ITALIAN JOURNAL OF ANIMAL SCIENCE
2019, VOL. 18, NO. 1, 1438–1444
https://doi.org/10.1080/1828051X.2019.1681910

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PAPER

Effects of fibrolytic enzymes and lactic acid bacteria on fermentation quality and *in vitro* digestibility of Napier grass silage

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ABSTRACT

An experiment was conducted to evaluate the effect of xylanase and cellulase derived from dried tomato pomace treated with *Aspergillus niger* and fermented juice of epiphytic of lactic acid bacteria (FJLB) on fermentative quality and *in vitro* digestibility of Napier grass silages. Thus, we treated Napier grass silage with or without fibrolytic enzymes (ENZ) in combination with or without FJLB. The pH values of silages treated with FJLB, but not ENZ, were found to be lower than the other silages during the ensiling process. The content of water soluble carbohydrates (WSC) of all silages was likely to decrease during fermentation in all silages. However, after 30 days of ensiling, the addition of ENZ resulted in higher WSC content when compared to the other treatments. At 30 day of ensiling, the crude protein of silage was higher in silage treated with FJLB. The gas production related to the soluble fraction was higher in silage treated with ENZ but total gas production was similar between treatments. The latter is in line the observation that the degradability of organic matter also was not different between treatments. It is concluded that neither the fermentation quality nor the *in vitro* digestibility of organic matter is improved when Napier grass is ensiled with both FJLB and fibrolytic enzymes compared with FJLB alone.

HIGHLIGHTS

- Ensiling Napier grass, which contains high water soluble carbohydrates, without additives results in an acceptable fermentation quality.
- A combination of fermented juice of epiphytic lactic acid bacteria (FJLB) and fibrolytic enzymes has no effect on quality of Napier grass silage.

ARTICLE HISTORY
Received 14 May 2019
Revised 23 September 2019
Accepted 14 October 2019

KEYWORDS
Gas production; inoculant; nutritive value; volatile fatty acid

Introduction

Napier grass (*Pennisetum purpureum*) is one of the most promising grasses available for ruminant production in tropical areas because of its high potential dry matter (DM) yield, i.e., around 71 tonnes DM/ha/year (Wijitphan et al. 2009). However, both the dry matter (DM) content and the content of water soluble carbohydrates (WSC) are considered too low for successful ensiling. Interestingly, in ruminant nutrition fibrolytic enzymes are currently applied due to their potency to improve fibre degradability and thus digestible energy intake in ruminants (Beauchemin et al. 2003). Indeed, enzymes such as xylanases and cellulases, can convert (hemi) cellulose into sugars (Stokes 1992) thereby rendering substrate available for fermentation. In line with this notion, it seems plausible that fibrolytic enzymes can also be applied in the process of ensiling and several authors (Sun et al. 2012; Khota et al. 2016; Desta et al. 2016) have already reported about the potency of fibrolytic enzymes to improve the quality of silage.

Apart from its low WCS content, the number of epiphytic lactic acid bacteria (LAB) in Napier grass also, may be too low for successful ensiling. Bureenok et al. (2005a, 2006) has shown that the addition of fermented juice of epiphytic LAB (FJLB) from tropical forages such as Guinea grass and Napier grass (i.e., grasses with a relatively low WSC content), already improved the quality of the respective silages. Interestingly, Sun et al. (2009) suggested that inoculants would be more effective in case the ensiling material has a high versus a low

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WSC content. It can thus be suggested that a combination of fibrolytic enzymes and FJLB versus FJLB alone, has an added value when ensiling grasses with a low WSC content. Currently, there is no information available on Napier silage fermentation and its digestibility when treated with fibrolytic enzymes in combination with FJLB. In the current study, we used "home-made" fibrolytic enzymes originating from *Aspergillus niger*. The aim of the current study is to evaluate the effects of FJLB and *A. niger* derived fibrolytic enzymes and their combination, on fermentation quality, chemical composition, and *in vitro* degradability of silage from Napier grass.

Materials and methods

Preparation of fibrolytic enzymes

A culture of *A. niger* was obtained from the National Centre for Genetic Engineering and Biotechnology (BIOTEC, Pathum Thani, Bangkok). Inoculum of *A. niger* was maintained on potato dextrose agar (PDA) at 30°C for 5 days and stored at 4°C until used. The spores from PDA plates were harvested by using sterile 0.01% Tween 80 (w/v) to obtain 1×10^7 spores mL^{-1}.

