Interpretive Summary

Short title: Tropical silage fermentation

The first author's last name: Khota

Summary: The natural lactic acid bacteria (LAB) population and silage fermentation of tropical grasses were studied. *Lactobacillus plantarum* and *L. casei* are the dominant species on tropical grasses; they could grow at lower pH and promote better lactic acid production than other isolates. When natural LAB is present but commercial inoculant is unavailable to improve silage fermentation, cellulase could improve tropical silage quality; inhibiting protein degradation and promoting fiber degradation.

Running head: Silage Fermentation of Tropical Grass

Natural Lactic Acid Bacteria Population of Tropical Grasses and their Fermentation Factor Analysis of Silage Prepared with Cellulase and Inoculant

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ABSTRACT

Natural lactic acid bacteria (LAB) populations in tropical grasses and their fermentation characteristics on silage prepared with cellulase enzyme and LAB inoculants were studied. A commercial inoculant *Lactobacillus plantarum* Chikuso 1 (CH), a local selected strain *Lactobacillus casei* TH14 (TH14), two cellulases, *Acremonium* cellulase (AC) and Maicelase (MC) were used as additives to silage preparation with fresh and wilted (6 h) Guinea grass and Napier grass. Silage was prepared using a laboratory-scale fermentation system. Treatments were CH, TH14, AC 0.01% fresh matter (FM), AC 0.1%, MC 0.01%, MC 0.1%, CH+AC 0.01%, CH+AC 0.1%, CH+MC 0.01%, CH+MC 0.1%, TH14+AC 0.1%, TH14+AC 0.01%, TH14+MC 0.1% and TH14+MC 0.01%. Microorganism counts of Guinea grass and Napier Grass prior to ensiling were 10^2 LAB and 10^6 aerobic bacteria; these increased during wilting. Based on morphological and biochemical characteristics, and 16S rRNA gene sequence analysis, natural strains from both grasses were identified as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidipiscis*, *Leuconostoc pseudomensentroides, Leuconostoc garlicum*, *Weissela confusa* and *Lactococcus lactis*. *L. plantarum* and *L. casei* are the dominant species, and could grow at lower pH and produce more lactic acid than other isolates. Crude protein (CP) and neutral detergent fiber (NDF) were 5.8% and 83.7% Dry matter (DM) for Guinea grass, and 7.5% and 77.1% DM for Napier grass. Guinea grass had a low level of water-soluble carbohydrate (WSC; 0.39% DM). Guinea grass silage treated with cellulase had a lower pH and higher lactic acid content than control and LAB treatments. 0.1% AC and MC treatments had the best result for fermentation quality. All high WSC (2.38% DM) Napier grass silages showed good fermentation quality. Compared to control and LAB-inoculated silage, the cellulase-treated silages had significantly higher CP content and lower NDF and acid detergent fiber contents.

The results confirmed that cellulase could improve tropical silage quality; inhibiting protein degradation and promoting fiber degradation.

Keywords: Cellulase, Fermentation factor, Lactic acid bacteria, Tropical silage

INTRODUCTION

The major constraint for dairying in the tropics is shortage of feed in terms of quality and quantity, especially in the dry season. The main feed sources for dairy cows are native grasses, and by-products from agriculture. Dairy cows fed on low quality roughage give low milk production. In order to establish a forage production system to cover the shortage of animal feed in the dry season, technologies using many grass varieties have been developed. These include the testing and cultivation of forages, studying their adaptability to various conditions and their nutritive value and productivity (Phaikaew et al., 2001). Purple guinea grass (*Panicum maximum* cv. TD 58) and Napier grass (*Pennisetum purpureum* cv. Pak Chong 1) are widely used for ruminant feed in the tropics, including Thailand. They can both grow well in the rainy season, are high in dry matter yield and also drought tolerant (Tudsri et al., 2002; Hare et al., 2009). They need to be conserved to supply feed for ruminants during the dry season.

Silage preparation and storage is one of the most effective techniques for animal feed supply in the dry season in the tropics. High quality tropical silages are difficult to create because of low LAB and water-soluble carbohydrate (WSC) contents in the forage (Pholsen et al., 2016). In this experiment, lactic acid bacteria (LAB) inoculants and cellulase enzyme were selected as microbial additives to improve silage quality. Cellulase improves fiber degradation, increasing WSC as a substrate for LAB to produce lactic acid (Cai et al., 1999).

The moisture content of the grass also directly affects bacterial activity during the fermentation phase. The activity of silage microorganisms slows as grass dry matter (DM) content increases and as silage pH decreases. Microorganism activity stops at a higher pH as grass DM content

increases (McDonald et al., 1991). Usually, tropical grass has a high moisture content (>80%) which causes butyric acid fermentation leading to unsuccessful ensiling (Pholsen et al., 2016). Grass wilting could inhibit undesirable microorganisms and reduce nutrient loss. However, the characteristics of LAB and cellulase, and their true function in silage making under different moisture conditions need further study.

In the present study, the natural lactic acid bacteria populations and fermentation quality of tropical grasses were examined. In order to analyze the fermentation factors, the fresh and wilted silages were also prepared with additives, with particular reference to cellulase enzyme and LAB inoculants; these are considered to be most important in silage fermentation quality improvement.

MATERIALS AND METHODS

Ensiling Materials and Silage Preparation

Purple guinea (*Panicum maximum* cv. TD 58) and Napier (*Pennisetum purpureum* x *Pennisetum americanum* cv. Pak Chong 1) grasses were grown in May, 2013 at the experimental farm, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand, in an area of 800 and 1,600 m^2 on Korat soil series (Oxic Paleustults), respectively. The plot of purple Guinea grass was ploughed twice and harrowed once. 17,778 populations of root stock of purple Guinea grass were planted into rows by hand at distances between and within rows of 75×75 cm, respectively. Cattle manure was applied at a rate of 24,000 kg/ha (4 equal portions of 6,000 kg/ha were split applied for 4 cuts) for high dry matter yield of organic grass (Yoottasanong et al., 2015). The plot of Napier grass was ploughed and harrowed once. 11,111 populations of stem cuttings of the Napier grass were planted into rows by hand at distances between and within rows of 120×75 cm, respectively. For high dry matter yield, basal dressing fertilizers of NPK (15-15-15) and cattle manure were applied at 300

and 12,500 kg/ha, and nitrogen fertilizer (urea) at a rate of 60 kg/ha was split applied (Kiyothong, 2014). On 10 April, 2014, both grasses were cut to adjust the height to 10 cm above ground level. The recommended rate of cattle manure was applied to purple Guinea and urea was applied to Napier grass. Both grasses were harvested at 60 days of regrowth on 10 June 2014. In order to study the relationship between moisture adjustment and silage fermentation, 50% of each grass was cut and chopped to 1 cm length (Supachai chopper, Kanchanaburi, Thailand) in the early morning and then wilted for 6 h in the shade. Another 50% was cut and chopped for fresh silage preparation.

A local selected lactic acid bacteria (LAB) *Lactobacillus casei* strain TH14 (Pholsen et al., 2016), a commercial inoculant strain Chikuso 1 (CH, *L. plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan) and two commercial cellulase enzymes (AC, *Acremonium* cellulase; MC, Maicelase, Meiji Seika Pharma Co., Ltd, Tokyo, Japan) were used as silage additives. The production strain, main composition and carboxymethyl-cellulase activity of cellulase used in this study are shown in Table 1. Strain TH 14 was isolated from sweet corn (*Zea mays* L.) stover silage. This strain grows well in a low pH environment and produces high lactic acid content (Pholsen et al., 2016). Lactobacilli de Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, Mich, USA) was inoculated with strains TH14 and CH and incubated overnight. After incubation, the optical density at 620 nm of the suspension was adjusted with sterile 0.85% NaCl solution to 0.42 nm. The LAB inoculum was 1 ml of suspension/kg of fresh matter (FM). The LAB was inoculated at 1.0×10^5 colony forming unit (cfu)/g FM. Both AC and MC cellulase were added at 0.01 and 0.1% FM. Four types of ensiled material (fresh and wilted Guinea grass and fresh and wilted Napier grass) were treated with 15 combinations of additives viz. control (untreated), CH, TH14, AC 0.01% FM, AC 0.1%, MC 0.01%, MC 0.1%, CH+AC 0.01%, CH+AC 0.1%, CH+MC 0.01%, CH+MC 0.1%, TH14+AC 0.1%, TH14+AC 0.01%, TH14+MC 0.1% and TH14+MC 0.01%. The experimental design was a 2×15 factorial

arrangement in a completely randomized design (grasses×additives) with three replications. 1000-g portions of grass, chopped to 20-mm length, were mixed well with additives, packed into a bag silo with laminated nylon and polyethylene (Hiryu KN, Asahikasei, Tokyo, Japan), and sealed using a vacuum sealer (SQ–303, Asahi Kasei Pax Corp., Tokyo, Japan). All silos were stored at room temperature (25 to 37 °C). At day 30 after ensiling, three bags per treatment were opened for evaluation of fermentation end-products, chemical and microorganism compositions.

