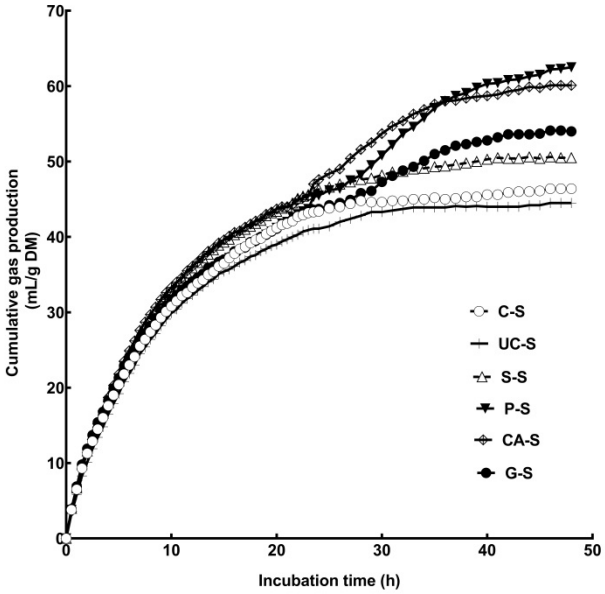

Figure 1 *In vitro* gas production curves of *Neolamarckia cadamba* leaf silages ensiled with additives
C-S: compacted silage (control); UC-S: uncompacted silage (negative control); S-S: silage with sodium sulfite (1% of fresh matter); P-S: silage with propionate acid (1% of fresh matter); CA-S: silage with citrate plus sodium acetate (1:1) (1% of fresh matter); G-S: silage with D-glucose (1% of fresh matter).

Table 4 *In vitro* fermentation profile of *Neolamarckia cadamba* leaf silages ensiled with additives

Item	Treatment						SEM	*P*-value
	C-S	UC-S	S-S	P-S	CA-S	G-S		
Gas production dynamics (mL/g, on a DM basis)								
GP_6	23.00	21.85	24.55	23.25	24.85	24.10	0.64	0.10
GP_{12}	33.30^{b}	32.30^{b}	35.95^{a}	33.75^{b}	36.10^{a}	34.15^{ab}	0.55	0.01
GP_{24}	43.45^{bc}	41.05^{c}	46.30^{ab}	45.50^{ab}	47.65^{a}	43.65^{bc}	0.89	0.02
GP_{48}	46.40^{d}	44.55^{d}	50.55^{c}	62.45^{a}	60.10^{a}	54.00^{b}	0.92	<0.01
b	46.43^{c}	44.56^{c}	50.58^{b}	63.69^{a}	60.21^{a}	54.41^{b}	1.17	<0.01
c (/h)	0.112^{a}	0.110^{a}	0.106^{a}	0.060^{d}	0.074^{c}	0.087^{b}	0.003	<0.01
Fermentation parameters								
pH	6.46	6.48	6.48	6.44	6.47	6.50	0.01	0.42
NH_3-N	19.10	21.60	18.58	18.22	19.88	19.15	1.30	0.30
GY_{24}	110.46	120.01	103.58	95.86	109.25	108.58	3.97	0.06
PF_{48}	9.05	8.35	9.70	10.44	9.16	9.23	0.35	0.06
SCFA	0.95^{bc}	0.89^{c}	1.01^{ab}	0.99^{ab}	1.04^{a}	0.95^{bc}	0.02	0.02
MCP	291.02^{abc}	244.86^{c}	337.04^{a}	337.69^{a}	303.91^{ab}	283.60^{bc}	14.56	0.03

C-S: compacted silage (control); UC-S: uncompacted silage (negative control); S-S: silage with sodium sulfite (1% of fresh matter); P-S: silage with propionate acid (1% of fresh matter); CA-S: silage with citrate plus sodium acetate (1:1) (1% of fresh matter); G-S: silage with D-glucose (1% of fresh matter); SEM: standard error of means
DM: dry matter; GP_t: cumulative gas production at incubation time t (h); b: asymptotic gas production; c: rate of gas production; pH: ruminal pH; NH_3-N (mg/100 mL): ammonium nitrogen; GY_{24} (mL gas/g DMD): gas yield at 24 h; PF_{48} (mg DMD/mL gas): partitioning factor; SCFA (mmol/g DM): short chain fatty acids; MCP (mg/g DM): microbial crude protein production
[a-d] Means followed by different superscripts in the same row are significantly different (P <0.05)