The fibrolytic enzymes were produced as described by Saithi et al. (2016). Briefly, the culture medium used for solid-state fermentation was dried tomato pomace (residue from the extraction of tomato sauce). The moisture content of dried tomato pomace was adjusted to about 50% and autoclaved (Nishio et al. 1979; Oriol et al. 1988; Hamidi-Esfahani et al. 2004; Hamidi-Esfahani et al. 2007). The substrate was cooled and mixed with 10% of an *A. niger* spore suspension (1×10^7 spores mL^{-1}) (w/w). The solid-state culture was incubated at 30 °C for 72 h (Szewczyk and Myszka 1994). Then, the substrate was dried at 50 °C for 3–5 days before being used as fibrolytic enzyme.

Dried tomato pomace treated by *A. niger* was milled through a 1 mm sieve. The moisture content of the culture medium was determined by drying in a hot-air oven at 80°C to derive a constant weight. The activity of xylanase and cellulase was according to a modified method of Bailey et al. (1992) and Mandels et al. (1976), respectively. The cellulase and xylanase activities from *A. niger* incubated with dried tomato pomace were 60,998 and 37,815 units g^{-1} DM, respectively.

Preparation of fermented juice of epiphytic lactic acid bacteria

Fermented juice of epiphytic lactic acid bacteria (FJLB) was prepared as described by Ohshima et al. (1997) and Bureenok et al. (2005a, 2005b). Briefly, 200 g of fresh Napier grass was macerated in 1000 mL of sterilised distilled water with a home blender. The juice was filtered through a double layer of cheesecloth; the filtrate was transferred to a glass bottle and 2% glucose was added. Then, the bottle was capped and incubated anaerobically at 30 °C for 2 days before being used as silage additive. The LAB counts of the FJLB were 5.5×10^8 colony forming unit (cfu) mL^{-1}. Contents of mould and/or yeast, if any, were not measured in the current study because it was considered unnecessary.

Silage making

Napier grass (*Pennisetum purpureum* x *Pennisetum americanum* 'Pak chong 1') was harvested after 70 day of regrowth. The experimental silages were prepared from fresh Napier grass (19.27% dry matter (DM)) and the chemical composition of the Napier grass was as follows (% DM): crude ash, 12.64; crude protein (CP), 5.3; neutral detergent fibre (NDF), 74.63; acid detergent fibre (ADF), 44.6; and water soluble carbohydrate (WSC), 9.13. Napier grass was chopped to 0.5–2 cm using a crop cutter. Right after cutting, all grass was thoroughly mixed and divided into four equal portions. Then, the four experimental silages were prepared with the treatments arranged in a 2×2 factorial fashion, i.e., forage with or without fibrolytic enzymes (ENZ) and with or without additional FJLB. The applied doses of forage specific FJLB was $\log_{10}$ 6.74 cfu g^{-1} fresh forage and ENZ was applied at a level of 0.5 g kg^{-1} fresh forage (contained 304,490 and 189,075 units of cellulase and xylanase activities, respectively). Thereafter, ~100 g of the mixed portions was packed tightly into plastic pouches (20.32 × 33 cm, 120 μ thickness; M-PLASPACK, Bangkok, Thailand). Air was withdrawn from the plastic pouches by means of a vacuum sealer. For each sampling day and each experimental forage, four replicate pouches ($n = 4$) were prepared and all pouches were kept at ambient temperature (27–35 °C). Pouches from each treatment were opened after 1, 7, 14 or 30 days of ensiling for chemical analysis.

Chemical analysis

After opening of the pouches on the specified days, subsamples (~20 g fresh material) were treated with 70 mL of distilled water and stored in a refrigerator at 4 °C for 12 h (Bureenok et al. 2006). Then, the extract was filtered (filter paper no.5; Whatman, England) and

Table 1. Fermentation profile and chemical composition of Napier grass silages treated with fibrolytic enzyme (ENZ), fermented juice of epiphytic lactic acid bacteria (FJLB) and its combination at 30 day of ensiling ($n = 4$; data are given in %DM, unless otherwise stated).