Microorganism Analysis of Pre-ensiled Grass and Silage

Pre-ensiled grasses and silage samples at 30 days (3 replications) after fermentation were used for microorganism analysis. The microorganism counts were done using the plate count method (Kozaki et al., 1992) and reported as colony forming unit per gram of fresh matter (cfu/g FM). 10 g FM was added to 90 ml sterilized distilled water, shaken well by hand and serial dilutions in 0.85% sodium chloride solution at 10^{-1} to 10^{-5}. Twenty microliters (µl) from each dilution was spread on agar plates. LAB were counted on MRS agar (Difco) after incubation at 30°C for 48 h in an anaerobic box (Sugiyamagen Ltd., Tokyo, Japan). For isolation of LAB, 10 to 20 strains on MRS agar medium were picked randomly from each silage sample, and a total of 172 isolates were collected, of which 107 isolates (21 and 37 isolates from fresh and wilted Guinea grass; 32 and 17 isolates from fresh and wilted Napier grass, respectively) were considered to be LAB, as determined by the Gram-stain appearance, catalase test and lactic acid productivity, their physiological properties including growth at different pH values, gas production from glucose and lactic acid isomer were then determined by the methods of Kozaki et al. (1992). The 16S rRNA gene sequence analysis was determined as described by Cai et al. (1998) and the sequence similarity of 16S rDNA gene of isolates was compared with sequences from the type strains of LAB held in the GenBank.

Coliform bacteria were counted on blue light broth agar (Nissui-seiyaku Ltd., Tokyo, Japan) after incubated at 30°C for 48 h. Aerobic bacteria and bacilli were counted on nutrient agar

(Difco), yeasts and mold were counted on potato dextrose agar (Nissui-seiyaku). The agar plates were incubated at 30°C for 2 to 7 days, although, at day 3 to 7 of incubation some colonies were too large and could not be counted. In this study, mold was counted at day 2 of incubation. Yeasts were distinguished from molds or bacteria by colony appearance and cell morphology observation.

Fermentation Quality of Silage

Silage fermentation end-products were analyzed from cold water extracts as described by Cai (2004). Silage (10 g FM) was added to 90 ml of sterilized distilled water (Cai et al., 1999). The pH was measured using a glass electrode pH meter (FiveGo; Mettler Toledo, Greifensee, Switzerland). Ammonia nitrogen content was determined using a spectrophotometer (UV/VIS Spectrometer, PG Instruments Ltd., London, UK) (Fawcett and Scott, 1960). Lactic acid buffer capacity (LBC) was measured by titrating with 0.1 M HCl to reduce pH from initial pH to pH 3 and then titrated to pH 6 with 0.1 M NaOH as described by McDonald et al. (1991). The organic acid and WSC contents were measured by HPLC methods as described by Cai (2004). For WSC extraction, 1 g of air dry plant material was extracted in 30 ml of 80% ethanol for 4 h at ambient temperature with continuous shaking (MaxQTM 2000, Thermo Fisher Scientific K.K., Yokohama, Japan). Residues were extracted twice with the 80% ethanol solution. Extracts were brought to 100 ml in volumetric flasks with 80% ethanol. Then 5ml of extract was transferred to a 15 ml uncapped tube and heated in a 95^{o}C water bath (BH401/501, Yamato Scientific Co., Ltd., Tokyo, Japan) for 15 min. Then 1 ml of distilled water, 1 ml of 0.3N $Ba(OH)_2$ and 1 ml of 5% $ZnSO_4$.7H2O were added to each sample. After shaking, samples were filtered through Whatman No 5 paper into 100 ml volumetric flasks. Tubes and filter paper were washed several times with distilled water and adjusted to volume. WSC including glucose, sucrose and fructose were determined by HPLC as described by Cai (2004). The analytical conditions were: SC1011

column (8.0 mm × 30 cm; Shoko, Tokyo, Japan); 80°C oven temperature; water mobile phase; and 1.0 mL/min detector (RI-1530; Jasco Corp., Tokyo, Japan).

Pre-ensiled grass and silage samples were dried in a forced air oven at 60^{o}C for 48 h, and ground to pass a 1 mm mesh screen for chemical composition analyses and gross energy (GE) determination. The DM, organic matter (OM), crude protein (CP) and ether extract (EE) were analyzed by the methods 934.01, 942.05, 976.05 and 920.39 of (AOAC, 1990), respectively. The NDF and acid detergent fiber (ADF) were analyzed according to Van Soest et al. (1991). Acid detergent lignin (ADL) was analyzed as described by Faichney and White (1983). GE was determined using an automatic adiabatic bomb calorimeter (AC 500, LECO, Michigan, USA).

Statistical Analysis

Data on the fermentation products and chemical composition of the 30-day silages were analyzed using a completely randomized design with a 2 × 15 [grass types (A) × additive treatment (B)] factorial treatment structure. The ANOVA procedure of SAS version 6.12 (SAS Institute Inc., Cary, NC) was used for the analysis and the statistical model is as follows:

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

where Y_{ijk} = observation; μ = overall mean, α_i = grass types effect (i = fresh and wilted), β_j = additive effect (j = 1 to 15), $\alpha\beta_{ij}$ = grass types × additive effect, and ε_{ijk} = error. The mean values were compared by Duncan's test (Steel and Torrie, 1980).

RESULTS

Population and Characteristics of Natural LAB

Population and characteristics of lactic acid bacteria isolated from Guinea grass and Napier grass and their silages are shown in Table 2. Fresh Guinea and Napier grass prior to ensiling both showed similar LAB counts (10^2 cfu/g FM). After 6 h of wilting, the LAB counts increased (10^4 and 10^5). *L. plantarum*, *L. casei* and *Weissela confusa* were the most frequent isolates from

both grasses; the counts of *L. plantarum* and *L. casei* were higher than other species on the two grasses. One hundred and seven strains of LAB were isolated from Guinea grass and Napier grass. All strains were Gram-positive, catalase-negative, homofermentative or heterofermentative bacteria. The cell forms were rod or cocci and produced L(+), D(-) or DL-lactic acid. Based on the morphological and biochemical characteristics, and 16S rRNA gene sequence analysis, these isolates were identified as *Lactobacillus plantarum* (48.7% of the total isolates), *Lactobacillus casei* (44.1%), *Lactobacillus acidipiscis* (0.9%), *Leuconostoc pseudomensentroides* (1.2%), *Leuconostoc garlicum* (1.1%), *Weissela confuse* (2.9%) and *Lactococcus lactis* (1.2%). In addition, *L. plantarum* and *L. casei* were dominantly present during fermentation process of Guinea grass and Napier grass silages, respectively. Both isolates could grow at a lower pH and produce more lactic acid than the others.

Chemical Composition of Grass Materials

Chemical composition, gross energy (GE), lactate buffer capacity (LBC) and WSC of Guinea grass and Napier grasses before ensiling are shown in Table 3. The DM of fresh Guinea grass and Napier grass were 20.18 and 17.88%, and increased by 7 and 12% during wilting, respectively. The OM, CP, EE, NDF, ADF and ADL were 90.35, 5.64, 1.53, 84.01, 51.71 and 3.42 % of DM, respectively for fresh Guinea grass, and 94.26, 7.69, 2.05, 76.06, 41.20 and 3.13 % of DM, respectively for fresh Napier grass. The chemical composition of both grasses exhibited no big changes during the wilting process. The GE contents of fresh Guinea grass and Napier grass were 4.08 and 4.34 kcal/g DM, respectively and showed a similar content in each wilted grass. The LBC of Guinea grass and Napier grass were 723.49 and 783.00 meq/kg of DM, respectively. The LBC of wilted grasses were lower than the fresh samples.

The ADL ranged from 3.13 to 3.97% and GE from 4.08 to 4.34 kcal/g DM for both fresh and wilted samples of both grasses. Napier grass was higher in OM, CP, EE, LBC and WSC, and lower in NDF and ADF than Guinea grass.

Fermentation Quality of Silages

Dry matter (DM), pH and fermentation products of Guinea and Napier grass silages at 30 days of ensiling are shown in Tables 4 and 5. In fresh and wilted Guinea grass, silages treated with cellulase at AC 0.1% or MC 0.1% had a significantly (P<0.05) lower pH and ammonia nitrogen content, and significantly (P<0.05) higher lactic acid content than those of control and LAB treatments. The grasses (A), additives (B) and their interaction (A×B) significantly (P<0.001) influenced silage pH, contents of lactic acid, propionic acid, butyric acid, and the B and AxB also influenced ammonia nitrogen content and the A influenced (P<0.001) acetic acid content. In addition, the AC 0.01% and MC 0.01% treatments were well preserved with lower (P<0.05) pH and higher (P<0.05) lactic acid content than control silages. The cellulase 0.1% treatments improved (P<0.05) fermentation quality more than cellulase 0.01% treatment.