Digestibility is one of the most important factors that affect forage intake and depends largely on the chemical composition (Huhtanen *et al*., 2007; He *et al*., 2018). Measurement of *in vitro* DM digestibility has been used widely to assess the nutritional quality of feeds because of its high correlation with *in vivo* digestibility (He *et al*., 2015). Typically, *in vitro* gas production at 24 hours of ruminant feed is highly correlated with its digestibility and available energy content (He *et al*., 2015). In the present study, silage S-S and CA-S had higher (P <0.01) OMD, and CPD was higher in S-S and P-S, and lower in UC-S (P <0.01) (Table 5). Applying additives at ensiling tended to improve the digestibility of the silages, especially the cell wall components, coinciding with the comparison of their gas production. The additives may destroy the complex structure of the fibre matrix and then improve its accessibility. Moreover, the CPD of S-S and P-S increased significantly, where the changes of tannin-protein complex should be to blame, at least in part. Unfortunately tannin content had not been monitored during silage processing in the present study. Tannins could combine with protein or bind digestive enzyme, exerting an intricate effect on protein digestibility (Tabacco *et al.*, 2006). The exact causes need further study, but these findings provide a possible method to use tannin-rich forages. The energy content of feedstuff is highly correlated with the digestibility of DM or OM (Wang, 2016). However, the differences in the predicted available energy (ME, NE_m and NE_g) of the silages were not large enough to be significant (P >0.05). The endpoint of *in vitro* incubation could also bias the comparison because different silages underwent various degradation rates. That is why one does not see consistent results with different evaluation methods.

Table 5 *In vitro* digestibility and available energy of *Neolamarckia cadamba* leaf silages ensiled with additives

Item	Treatment						SEM	*P*-value
	C-S	UC-S	S-S	P-S	CA-S	G-S		
In vitro digestibility (%, on a DM basis)								
Dry matter digestibility	50.42	47.73	55.43	54.84	53.32	50.51	1.72	0.06
Neutral detergent fibre digestibility	31.61	34.01	42.68	47.51	46.15	35.12	4.82	0.08
Acid detergent fibre digestibility	18.85	23.83	25.10	27.76	31.53	18.26	4.58	0.26
Organic matter digestibility	36.43^{c}	36.34^{c}	38.82^{a}	36.64^{c}	37.89^{ab}	37.12^{bc}	0.33	0.01
Crude protein digestibility	37.74^{b}	30.73^{c}	42.80^{a}	43.99^{a}	39.59^{b}	38.81^{b}	0.66	<0.01
Available energy (MJ/kg, on a DM basis)								
Metabolizable energy	9.58	9.46	9.58	9.41	9.72	9.92	0.21	0.61
Net energy for maintenance	5.94	5.83	5.94	5.78	6.06	6.24	0.19	0.61
Net energy for gain	3.49	3.40	3.50	3.36	3.61	3.77	0.18	0.62

C-S: compacted silage (control); UC-S: uncompacted silage (negative control); S-S: silage with sodium sulfite (1% of fresh matter); P-S: silage with propionate acid (1% of fresh matter); CA-S: silage with citrate plus sodium acetate (1:1) (1% of fresh matter); G-S: silage with D-glucose (1% of fresh matter); SEM: standard error of means.
[a-c] Means followed by different superscripts (a, b and c) in the same row are significantly different (P <0.05)

Conclusions

The results of the present study showed that applying sodium sulphite or glucose to *N. cadamba* leaf silage at ensiling promoted lactate production, while the additions of propionate and citric acid plus sodium acetate decreased lactate concentration. The application of additives resulted in higher WSC content, and higher *in vitro* gas production with improved digestibility. The low NH_3-N ratio and the high proportion of true protein inferred that the protein was well preserved in these silages, even the uncompacted silage. These findings suggest that sulphite and organic acids such as propionate and citrate acid could be an option to improve the feed quality of *N. cadamba* leaf silage, which is high in true protein proportion.

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Authors' Contributions

LH and XC conceived and designed the experiments. LH, CW and WZ conducted the field trial, and collected and analysed the samples. LH wrote the paper. QZ and LH discussed and reviewed the paper.

Conflict of Interest Declaration

The authors have no conflict of interest to declare.

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