	No ENZ		With ENZ			*p*-Values		
	No FJLB	With FJLB	No FJLB	With FJLB	SEM	ENZ	FJLB	ENZ × FJLB
Fermentation profile								
pH	3.98^{ab}	3.70^{b}	4.26^{a}	3.71^{b}	0.08	.257	.004	.358
LAB (log_{10} cfu/g FW)	6.31	6.61	6.33	6.14	0.07	.984	.292	.898
Lactic acid (LA)	3.97^{b}	5.25^{a}	3.70^{b}	4.95^{a}	0.12	.251	.001	.949
Acetic acid (AA)	2.17^{a}	1.07^{b}	2.36^{a}	1.56^{ab}	0.10	.120	.001	.461
Propionic acid	0.21^{ab}	0.09^{b}	0.23^{a}	0.13^{ab}	0.02	.338	.004	.739
Butyric acid	0.36	0.36	0.47	0.27	0.03	.904	.164	.155
LA:AA	1.86^{c}	4.98^{a}	1.60^{c}	3.41^{b}	0.16	.017	<.001	.071
NH_3–N (% total N)	8.85^{a}	6.14^{c}	8.09^{ab}	6.47^{bc}	0.18	.589	.001	.185
WSC	0.84^{a}	0.41^{b}	0.77^{a}	0.46^{b}	0.07	.799	<.001	.280
Chemical composition								
Dry matter (% FW)	19.01	18.63	18.35	19.52	0.19	.759	.285	.051
Crude protein	5.67^{b}	6.16^{a}	6.03^{ab}	6.04^{ab}	0.53	.380	.030	.038
Neutral detergent fibre	77.59	65.69	76.56	78.09	0.43	.423	.829	.060
Acid detergent fibre	49.17^{a}	47.12^{ab}	48.65^{ab}	46.39^{b}	0.32	.006	.323	.870
Hemicellulose	28.41	28.57	27.69	31.69	0.53	.242	.087	.112

$^{a–d}$Means with different superscripts within rows significantly differed ($p < .05$). SEM: standard error of the means; LAB: lactic acid bacteria; FW: fresh weight; NH_3-N: ammonia-nitrogen; WSC: water soluble carbohydrate; Hemicellulose: NDF-ADF; ENZ: Effect of ENZ addition; FJLB: Effect of FJLB addition; ENZ × FJLB: Interaction effect between ENZ and FJLB addition.

the pH of the extract was recorded. Thereafter, the filtrate was stored at −20 °C until the analysis of lactic acid, volatile fatty acids and NH_3–N (Cai 2004). Lactic acid and volatile fatty acids were determined by means of HPLC. The dry matter (DM) content and CP (N-Kjeldahl × 6.25) and ash were determined as described by the AOAC (1999, methods no. 935.29, 990.03 and 942.05, respectively). NDF and ADF were determined according to the method of Van Soest et al. (1991) and were expressed exclusive of residual ash (NDFom and ADFom). WSC content was determined by the method of Dubois et al. (1956). The numbers of lactic acid bacteria (LAB) were measured by the plate count method on MRS agar. Colonies were counted from the plates at appropriate dilutions and the number of colony forming units (cfu) was expressed per gram of fresh weight.

In vitro *gas production*

The sampling of rumen fluid was approved by the Ethical Committee of the University of Technology Isan, Thailand. The rumen fluid was used as inoculant and was obtained from two adult goats. The animals were fed Napier grass silage supplemented with commercial concentrate. Kinetics of degradation and gas production were evaluated after 30 days of fermentation using semi-automatic equipment to measure *in vitro* gas production. The *in vitro* digestibility was determined as described by Menke and Steingass (1988).

Briefly, samples of each grass silage (0.2 g DM) were accurately weighed into a 100 mL syringe. Subsequently, a mixture composed of 20 mL of anaerobic buffer solution and 10 mL of ruminal fluid was added to each syringe. Blank (rumen fluid without sample) were run in triplicate in each series. Then, the syringes were placed into a water bath and automatically stirred at 39 °C. Gas production (GP) was measured at distinct incubation times (0, 1, 2, 4, 6, 8, 12, 18, 24, 36, 48, 60, 72 84 and 96 h). Total gas productions were corrected for the blank incubation. Cumulative gas production data were fitted as described by Ørskov and McDonald (1979);

$$y = a + b\ (1 - e^{-ct})$$

where: a = the gas production from the soluble fraction (mL), b = the gas production from the insoluble fraction (mL), c = the gas production rate constant for the insoluble fraction b (mL/h), t = the incubation time (h). $a + b$ = the potential gas production (mL), gas production at the time "t".