In fresh and wilted Napier grass, all silages were well preserved with relatively low pH (<4.06), butyric acid (<0.2 g/kg DM) and ammonia nitrogen (<0.92 g/kg DM) and high lactic acid content (>4.21 g/kg DM). The average pH values were 3.69 for fresh Napier grass and 4.07 for wilted, the propionic acid content in all Napier grass silages was below the detectable level (<0.001% of FM). AC 0.1%, MC 0.1% or their combination with LAB treatments also showed higher (P<0.05) fermentation quality than other treatments. The A, B and A×B significantly (P<0.001) influenced silage pH and butyric acid content. A and B also influenced (P<0.001) the ammonia nitrogen content and the A influenced (P<0.001) acetic acid content.

Microorganisms Counts and Chemical Composition of Silages

Microbiological analysis of Guinea grass and Napier grass silages are shown in Table 6 and Table 7. At day 30 of fermentation, the LAB colonies of both silages were the dominant population and their counts ranged from 10^6 to 10^9, aerobic bacteria from 10^3 to 10^5 cfu/g FM, the molds were below the detectable level (10 cfu/g FM) in all silages. The grasses (A), additives (B) and their interaction (A×B) did not influence (P=0.033-0.938) counts of LAB, but

influenced (P<0.001) count of coliform bacteria. Aerobic bacteria and yeast counts were similar in Guinea grass and Napier grass silages. Coliform bacteria (10^4 to 10^7) were found in control, LAB, AC 0.01% and MC 0.01% treatments in fresh and wilted Guinea grass silages. But in the all Napier grass silages, coliform bacteria decreased to below the detectable level (<10^2 cfu/g FM).

Chemical composition and GE of Guinea and Napier grass silages at 30 days of fermentation are shown in Tables 8 and 9. The CP contents of the two wilted grass silages were significantly (P<0.05) higher than the fresh. When Guinea grass silages were treated with AC 0.1%, MC 0.1% or their combination with LAB, the CP contents were significantly (P<0.05) higher and the NDF and ADF contents were significantly lower (P<0.05) than the control. In the Napier grass silages, the CP content of TH14-treatment was significantly (P<0.05) higher, and the NDF content of AC 0.1% or AC 0.1% + LAB-treatments were significantly (P<0.05) lower than the control. The Napier grass silages showed higher OM, CP, EE, NDF, ADL and GE than Guinea grass silages.

Grasses (A), additives (B) and their interaction (A x B) in both silages significantly (P<0.001) influenced CP, ADF, ADL and GE, but did not influence EE (P=0.002-0.585). A and A x B of both silages and B of Guinea grass silages also significantly (P<0.001) influenced OM while B of Napier grass did not (P=0.014). B of both silages, A x B of Guinea grass silages and A of Napier grass silages influenced NDF (P<0.001), but A of Guinea grass silages and A x B of Napier grass silages did not (P=0.005-0.030).

DISCUSSION

Epiphytic LAB naturally presents on forage crops, is responsible for silage fermentation and also influences silage quality (Lin et al., 1992; Cai et al., 1998). Addition of cellulase potentially increases the amount of substrate for LAB and thus may be a practical tool to enhance the

ensiling process (McDonald et al., 1991; Eun and Beauchemin, 2008; Xing et al., 2009). During silage fermentation, LAB converts sugar into lactic acid. As a result, the pH is reduced, and the forage is preserved (Cai et al., 1999). Among epiphytic LAB, Cai et al. (1998) reported that lactic acid-producing cocci e.g. heterofermentative weissela, leuconostocs, and homofermentative pediococci, lactococci, enterococci initiated lactate fermentation during ensiling, creating a suitable anaerobic environment for the development of lactobacilli, although it was shown that they grew vigorously only in the early stage of the ensiling process. When the heterofermentative cocci or lactobacilli dominated the silage fermentation, they did not improve silage quality and may cause some fermentation loss (Cai et al., 1998). In contrast with these cocci, lactic acid-producing rods e.g. lactobacilli play an important role in promoting lactic acid production for a longer time during silage fermentation. However, as shown in Table 2, the low number of LAB ($<10^3$ cfu/g FM) and high numbers of aerobic bacteria (10^5 cfu/g FM) present in both fresh grass materials suggested that the silage fermentation should be controlled by using LAB inoculant or cellulase.

There are several reports of LAB, especially lactobacilli, composing the major microbial population of forage crops and silage. Some lactobacilli isolated from silage have been characterized by phenotypic features and 16S rRNA gene sequences and have been described as novel species e.g. *L. plantarum*, and *L. casei* (Cai et al., 1999, 1998; Ennahar et al., 2003; Pang et al., 2011) where they may contribute to silage fermentation. In the present study, following biochemical and phylogenetic analyses, isolates from tropical silage characterization belonged to the genera *Lactobacillus*, *Weissela*, *Leuconostoc*, *Lacotococcus* and *Enterococcus*. Among seven identified species, *L. plantarum* and *L. casei* were the dominants in isolates from four kinds of silage. To our knowledge, this is also the first report of natural *Leuconostoc garlicum* and *Lactobacillus acidipiscis* on silage.

The chemical compositions of the tropical grasses used in this study were different, especially DM, CP, NDF and WSC contents. Pholsen et al. (2016) found that it is usually difficult to make good quality silage with some tropical grasses because of their high moisture and low WSC content. In the present study, lower LAB numbers and WSC content were presented in Guinea grass compared to Napier grass. Both fresh and wilted Guinea grass silages were poor quality in control and LAB-inoculated treatments because they had low lactic acid contents, high pH and ammonia nitrogen. As shown in Table 4, the Guinea grass has relatively low WSC content (<0.42% of DM), and the LAB could not ferment sufficient sugar to produce lactic acid. In addition, the pH of silage did not decline below 4.0, allowing butyric fermentation and ammonia nitrogen production by clostridia. However, when Guinea grass silages were treated with cellulase, especially at 0.1%, they had the best result for fermentation quality with low pH and high lactic acid content compared to control or LAB treatments.

All Napier grass silages had significantly (P<0.05) lower pH, ammonia nitrogen and significantly (P<0.05) higher lactic acid content compared with Guinea grass silages. The most plausible explanation lies in the physiological properties of natural LAB strains and the chemical composition of Napier grass that contained a relatively high level of WSC (>2.31% of DM). The natural strains *L. casei* and *L. plantarum* were homofermentative types of LAB which grew well under low pH conditions. Both strains have high lactic acid production capacity and could produce more lactic acid than the others (Pholsen et al., 2016). During silage fermentation, these natural LAB could produce beneficial effects by promoting the propagation of LAB and inhibiting the growth of aerobic bacteria, as well as improving silage quality (Cai et al., 1999; Nadeau et al., 2000).

The cellulase-treated silages had significantly higher (P < 0.05) CP content and lower (P < 0.05) NDF and ADF contents compared to control and LAB-inoculated silages. The significantly higher CP contents in the cellulase-treated silages could be attributable to cellulase degradation

of plant fiber increasing sugar for LAB to produce lactic acid. As a result, the pH decreases sharply which inhibits the growth of Clostridium spp. (Nadeau et al., 2000; Tian et al., 2014). Clostridium spp. usually produce ammonia nitrogen from decomposed protein in the silage materials (Xing et al., 2009).

The LAB inoculation had no further beneficial effect on promoting lactic acid fermentation. This could be attributed to the natural strains *L. casei* and *L. plantarum* (which were most frequently isolated from both grasses) having the ability to produce more lactic acid and more WSC than other strains (Pholsen et al., 2016). Therefore, when sufficient LAB is present on grass, there is no need to use LAB as inoculant for silage making. This indicates that in any future experiments it may be necessary to study the relationships between grass condition and fermentation quality. Finally, our results indicate that addition of cellulase may result in beneficial effects by promoting the propagation of LAB and by inhibiting the growth of clostridia, as well as decreasing the NDF content and CP loss. The results confirmed that cellulase could improve tropical silage quality; inhibiting protein degradation and promoting fiber degradation, especially in tropical grasses containing low WSC.

CONCLUSIONS

Natural lactic acid bacteria populations of Guinea and Napier grass and their fermentation factor analysis in silage prepared with cellulase and Inoculant were studied in tropical conditions. The natural strains *L. plantarum* and *L. casei* are the most frequently isolated from both tropical grasses; they could grow at low pH and promote lactic acid production during silage fermentation. Based on the analysis of silage fermentation and chemical composition, we have found that the natural LAB population showed positive relationships with silage fermentation quality. When natural LAB is present on grass in sufficient quantity, commercial inoculant is unlikely to improve silage fermentation. We have also shown that cellulase enzyme can inhibit protein

degradation and promote fiber degradation; thus it has great potential as an additive for tropical silage fermentation.