The organic matter digestibility (OMD) was calculated according to Kamalak et al. (2005):

$$\begin{aligned} \text{OMD}\ (\%) = 16.149 &+ (\text{GP24h} * 0.9042) \\ &+ \left(\text{CP}\ (\%) * 0.0492\right) + \left(\text{EE}\ (\%) * 0.0387\right) \\ &+ \left(\text{Ash}\ (\%) * 0.0387\right) \end{aligned}$$

Statistical analysis

All data of Napier grass silage were subjected to ANOVA using SAS (1994), based on the model:

$$Y_{ij} = \mu + ENZ_i + FJLB_j + (ENZ \times FJLB)_{ij} + e_{ij}$$

where Y_{ij} = response variable, μ = overall mean, ENZ_i = fibrolytic enzyme (i = with or without), $FJLB_j$ =

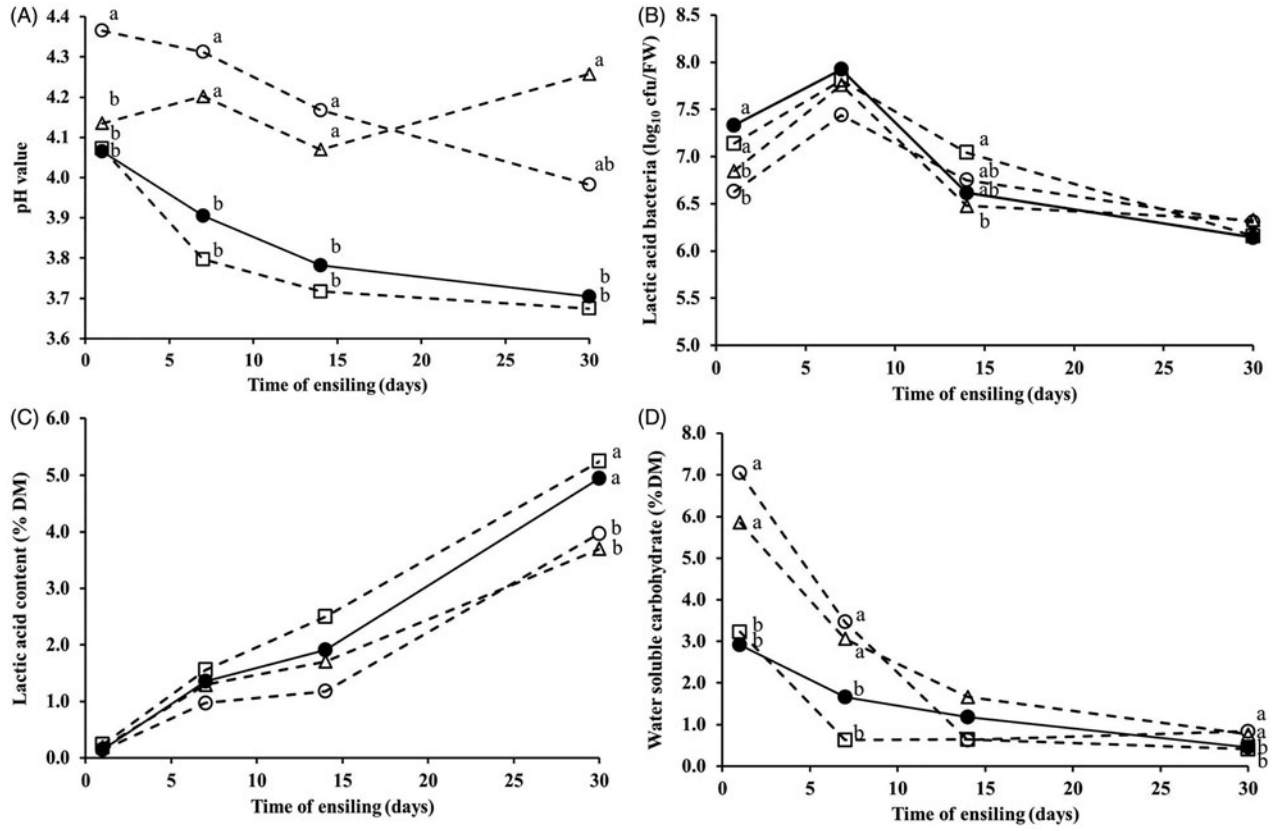

Figure 1. Time courses on pH values, (panel a), lactic acid bacteria counts (panel b), lactic acid contents (panel c), and water soluble carbohydrate contents (panel d) during the ensiling of Napier grass. symbols: o: untreated silage (no fibrolytic enzyme (ENZ) or fermented juice of epiphytic lactic acid bacteria (FJLB)); □: silage treated with FJLB; Δ: silage treated with ENZ; •: silage treated with ENZ and FJLB.

fermented juice of lactic acid bacteria (j = with or without), $(ENZ \times FJLB)_{ij}$ = interaction term between ENZ and FJLB and e_{ij} = residual error. Tukey test was used to determine the significant difference between means. The level of statistical significance was declared at $p < .05$.