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Table 1 CMCase activity of cellulase used in this study

	Acremonium cellulase	Meicelase
Production strain	*Acremonium cellulolyticus*	*Tricoderma viride*
Main composition	Glucanase, Pectinase	Xylanase, Glucanase
CMCase activity	7,350 U/g	2,720 U/g

CMCase, carboxymethyl-cellulase.

Table 2 Population and characteristics of lactic acid bacteria isolated from Guinea grass and Napier grass and their silages

	Lactobacillus plantarum	*Lactobacillus casei*	*Lactobacillus acidipiscis*	*Leuconostoc pseudomensentroides*	*Leuconostoc garlicum*	*Weissela confusa*	*Lactococcus lactis*
Representative strain	KK2	KK10	KK16	KK63	KK56	KK87	KK25
LAB count							
Fresh Guinea grass	$1.9x10^2$	$<10^2$	$<10^2$	nd	$<10^2$	$<10^2$	nd
Wilted Guinea grass	$1.1x10^4$	$4.9x10^3$	$4.6x10^2$	nd	$2.5x10^2$	$1.4x10^3$	nd
Fresh Napier grass	<102	$1.1x10^2$	nd	<102	nd	<102	<102
Wilted Napier grass	$6.6x10^4$	$1.8x10^5$	nd	$1.8x10^4$	nd	$1.5x10^4$	$1.2x10^4$
LAB proportion (% of total isolates)							
Grass prior to ensiling							
Fresh Guinea grass	75.2	11.6	2.1	nd	5.0	6.1	nd
Wilted Guinea grass	60.5	27.4	2.6	nd	1.4	8.1	nd
Fresh Napier grass	40.0	50.4	nd	3.4	nd	2.7	3.5
Wilted Napier grass	22.7	62.1	nd	6.0	nd	5.2	4.0
Silage without additives at day 30							
Fresh Guinea grass	80.2	18.7	nd	nd	nd	1.1	nd
Wilted Guinea grass	87.6	10.1	nd	nd	2.3	nd	nd
Fresh Napier grass	8.0	90.2	nd	nd	nd	nd	1.8
Wilted Napier grass	15.3	82.5	2.2	nd	nd	nd	nd
Total (% of total isolates)	48.7	44.1	0.9	1.2	1.1	2.9	1.2
Characteristics							
Shape	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci
Gram stain	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Gas from glucose	-	-	-	+	+	+	-
Lactate production in MRS broth (%)	1.63	1.55	1.03	0.65	0.45	0.52	0.87
Final pH in MRS broth	3.52	3.55	4.30	4.60	4.75	4.80	4.53
Fermentation type	Homo	Homo	Homo	Hetero	Hetero	Hetero	Homo
Optical form of lactate	DL	L(+)	L(+)	D(-)	D(-)	D(-)	L(+)
Growth at pH							
3.0	-	-	-	-	-	-	-
3.5	+	+	-	-	-	-	-
4.0	+	+	w	-	-	-	w
4.5	+	+	+	-	-	-	+
5.0	+	+	+	+	+	+	+
16S rDNA similarity with each type strain (%)[a]	99.8	99.9	99.8	99.7	99.9	99.7	99.9

+, positive; -, negative; w, weakly positive; nd, not detected; [a]The sequence similarity of 16S rDNA gene of isolates were compared with sequences from each type strains of LAB held in the GenBank.

Table 3 Chemical composition, gross energy (GE), lactate buffer capacity (LBC) and WSC of Guinea grass and Napier grasses before ensiling

Items	DM	OM	CP	EE	NDF	ADF	ADL	GE	LBC	Total WSC
	(%)	% DM						(kcal/g)	(meq/kgDM	% DM
Guinea grass										
Fresh	20.18	90.35	5.64	1.53	84.01	51.71	3.42	4.08	723.49	0.42
Wilted	28.69	91.13	6.05	1.55	83.45	47.16	3.82	4.18	571.63	0.35
Napier grass										
Fresh	17.88	94.26	7.69	2.05	76.06	41.20	3.13	4.34	783.00	2.44
Wilted	29.03	93.23	7.37	1.91	78.05	41.65	3.97	4.31	606.27	2.31

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; meq, milliequivalents; WSC, water-soluble carbohydrate including glucose, sucrose and fructose.

Table 4 Dry matter (DM), pH and fermentation products of Guinea grass silages at 30 days of ensiling

Item		DM	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Ammonia-N
		%		g/kg DM				
Fresh	Control	19.65	4.67	0.09	2.38	0.15	1.81	1.94
	CH	19.88	5.01	ND	1.93	0.14	1.21	2.29
	TH14	19.21	4.99	ND	1.84	0.15	1.44	2.45
	AC 0.01%	19.57	4.47	0.61	0.95	0.02	2.21	1.28
	AC 0.1%	19.57	3.70	4.96	0.86	ND	0.01	0.48
	MC 0.01%	18.92	4.72	0.10	1.76	0.14	3.25	1.41
	MC 0.1%	19.58	3.84	5.14	1.07	ND	0.03	0.53
	CH+ AC 0.01%	19.04	3.89	4.87	1.33	ND	0.49	0.77
	CH+ AC 0.1%	18.65	3.61	6.02	0.86	ND	0.01	0.19
	CH+ MC 0.01%	18.19	4.53	0.23	1.42	0.14	3.09	1.15
	CH+ MC 0.1%	18.84	3.77	5.20	1.05	ND	0.02	0.55
	TH14+ AC 0.01%	18.60	3.94	4.12	1.31	ND	0.46	0.90
	TH14+ AC 0.1%	18.83	3.64	6.67	1.34	ND	0.01	0.44
	TH14+ MC 0.01%	17.96	4.59	0.13	1.72	0.11	2.63	1.32
	TH14+ MC 0.1%	19.32	3.85	4.78	1.21	ND	0.04	0.65
Wilted	Control	28.43	5.16	0.23	0.77	0.04	0.06	1.52
	CH	28.37	4.88	0.57	1.33	0.03	0.10	1.30
	TH14	28.45	5.25	0.24	0.90	0.04	0.04	1.86
	AC 0.01%	27.38	4.31	1.51	0.68	0.02	0.13	1.32
	AC 0.1%	27.64	4.09	2.37	0.60	ND	0.13	1.02
	MC 0.01%	28.20	4.69	1.03	0.89	0.03	0.09	1.33
	MC 0.1%	26.95	4.23	2.05	0.69	ND	0.21	1.24
	CH+ AC 0.01%	28.44	4.14	1.40	0.48	ND	0.08	0.98
	CH+ AC 0.1%	26.88	3.80	3.35	0.56	ND	0.01	0.59
	CH+ MC 0.01%	27.79	4.44	0.85	0.51	0.01	0.08	1.33
	CH+ MC 0.1%	27.34	4.02	3.18	0.65	ND	0.05	1.05
	TH14+ AC 0.01%	27.19	4.25	2.23	0.78	0.01	0.26	1.68
	TH14+ AC 0.1%	26.29	4.00	2.92	0.63	ND	0.12	1.11
	TH14+ MC 0.01%	27.61	4.74	1.31	1.08	0.06	0.19	1.86
	TH14+ MC 0.1%	26.45	4.22	1.90	0.49	ND	0.29	1.42
SEM		0.561	0.055	0.727	0.349	0.022	0.340	0.217
Grass means								
	Fresh	19.05^{b}	4.21^{b}	2.86^{a}	1.40^{a}	0.06^{a}	1.11^{a}	1.09^{b}
	Wilted	27.56^{a}	4.41^{a}	1.68^{b}	0.74^{b}	0.02^{b}	0.12^{b}	1.31^{a}
Additive means								
	Control	24.04^{ab}	4.92^{b}	0.16^{d}	1.57^{ab}	0.10^{a}	0.94^{bcd}	1.73^{bc}
	CH	24.12^{a}	4.95^{b}	0.29^{d}	1.63^{a}	0.09^{a}	0.65^{cdef}	1.79^{ab}
	TH14	23.83^{abc}	5.12^{a}	0.12^{d}	1.37^{abc}	0.10^{a}	0.74^{cde}	2.15^{a}
	AC 0.01%	23.47^{abcd}	4.34^{e}	1.06^{d}	0.82^{c}	0.02^{b}	1.17^{abc}	1.30^{de}
	AC 0.1%	23.61^{abcd}	3.90^{g}	3.67^{abc}	0.74^{c}	ND	0.07^{ef}	0.75^{gh}
	MC 0.01%	23.56^{abcd}	4.70^{c}	0.56^{d}	1.32^{abc}	0.09^{a}	1.67^{a}	1.37^{cde}
	MC 0.1%	23.27^{abcd}	4.04^{f}	3.59^{abc}	0.88^{bc}	ND	0.12^{ef}	0.88^{fg}
	CH+ AC 0.01%	23.74^{abc}	4.01^{f}	3.13^{c}	0.91^{bc}	ND	0.28^{ef}	0.87^{fg}
	CH+ AC 0.1%	22.77^{cd}	3.70^{h}	4.69^{ab}	0.71^{c}	ND	0.01^{f}	0.39^{h}
	CH+ MC 0.01%	22.99^{abcd}	4.49^{d}	0.54^{d}	0.97^{abcd}	0.08^{a}	1.59^{a}	1.24^{def}
	CH+ MC 0.1%	23.09^{abcd}	3.89^{g}	4.19^{abc}	0.85^{c}	ND	0.04^{f}	0.80^{gh}
	TH14+ AC 0.01%	22.90^{bcd}	4.10^{f}	3.17^{c}	1.05^{abc}	0.01^{b}	0.36^{def}	1.29^{de}
	TH14+ AC 0.1%	22.56^{d}	3.82^{g}	4.80^{a}	0.99^{abc}	ND	0.07^{ef}	0.77^{gh}
	TH14+ MC 0.01%	22.79^{cd}	4.66^{c}	0.72^{d}	1.40^{abc}	0.08^{a}	1.41^{ab}	1.59^{bcd}
	TH14+ MC 0.1%	22.89^{bcd}	4.04^{f}	3.35^{bc}	0.85^{c}	ND	0.16^{ef}	1.04^{efg}
Significance of main effect and interaction								
	Grasses (A)	<.001	<.001	<.001	<.001	<.001	<.001	0.003
	Additives (B)	0.026	<.001	<.001	0.029	<.001	<.001	<.001
	A x B	0.160	<.001	<.001	0.783	<.001	<.001	<.001