Results

Fermentation profile and chemical composition

After 30 days of ensiling, the fermentation profile was not affected ($p \geq .071$) by an interaction between the fibrolytic enzymes and FJLB (Table 1). Addition of ENZ had not affect ($p > .120$) the values of pH, AA, LA, and PA contents of Napier grass silages were not significantly different from the untreated silage. The FJLB treated silages had significantly ($p < .05$) decreased pH, AA content and PA content and significantly ($p < .05$) increased LA content and LA:AA ratio of Napier grass silage compared with the other treatment. The addition of ENZ caused a decrease ($p = .017$) in the LA:AA ratio of silages. The butyric acid contents of all silages were less than 0.5%, and not significantly different among treatments. The use of FJLB caused lower ($p = .001$) NH_3–N content. The WSC contents were not affected by ENZ × FJLB ($p = .185$). The addition of FJLB caused a 46% decrease in WSC contents in silages ($p < .001$).

In the course time (Figure 1), after the first day of ensiling, the FJLB and ENZ combined with FJLB treated silages gradually decreased the pH value throughout the ensiling period and the values were lower than the ENZ treated silage at the end of ensiling (panel A). At the first day of ensiling, LAB colonies of untreated silages and ENZ alone were lower ($p < .05$) than the FJLB and ENZ combined with FJLB treated silages. LAB counts were reached to $\log_{10}$ 8 cfu g^{-1} fresh weight after 7 days of ensiling and declined to approximately $\log_{10}$ 6 cfu g^{-1} fresh weight in all silage and were not different ($p > .05$) among the silage groups (panel B). Lactic acid contents gradually increased during the first day of fermentation in all silages (panel C). Although the levels of lactic acid were not significantly different during the early ensiling, pH values were significantly different among silages. The WSC content of all silages tended to decrease in the beginning of ensiling day (panel D). All FJLB additives rapidly decreased WSC content and reached the lowest value within 7 days of the

Table 2. *In vitro* degradability coefficients of the dry matter (DM) of 30-days Napier grass silages treated with fibrolytic enzyme (ENZ) and fermented juice of epiphytic lactic acid bacteria (FJLB) ($n = 4$).

	No ENZ		With ENZ			*p*-Values		
	No FJLB	With FJLB	No FJLB	With FJLB	SEM	ENZ	FJLB	ENZ x FJLB
a	3.29[b]	5.67[ab]	8.92[a]	5.93[ab]	0.47	.009	.757	.014
b	86.03	78.58	86.47	76.33	2.47	.859	.101	.791
c (mL/h)	0.04	0.04	0.04	0.04	0.01	.329	.699	.062
a+*b*	89.32	84.26	95.39	82.26	2.71	.714	.120	.471
OMD (%DM)	42.74	42.93	47.06	38.21	1.72	.954	.232	.214

[a]Soluble fraction; [b]insoluble fraction, but degradable; [c]degradation rate of fraction; a + b, potential gas production; OMD: organic matter digestibility; SEM: standard error of the means; ENZ: Effect of ENZ addition; FJLB: Effect of FJLB addition; ENZ × FJLB: Interaction effect between ENZ and FJLB addition.

start of fermentation, as compared to the control and ENZ additive.

The combination of ENZ and FJLB tended to increase the DM content of silages ($p = .051$). Addition of FJLB caused higher ($p = .03$) CP contents in silages, but the addition of both ENZ and FJLB decreased the CP contents of silages ($p = .038$). The NDF contents of silages did not show significant ($p > .06$) difference among different treatments. The ADF contents was not affected by ENZ × FJLB ($p = .870$). The addition of FJLB decreased in ADF contents ($p = .006$).