$^{a\ to\ h}$, Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; ND, not detected.

Table 5 Dry matter (DM), pH and fermentation products of Napier grass silages at 30 days of ensiling

Item		DM	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Ammonia-N
		%		g/kg DM				
Fresh	Control	16.03	3.80	5.05	1.11	ND	0.01	0.87
	CH	16.10	3.74	7.06	0.67	ND	ND	0.61
	TH14	16.35	3.80	4.86	1.24	ND	ND	0.85
	AC 0.01%	15.84	3.69	6.57	1.900	ND	0.01	0.84
	AC 0.1%	14.80	3.66	6.62	2.26	ND	ND	0.68
	MC 0.01%	14.29	3.70	8.02	2.50	ND	0.01	0.73
	MC 0.1%	14.80	3.68	5.91	1.76	ND	0.01	0.65
	CH+ AC 0.01%	15.40	3.66	7.55	0.96	ND	0.01	0.60
	CH+ AC 0.1%	14.96	3.63	7.93	1.48	ND	ND	0.46
	CH+ MC 0.01%	14.62	3.65	9.00	1.22	ND	0.01	0.59
	CH+ MC 0.1%	14.17	3.62	8.05	1.27	ND	ND	0.44
	TH14+ AC 0.01%	14.69	3.64	6.72	2.28	ND	ND	0.62
	TH14+ AC 0.1%	14.99	3.68	6.69	2.69	ND	ND	0.59
	TH14+ MC 0.01%	14.28	3.72	6.55	2.01	ND	ND	0.78
	TH14+ MC 0.1%	15.32	3.67	6.78	2.05	ND	0.01	0.69
Wilted	Control	27.21	4.32	3.38	0.76	ND	0.4	0.97
	CH	27.15	4.15	4.60	0.84	ND	0.06	0.91
	TH14	26.77	4.26	3.81	0.97	ND	0.05	0.80
	AC 0.01%	24.42	3.98	5.15	0.95	ND	0.01	0.85
	AC 0.1%	22.86	3.94	6.77	1.43	ND	ND	0.76
	MC 0.01%	26.01	4.10	5.26	0.95	ND	0.01	0.84
	MC 0.1%	25.95	4.02	5.36	0.93	ND	ND	0.64
	CH+ AC 0.01%	26.70	4.02	4.93	0.78	ND	0.01	0.62
	CH+ AC 0.1%	22.92	3.93	6.19	1.05	ND	ND	0.47
	CH+ MC 0.01%	26.93	4.13	5.22	1.01	ND	0.03	0.85
	CH+ MC 0.1%	27.92	4.02	5.66	0.83	ND	ND	0.51
	TH14+ AC 0.01%	26.27	4.02	4.66	0.84	ND	0.03	0.87
	TH14+ AC 0.1%	25.98	3.99	5.87	1.16	ND	ND	0.73
	TH14+ MC 0.01%	24.94	4.12	4.78	1.02	ND	0.11	0.82
	TH14+ MC 0.1%	28.13	4.05	5.58	0.85	ND	ND	0.61
	SEM	0.846	0.032	1.327	0.365	0.000	0.037	0.087
Grass means								
	Fresh	15.11^{b}	3.69^{b}	6.89^{a}	1.69^{a}	ND	0.01^{b}	0.67^{b}
	Wilted	26.01^{a}	4.07^{a}	5.15^{b}	0.96^{b}	ND	0.05^{a}	0.75^{a}
Additive means								
	Control	21.63^{a}	4.06^{a}	4.21^{c}	0.94^{de}	ND	0.20^{a}	0.92^{a}
	CH	21.63^{a}	3.94^{b}	5.83^{abc}	0.76^{e}	ND	0.03^{b}	0.76^{abcde}
	TH14	21.56^{a}	4.03^{a}	4.33^{bc}	1.10^{bcde}	ND	0.02^{b}	0.83^{abc}
	AC 0.01%	20.13^{abc}	3.84^{def}	5.86^{abc}	1.43^{abcde}	ND	0.01^{b}	0.85^{ab}
	AC 0.1%	18.83^{c}	3.80^{ef}	6.70^{abc}	1.84^{ab}	ND	0.01^{b}	0.72^{bcde}
	MC 0.01%	20.15^{abc}	3.90^{bc}	6.64^{abc}	1.72^{abc}	ND	0.01^{b}	0.79^{abcd}
	MC 0.1%	20.38^{abcc}	3.85^{cde}	5.63^{abc}	1.35^{abcde}	ND	0.01^{b}	0.65^{de}
	CH+ AC 0.01%	21.05^{ab}	3.84^{def}	6.24^{abc}	0.87^{de}	ND	0.01^{b}	0.61^{ef}
	CH+ AC 0.1%	18.94^{c}	3.78^{f}	7.06^{ab}	1.27^{abcde}	ND	ND	0.47^{f}
	CH+ MC 0.01%	20.77^{ab}	3.89^{bcd}	7.11^{a}	1.11^{bcde}	ND	0.02^{b}	0.73^{bcde}
	CH+ MC 0.1%	21.045^{ab}	3.82^{ef}	6.86^{abc}	1.05^{cde}	ND	ND	0.48^{f}
	TH14+ AC 0.01%	20.48^{abc}	3.83^{def}	5.69^{abc}	1.56^{abcd}	ND	0.02^{b}	0.75^{bcde}
	TH14+ AC 0.1%	20.48^{abc}	3.83^{def}	6.28^{abc}	1.93^{a}	ND	ND	0.66^{cde}
	TH14+ MC 0.01%	19.62^{bc}	3.92^{b}	5.67^{abc}	1.52^{abcd}	ND	0.06^{b}	0.81^{abcd}
	TH14+ MC 0.1%	21.73^{a}	3.86^{cde}	6.18^{abc}	1.45^{abcde}	ND	0.01^{b}	0.65^{de}
Significance of main effect and interaction								
	Grass (A)	<.001	<.001	<.001	<.001	0.000	<.001	<.001
	Additives (B)	0.001	<.001	0.346	0.007	0.000	<.001	<.001
	A x B	0.007	<.001	0.978	0.151	0.000	<.001	0.347

$^{a\ to\ f}$, Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; ND, not detected.