In vitro *kinetics of degradation and gas production*

The gas production kinetics and some estimated parameters in all silages are given in Table 2. As can be seen from the result, addition of ENZ caused an increase in ($p = .009$) gas production of the immediate soluble fractions (a) but the addition of both ENZ and FJLB decreased the gas production from these fractions of silages ($p = .014$). Gas production from slowly fermentable fraction (b), the potential gas production ($a + b$) and the gas production rate (c) were not significantly different among treatments. The organic matter digestibility (OMD) were not different in all silages.

Discussion

Fermentation quality and nutritive value

Usually, good silage should have pH value of 4.20 or less, butyric acid content less than 10 g kg^{-1} DM, and NH_3-N content less than 100 g kg^{-1} total nitrogen (McDonald et al. 1991). Therefore, these silage were well preserved. Since the FJLB used in our study contained LAB with producing lactic acid to inhibit growth of clostridia and aerobic bacteria, it improved silage quality (Wang et al. 2009). The significantly higher CP contents in FJLB treated silages could be attributable to a high LA content. As a result, the pH dropped sharply, which inhibited the growth of Clostridium spp. (Nadeau et al. 2000; Tian et al. 2014). Clostridium spp. usually produce NH_3-N from protein in the silage materials (Xing et al. 2009). In this study, the ENZ treatment had no effect on degradation of plant fibre to increase sugar for promoting lactic acid fermentation. This could be attributed to the level of WSC (91.3 g kg^{-1} DM) in the material. Generally, the content of WSC available in forage materials is greatly related to silage fermentation and good silage quality is readily made from forage crops with high WSC content. Zhang et al. (2016) suggested that a WSC content of about 60–70 g kg^{-1} DM should be sufficient for the fermentation process. Thus, the WSC contents of the Napier grass used in the current study may have been enough to stimulate the growth of LAB for producing LA. Therefore, the addition of ENZ may not have beneficial effects on promoting the propagation of LAB. Previous studies, have suggested that addition of cellulase could improve the fermentation quality by degrading NDF and ADF of tropical crop and by-product silages (Khota et al. 2016; Li et al. 2017; Wang et al. 2019). This result was not evident in our study, this could be attributed to the fibrolytic enzyme activity which depends on the temperature and pH condition (Colombatto et al. 2004). Cellulase requires a pH of 5.0–6.5 and temperature of 39 °C–50 °C for optimal activity (Chung et al. 2012; Kung et al. 2002). Therefore, the rapid decrease in pH from the fresh Napier grass (5.5) to below 4.2 within first day after ensiling in all treated silages (Figure 1) could have inhibited the activity of cellulase. This study agreed with Khota et al. (2017) who found that fibrolytic enzyme did not significantly decrease fibre contents in sorghum silages which caused by the rapid decreasing pH value to below 4 at the early ensiling process.

In vitro *gas production*

Nagadi et al. (2000) suggested that the *in vitro* gas production is highly dependent on the availability of

fermentable carbohydrate and nitrogen. Addition of FJLB resulted in a higher content of crude protein in silages compared with the silage without any additive. However, the gas production from soluble fraction was not different. This is in line with Akinfemi et al. (2009) who suggested that protein fermentation gives a relatively small gas production when compared to carbohydrate fraction. The ENZ treated silages had a higher residual of WSC content, but the gas production of soluble fraction was higher in ENZ silages. This may have been caused by the non-soluble carbohydrate fraction in ENZ treated silage. Although, lower in WSC content, the gas production of soluble fraction in silages treated with FJLB was not different from the ENZ treated silages. The most plausible explanation is the high content of lactic acid in the FJLB treated silages. Kondo et al. (2004) reported that the lactic acid content showed a positive relationship with gas production which silage could preserve more fermentable nutrients at low pH and high lactic acid and suggested that lactic acid itself might be metabolised *in vitro* rumen system. The combination of ENZ and FJLB did not improve the nutrient digestion. A possible reason for this is that fibre-degrading enzymes predigested the readily digestible fibre leaving a slower and less degradable fraction. Moreover, the LAB also used WSC to produce the lactic acid. Therefore, the leftover of readily digestible fraction was smaller than the others.

Conclusions

This study confirmed that addition of fibrolytic enzyme and its combined with lactic acid bacteria (FJLB) did not improve the fermentation and chemical composition of Napier grass silage which contained enough WSC. The FJLB improved the fermentation quality and inhibited protein degradation of Napier grass silage.

Disclosure statement

The authors declare no conflict of interest in this paper.

Ethical Approval

The experimental protocol was approved by the Ethical Committee of the University of Technology Isan, Thailand.

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