Table 6 Microbiological analysis of Guinea grass silages at 30 days of fermentation

Item		Microorganism (cfu/g FM)				
		Lactic acid bacteria	Coliform bacteria	Aerobic bacteria	Yeast	Mold
Fresh	Control	$5.1x10^{7}$	$2.4\ x10^{4}$	$8.9x10^{5}$	ND	ND
	CH	$1.2x10^{9}$	$7.2\ x10^{4}$	$1.9x10^{5}$	ND	ND
	TH14	$2.1x10^{8}$	$5.0\ x10^{4}$	$5.7x10^{5}$	ND	ND
	AC 0.01%	$9.5x10^{8}$	ND	$2.2x10^{6}$	ND	ND
	AC 0.1%	$4.9x10^{7}$	ND	$6.2x10^{4}$	$1.4x10^{4}$	ND
	MC 0.01%	$3.0x10^{8}$	ND	$5.7x10^{5}$	ND	ND
	MC 0.1%	$9.4x10^{7}$	$1.0x10^{4}$	$4.4x10^{4}$	$3.5x10^{3}$	ND
	CH+ AC 0.01%	$6.1x10^{7}$	ND	$3.6x10^{4}$	ND	ND
	CH+ AC 0.1%	$7.0x10^{6}$	ND	$1.1x10^{4}$	$2.0x10^{4}$	ND
	CH+ MC 0.01%	$8.3x10^{8}$	ND	$5.5x10^{5}$	$7.2x10^{3}$	ND
	CH+ MC 0.1%	$1.6x10^{7}$	$9.0x10^{4}$	$1.0x10^{4}$	$8.3x10^{3}$	ND
	TH14+ AC 0.01%	$5.6x10^{8}$	ND	$9.2x10^{5}$	ND	ND
	TH14+ AC 0.1%	$2.0x10^{6}$	ND	$2.0x10^{3}$	$1.3x10^{4}$	ND
	TH14+ MC 0.01%	$2.1x10^{8}$	ND	$2.6x10^{5}$	ND	ND
	TH14+ MC 0.1%	$2.9x10^{9}$	ND	$6.5x10^{4}$	$1.5x10^{3}$	ND
Wilted	Control	$2.5x10^{9}$	$2.7x10^{7}$	$8.6x10^{5}$	$3.0x10^{2}$	ND
	CH	$2.7x10^{9}$	$2.6x10^{7}$	$2.9x10^{6}$	$7.3x10^{4}$	ND
	TH14	$3.3x10^{8}$	$9.0x10^{7}$	$9.8x10^{5}$	$6.0x10^{4}$	ND
	AC 0.01%	$1.1x10^{8}$	$4.7x10^{6}$	$1.0x10^{5}$	$7.0x10^{2}$	ND
	AC 0.1%	$9.8x10^{7}$	ND	$4.1x10^{4}$	$5.8x10^{3}$	ND
	MC 0.01%	$3.4x10^{8}$	$1.2x10^{7}$	$9.0x10^{5}$	$3.8x10^{3}$	ND
	MC 0.1%	$1.4x10^{8}$	ND	$6.8x10^{5}$	$3.2x10^{3}$	ND
	CH+ AC 0.01%	$5.2x10^{7}$	$5.0x10^{4}$	$7.7x10^{4}$	$8.0x10^{2}$	ND
	CH+ AC 0.1%	$4.0x10^{6}$	ND	$5.0x10^{3}$	$8.9x10^{3}$	ND
	CH+ MC 0.01%	$4.3x10^{8}$	$7.8x10^{5}$	$2.5x10^{6}$	ND	ND
	CH+ MC 0.1%	$9.0x10^{6}$	$3.0x10^{4}$	$2.3x10^{4}$	$1.0x10^{3}$	ND
	TH14+ AC 0.01%	$8.4x10^{7}$	$2.1x10^{7}$	$8.0x10^{4}$	$3.0x10^{2}$	ND
	TH14+ AC 0.1%	$3.0x10^{6}$	ND	$2.0x10^{3}$	$7.8x10^{4}$	ND
	TH14+ MC 0.01%	$9.0x10^{8}$	$9.1x10^{5}$	$1.1x10^{6}$	$1.0x10^{2}$	ND
	TH14+ MC 0.1%	$6.4x10^{7}$	$5.1x10^{5}$	$6.3x10^{4}$	$2.1x10^{3}$	ND
SEM		9.59	11.73	5.15	2.57	ND
Grass means						
	Fresh	$5.0x10^{8}$	$1.6x10^{4b}$	$4.3x10^{5}$	$4.5x10^{3}$	ND
	Wilted	$5.2x10^{8}$	$1.2x10^{7a}$	$6.9x10^{5}$	$1.5x10^{4}$	ND
Additive means						
	Control	$1.3x10^{9}$	$1.4x10^{7b}$	$5.5x10^{5bcd}$	$1.7x10^{2}$	ND
	CH	$1.9x10^{9}$	$1.3x10^{7b}$	$1.8x10^{6a}$	$3.7x10^{4}$	ND
	TH14	$2.7x10^{8}$	$4.5x10^{7a}$	$7.4x10^{5bcd}$	$3.0x10^{4}$	ND
	AC 0.01%	$5.3x10^{8}$	$2.4x10^{6b}$	$1.2x10^{6abc}$	$3.7x10^{2}$	ND
	AC 0.1%	$7.4x10^{7}$	ND	$5.1x10^{4d}$	$1.0x10^{4}$	ND
	MC 0.01%	$3.2x10^{8}$	$6.2x10^{6}$	$7.4x10^{5bcd}$	$1.9x10^{3}$	ND
	MC 0.1%	$1.2x10^{8}$	ND	$3.7x10^{5cd}$	$3.4x10^{3}$	ND
	CH+ AC 0.01%	$5.7x10^{7}$	$2.0x10^{4b}$	$5.7x10^{4d}$	$3.8x10^{2}$	ND
	CH+ AC 0.1%	$5.3x10^{6}$	ND	$8.0x10^{4d}$	$1.4x10^{4}$	ND
	CH+ MC 0.01%	$6.4x10^{8}$	$3.9x10^{5b}$	$1.5x10^{6ab}$	$3.6x10^{3}$	ND
	CH+ MC 0.1%	$1.3x10^{7}$	$6.0x10^{4b}$	$1.7x10^{4d}$	$4.7x10^{3}$	ND
	TH14+ AC 0.01%	$3.2x10^{8}$	$1.1x10^{7b}$	$5.0x10^{5cd}$	$1.7x10^{2}$	ND
	TH14+ AC 0.1%	$2.7x10^{6}$	ND	$2.0x10^{4d}$	$4.6x10^{4}$	ND
	TH14+ MC 0.01%	$5.6x10^{8}$	$4.6\ x10^{5b}$	$7.1x10^{5bcd}$	$5.0x10^{2}$	ND
	TH14+ MC 0.1%	$1.5x10^{9}$	$2.5\ x10^{5b}$	$6.4x10^{4d}$	$1.8x10^{3}$	ND
Significance of main effect and interaction						
	Grasses (A)	0.938	0.002	0.091	0.166	ND
	Additives (B)	0.384	0.003	<0.001	0.558	ND
	A x B	0.499	0.003	0.004	0.625	ND

[a to d], Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; cfu, colony forming unit; FM, fresh matter; ND, not detected.

Table 7 Microbiological analysis of Napier grass silages at 30 days of fermentation

Item		Microorganism (cfu/g FM)				
		Lactic acid bacteria	Coliform bacteria	Aerobic bacteria	Yeast	Mold
Fresh	Control	$4.6x10^{7}$	ND	$2.6x10^{4}$	$9.3x10^{4}$	ND
	CH	$1.9x10^{7}$	ND	$1.6x10^{4}$	$1.2x10^{4}$	ND
	TH14	$3.8x10^{7}$	ND	$3.0x10^{4}$	$2.5x10^{3}$	ND
	AC 0.01%	$2.0x10^{6}$	ND	$1.6x10^{4}$	$1.0x10^{4}$	ND
	AC 0.1%	$7.0x10^{6}$	ND	$1.5x10^{4}$	$9.6x10^{3}$	ND
	MC 0.01%	$1.7x10^{7}$	ND	$1.5x10^{4}$	$4.7x10^{3}$	ND
	MC 0.1%	$1.1x10^{7}$	ND	$1.0x10^{4}$	$1.0x10^{4}$	ND
	CH+AC 0.01%	$7.0x10^{6}$	ND	$7.0x10^{3}$	$1.8x10^{4}$	ND
	CH+AC 0.1%	$7.4x10^{7}$	ND	$9.2x10^{4}$	$1.4x10^{6}$	ND
	CH+MC 0.01%	$1.7x10^{7}$	ND	$1.4x10^{4}$	$2.0x10^{4}$	ND
	CH+MC 0.1%	$2.1x10^{7}$	ND	$1.6x10^{4}$	$2.5x10^{4}$	ND
	TH14+AC 0.01%	$2.0x10^{7}$	ND	$1.1x10^{4}$	$7.8x10^{3}$	ND
	TH14+AC 0.1%	$2.0x10^{6}$	ND	$2.0x10^{3}$	$1.6x10^{4}$	ND
	TH14+MC 0.01%	$2.0x10^{7}$	ND	$5.0x10^{3}$	$1.0x10^{4}$	ND
	TH14+MC 0.1%	$5.0x10^{6}$	ND	$7.0x10^{3}$	$9.5x10^{3}$	ND
Wilted	Control	$1.7x10^{8}$	ND	$6.0x10^{5}$	$4.3x10^{3}$	ND
	CH	$3.3x10^{7}$	ND	$5.6x10^{4}$	$6.0x10^{4}$	ND
	TH14	$1.3x10^{9}$	ND	$1.3x10^{6}$	$2.5x10^{5}$	ND
	AC 0.01%	$1.5x10^{7}$	ND	$1.9x10^{5}$	$6.7x10^{5}$	ND
	AC 0.1%	$2.0x10^{6}$	ND	$9.9x10^{5}$	$7.1x10^{3}$	ND
	MC 0.01%	$7.7x10^{8}$	ND	$1.9x10^{6}$	$5.3x10^{5}$	ND
	MC 0.1%	$7.6x10^{8}$	ND	$3.9x10^{4}$	$1.4x10^{5}$	ND
	CH+AC 0.01%	$1.3x10^{7}$	ND	$2.4x10^{4}$	$2.3x10^{4}$	ND
	CH+AC 0.1%	$5.0x10^{6}$	ND	$2.4x10^{5}$	$6.3x10^{4}$	ND
	CH+MC 0.01%	$4.8x10^{7}$	ND	$1.0x10^{6}$	$1.3x10^{5}$	ND
	CH+MC 0.1%	$9.3x10^{7}$	ND	$7.2x10^{5}$	$8.7x10^{4}$	ND
	TH14+AC 0.01%	$2.7x10^{9}$	ND	$3.5x10^{6}$	$9.4x10^{4}$	ND
	TH14+AC 0.1%	$1.0x10^{9}$	ND	$6.4x10^{4}$	$2.0x10^{5}$	ND
	TH14+MC 0.01%	$8.6x10^{8}$	ND	$8.7x10^{5}$	$1.2x10^{4}$	ND
	TH14+MC 0.1%	$4.4x10^{9}$	ND	$1.5x10^{6}$	$1.4x10^{6}$	ND
	SEM	11.65	ND	9.62	37.51	ND
Grass means						
	Fresh Napier	$2.0x10^{7}$	ND	$1.8x10^{4b}$	$1.1x10^{5}$	ND
	Wilted Napier	$8.2x10^{8}$	ND	$8.8x10^{5a}$	$2.4x10^{5}$	ND
Additive means						
	Control	$1.1x10^{8}$	ND	$3.2x10^{5}$	$4.9x10^{4}$	ND
	CH	$2.6\ x10^{7}$	ND	$3.6x10^{4}$	$3.7x10^{4}$	ND
	TH14	$6.8x10^{8}$	ND	$6.7x10^{5}$	$1.3x10^{5}$	ND
	AC 0.01%	$9.0x10^{6}$	ND	$1.0x10^{5}$	$3.4x10^{5}$	ND
	AC 0.1%	$5.0x10^{6}$	ND	$5.0x10^{5}$	$8.4x10^{3}$	ND
	MC 0.01%	$3.9x10^{8}$	ND	$9.8x10^{5}$	$2.7x10^{5}$	ND
	MC 0.1%	$3.9x10^{8}$	ND	$2.5x10^{4}$	$7.9x10^{4}$	ND
	CH+AC 0.01%	$1.0x10^{7}$	ND	$1.6x10^{4}$	$2.1x10^{4}$	ND
	CH+AC 0.1%	$4.0x10^{7}$	ND	$1.7x10^{5}$	$7.4x10^{5}$	ND
	CH+MC 0.01%	$3.3x10^{7}$	ND	$5.4x10^{5}$	$7.6x10^{4}$	ND
	CH+MC 0.1%	$5.7x10^{7}$	ND	$3.7x10^{5}$	$5.7\ x10^{4}$	ND
	TH14+AC 0.01%	$1.3x10^{9}$	ND	$1.7x10^{6}$	$5.1x10^{4}$	ND
	TH14+AC 0.1%	$5.3x10^{8}$	ND	$3.3x10^{4}$	$1.1x10^{5}$	ND
	TH14+MC 0.01%	$4.5x10^{8}$	ND	$4.4x10^{5}$	$1.1x10^{4}$	ND
	TH14+MC 0.1%	$2.2x10^{9}$	ND	$7.7x10^{5}$	$7.1x10^{5}$	ND
Significance of main effect and interaction						
	Grasses (A)	0.033	ND	0.006	0.252	ND
	Additives (B)	0.706	ND	0.817	0.383	ND
	A x B	0.697	ND	0.809	0.146	ND

[a to b], Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; cfu, colony forming unit; FM, fresh matter; ND, not detected.

Table 8 Chemical composition and gross energy (GE) of Guinea grass silages at 30 day of fermentation

Item		OM	CP	EE	NDF	ADF	ADL	GE
		% DM						(kcal/g)
Fresh	Control	89.78	5.87	2.15	73.71	47.86	4.81	4.27
	CH	89.87	5.67	1.98	73.87	47.02	4.46	4.14
	TH14	89.81	5.53	1.90	74.44	48.94	5.02	4.28
	AC 0.01%	89.98	6.06	2.02	70.94	44.45	4.55	4.31
	AC 0.1%	90.94	6.26	1.87	68.73	43.26	5.14	4.40
	MC 0.01%	88.69	5.77	1.81	73.62	47.18	5.09	4.34
	MC 0.1%	89.27	6.16	1.70	69.68	42.54	4.59	4.28
	CH+ AC 0.01%	89.20	5.85	2.02	71.67	45.03	4.81	4.29
	CH+ AC 0.1%	91.12	5.68	1.98	71.72	44.62	6.09	4.43
	CH+ MC 0.01%	89.26	5.37	1.82	74.27	47.18	5.73	4.25
	CH+ MC 0.1%	89.57	6.07	1.68	70.18	43.20	4.82	4.24
	TH14+ AC 0.01%	89.27	5.77	2.07	70.12	44.08	4.90	4.25
	TH14+ AC 0.1%	91.00	5.54	1.92	70.47	43.86	6.04	4.38
	TH14+ MC 0.01%	89.19	5.20	2.00	74.27	48.24	5.76	4.24
	TH14+ MC 0.1%	89.37	6.00	2.15	69.15	42.35	4.61	4.23
Wilted	Control	90.50	5.42	1.56	76.12	47.45	4.45	4.16
	CH	90.52	5.78	1.29	75.55	47.53	4.42	4.23
	TH14	90.58	5.44	1.52	76.69	47.58	4.46	4.21
	AC 0.01%	90.36	6.21	1.78	71.78	45.40	4.56	4.18
	AC 0.1%	90.42	6.79	1.66	66.68	42.47	5.23	4.31
	MC 0.01%	90.45	5.82	1.73	74.23	46.52	4.70	4.21
	MC 0.1%	90.42	6.60	1.78	70.39	43.81	4.83	4.26
	CH+ AC 0.01%	90.71	6.36	1.44	69.99	44.22	4.61	4.11
	CH+ AC 0.1%	90.52	6.99	1.85	65.64	40.07	4.75	4.33
	CH+ MC 0.01%	90.63	5.63	2.00	73.10	46.06	4.46	4.22
	CH+ MC 0.1%	91.12	6.63	1.99	68.63	42.51	4.28	4.32
	TH14+ AC 0.01%	90.82	6.11	1.78	71.40	45.30	4.58	4.28
	TH14+ AC 0.1%	90.71	6.66	1.88	65.12	41.60	5.26	4.33
	TH14+ MC 0.01%	90.97	5.76	2.10	74.03	46.05	4.43	4.27
	TH14+ MC 0.1%	90.78	6.46	2.00	69.46	43.36	4.39	4.28
SEM		0.150	0.129	0.180	0.760	0.517	0.215	0.033
Grass means								
	Fresh	89.75^{b}	5.79^{b}	1.94^{a}	71.79^{a}	45.32^{a}	5.09^{a}	4.29^{a}
	Wilted	90.63^{a}	6.18^{a}	1.76^{b}	71.25^{b}	44.66^{b}	4.62^{b}	4.25^{b}
Additive means								
	Control	90.14^{bcd}	5.65^{fg}	1.86^{ab}	74.91^{ab}	47.66^{ab}	4.63^{ef}	4.22^{bcd}
	CH	90.20^{bc}	5.72^{efg}	1.64^{b}	74.71^{ab}	47.28^{bc}	4.44^{f}	4.19^{d}
	TH14	90.20^{bc}	5.48^{g}	1.71^{ab}	75.56^{a}	48.26^{a}	4.74^{def}	4.25^{bcd}
	AC 0.01%	90.17^{bc}	6.13^{bcd}	1.90^{ab}	71.36^{c}	44.93^{d}	4.56^{ef}	4.25^{bcd}
	AC 0.1%	90.68^{a}	6.53^{a}	1.77^{ab}	67.71^{f}	42.86^{e}	5.19^{bc}	4.36^{a}
	MC 0.01%	89.57^{e}	5.80^{ef}	1.77^{ab}	73.93^{b}	46.85^{bc}	4.90^{cde}	4.28^{b}
	MC 0.1%	89.85^{d}	6.38^{ab}	1.74^{ab}	70.03^{cde}	43.17^{e}	4.71^{def}	4.27^{b}
	CH+ AC 0.01%	89.96^{cd}	6.11^{cd}	1.73^{ab}	70.83^{cd}	44.62^{d}	4.71^{def}	4.20^{cd}
	CH+ AC 0.1%	90.82^{a}	6.34^{abc}	1.92^{ab}	68.68^{ef}	42.35^{e}	5.42^{ab}	4.38^{a}
	CH+ MC 0.01%	89.95^{cd}	5.50^{g}	1.91^{ab}	73.68^{b}	46.62^{c}	5.10^{bcd}	4.24^{bcd}
	CH+ MC 0.1%	90.34^{b}	6.35^{abc}	1.84^{ab}	69.40^{de}	42.86^{e}	4.55^{ef}	4.28^{b}
	TH14+ AC 0.01%	90.04^{bcd}	5.94^{de}	1.93^{ab}	70.76^{cd}	44.69^{d}	4.74^{def}	4.27^{b}
	TH14+ AC 0.1%	90.86^{a}	6.10^{cd}	1.90^{ab}	67.79^{f}	42.73^{e}	5.65^{a}	4.35^{a}
	TH14+ MC 0.01%	90.08^{bcd}	5.48^{g}	2.05^{a}	74.15^{ab}	47.14^{bc}	5.09^{bcd}	4.25^{bcd}
	TH14+ MC 0.1%	90.08^{bcd}	6.23^{bc}	2.07^{a}	69.30^{e}	42.85^{e}	4.50^{ef}	4.25^{bcd}
Significance of main effect and interaction								
	Grasses (A)	<.001	<.001	0.002	0.030	<.001	<.001	<.001
	Additives (B)	<.001	<.001	0.264	<.001	<.001	<.001	<.001
	A x B	<.001	<.001	0.068	<.001	<.001	<.001	<.001

[a to g], Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

Table 9 Chemical composition and gross energy (GE) of Napier grass silages at 30 day of fermentation

Item		OM	CP	EE	NDF	ADF	ADL	GE
		% DM						(kcal/g)
Fresh	Control	93.48	6.85	2.14	73.97	38.00	4.74	4.43
	CH	93.40	7.01	2.20	75.15	40.90	4.64	4.43
	TH14	93.56	6.91	2.37	83.42	47.48	5.17	4.44
	AC 0.01%	94.18	6.56	1.95	75.30	43.28	6.37	4.55
	AC 0.1%	94.92	6.95	2.16	74.25	38.15	8.50	4.62
	MC 0.01%	93.71	6.23	2.12	72.92	36.39	5.76	4.44
	MC 0.1%	94.28	6.35	2.23	75.17	41.68	6.15	4.56
	CH+ AC 0.01%	94.10	6.62	2.21	79.29	42.22	5.42	4.53
	CH+ AC 0.1%	94.80	6.54	2.39	75.61	36.59	7.56	4.59
	CH+ MC 0.01%	93.43	6.52	2.22	74.88	43.31	5.41	4.50
	CH+ MC 0.1%	93.93	6.88	2.43	79.05	37.70	6.64	4.63
	TH14+ AC 0.01%	94.65	5.68	2.24	76.60	45.63	7.38	4.69
	TH14+ AC 0.1%	94.58	6.48	2.26	73.44	46.61	8.18	4.38
	TH14+ MC 0.01%	93.92	6.67	2.16	74.64	50.22	5.99	4.98
	TH14+ MC 0.1%	94.34	7.14	2.43	74.83	48.22	6.31	4.58
Wilted	Control	92.35	7.22	2.24	77.72	51.31	4.54	4.38
	CH	92.59	7.28	2.19	78.29	51.42	5.15	4.44
	TH14	92.40	8.33	2.27	76.15	41.62	4.99	4.40
	AC 0.01%	91.96	7.54	2.11	74.36	46.87	5.96	4.48
	AC 0.1%	91.57	8.15	2.02	68.45	47.76	7.69	4.51
	MC 0.01%	91.83	7.30	2.17	72.06	49.95	5.67	4.41
	MC 0.1%	91.64	7.62	2.22	72.27	50.76	5.62	4.44
	CH+ AC 0.01%	91.49	8.06	2.00	73.45	45.20	5.43	4.45
	CH+ AC 0.1%	91.23	7.31	2.12	67.85	50.65	6.61	4.53
	CH+ MC 0.01%	91.96	7.78	2.24	75.77	45.99	5.17	4.46
	CH+ MC 0.1%	91.84	7.90	2.29	73.40	52.03	5.67	4.46
	TH14+ AC 0.01%	91.83	7.62	2.26	73.76	46.45	5.74	4.47
	TH14+ AC 0.1%	91.59	8.55	2.42	66.87	44.25	6.76	4.43
	TH14+ MC 0.01%	91.93	7.78	2.28	75.81	42.83	5.49	4.39
	TH14+ MC 0.1%	91.62	8.32	2.27	72.59	38.79	5.53	4.45
	SEM	0.167	0.112	0.119	1.912	0.908	0.190	0.065
Grass means								
	Fresh	94.09^{a}	6.58^{b}	2.23	75.90^{a}	42.42^{b}	6.28^{a}	4.56^{a}
	Wilted	91.86^{b}	7.75^{a}	2.21	73.25^{b}	47.05^{a}	5.73^{b}	4.45^{b}
Additive means								
	Control	92.91^{abc}	7.03^{de}	2.19	75.85^{bc}	44.65^{bcdef}	4.64^{j}	4.40^{e}
	CH	93.00^{abc}	7.14^{cde}	2.20	76.73^{ab}	46.16^{ab}	4.90^{ij}	4.43^{cde}
	TH14	92.98^{abc}	7.62^{a}	2.32	79.78^{a}	44.55^{bcdef}	5.08^{hi}	4.42^{de}
	AC 0.01%	93.07^{ab}	7.05^{de}	2.03	74.83^{bcde}	45.07^{abcd}	6.17^{e}	4.51^{bcde}
	AC 0.1%	93.25^{a}	7.55^{a}	2.09	71.35^{ef}	42.95^{f}	8.10^{a}	4.57^{b}
	MC 0.01%	92.77^{bc}	6.77^{fg}	2.15	72.50^{cdef}	43.17^{ef}	5.72^{fg}	4.43^{de}
	MC 0.1%	92.96^{abc}	6.99^{e}	2.22	73.72^{bcdef}	46.22^{ab}	5.89^{ef}	4.50^{bcde}
	CH+ AC 0.01%	92.80^{bc}	7.34^{bc}	2.11	76.36^{abc}	43.71^{cdef}	5.43^{gh}	4.49^{bcde}
	CH+ AC 0.1%	93.01^{abc}	6.93^{ef}	2.26	71.73^{def}	43.62^{cdef}	7.09^{c}	4.56^{bc}
	CH+ MC 0.01%	92.70^{c}	7.15^{cde}	2.23	75.33^{bcd}	44.65^{bcdef}	5.29^{h}	4.48^{bcde}
	CH+ MC 0.1%	92.89^{bc}	7.38^{b}	2.36	76.23^{abc}	44.87^{abcde}	6.15^{e}	4.54^{bcd}
	TH14+ AC 0.01%	93.24^{a}	6.65^{g}	2.25	75.18^{bcd}	46.04^{ab}	6.56^{d}	4.58^{ab}
	TH14+ AC 0.1%	93.09^{ab}	7.52^{a}	2.34	70.16^{f}	45.43^{abc}	7.47^{b}	4.41^{e}
	TH14+ MC 0.01%	92.93^{abc}	7.23^{bcd}	2.22	75.23^{bcd}	46.53^{a}	5.74^{fg}	4.69^{a}
	TH14+ MC 0.1%	92.98^{abc}	7.73^{a}	2.35	73.71^{bcdef}	43.50^{def}	5.92^{ef}	4.52^{bcde}
Significance of main effect and interaction								
	Grasses (A)	<.001	<.001	0.477	<.001	<.001	<.001	<.001
	Additives (B)	0.014	<.001	0.056	<.001	<.001	<.001	<.001
	A x B	<.001	<.001	0.585	0.005	<.001	<.001	<.001

$^{a\ to\ j}$, Means within columns with difference superscript letters differ at $P < 0.05$; Values are means of three silage samples; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.