



# Effects of fibrolytic enzyme supplementation on feed intake, digestibility and rumen fermentation characteristics in goats fed with Leucaena silage

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## ABSTRACT

The primary objective of this study was to determine the effect of adding fibrolytic enzyme cocktail, which was derived from dried tomato pomace treated with *Aspergillus niger*, to Leucaena silage on total gas production by using in vitro gas techniques. In addition, the effect of these enzymes on voluntary feed intake, nutrient digestibility, and rumen fermentation were determined using 8 male, crossbred Anglo Nubian x Native goats. The experiment was designed as a replicated 4 × 4 Latin square. The experimental treatments were randomly assigned to each sequence of feeding of the 4 experimental treatments, i.e., 0%, 0.2%, 0.4%, and 0.8% (dry matter) of enzyme cocktail supplemented to Leucaena silage. The addition of 0.4% enzyme cocktail increased total gas production at 96 h, which was not different from the addition of commercial cellulase. Gas production from the insoluble fraction (b) was improved by increasing the enzyme cocktail levels ( $P = 0.039$ ). The voluntary intake of Leucaena silage increased with an increase in the addition of enzyme level ( $P = 0.025$ ). The dry matter, organic matter, crude protein, NDF and ADF digestibility's were also increased by increasing the addition of enzyme cocktail level. The addition of enzyme cocktail increased the *Prevotella\_1* in rumen fluid compared with the untreated group. In conclusion, at 0.4%, the fibrolytic enzyme cocktail derived from *A. niger* increased feed intake, nutrient digestibility, and ruminal fibrolytic bacteria community in goats.

## 1. Introduction

Exogenous fibrolytic enzymes such as xylanases and cellulases are widely used to improve the fibre digestibility of roughage in ruminants to improve the fibre digestibility of roughage (Arif et al., 2019; Saleem et al., 2019; Kholif et al., 2018). Furthermore, the use of exogenous fibrolytic enzymes are considered a sustainable strategy to improve the degradability of dietary fibre because these enzymes are produced by micro-organisms, which can grow on low-quality substrates and

agro-industrial wastes (Sakita et al., 2020; Jabri et al., 2022). Tomato pomace, which makes up to 4% of the original fruit weight, is a residue of the extraction of tomato sauce from tomatoes and is produced in large quantities each year. Tomato pomace is considered a highly suitable substrate for fungal growth due to the abundance of pectin, hemicellulose, and cellulose and a great variety of biologically active compounds such as carotenoids, proteins and minerals. Moreover, the use of tomato pomace is opportune because it substantially reduces the enzyme production cost (Zuorro et al., 2013; Grassino et al., 2020). To date, tomato

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pomace is commonly used as substrate for the production of some hydrolytic enzymes, including xylanase, exo-polygalacturonase (exo-PG), cellulase (CMCase) and  $\alpha$ -amylase (Umsza-Guez et al., 2011).

*Aspergillus* sp. has been reported for its capacity to produce cellulase under solid state fermentation of corn stover (Gao et al., 2008). In a previous research, we used tomato pomace as a substrate for *A. niger* to produce cocktail enzymes, mainly xylanases and cellulases, and found that gas production from the soluble fraction increased in silage treated with this enzyme (Bureenok et al., 2019). However, the efficacy of these fibrolytic enzymes in enhancing fibre digestion has only been tested under in-vitro conditions but not under feeding conditions in ruminants. Moreover, enzyme effectiveness under in-vivo conditions is also influenced by dietary composition and enzyme dosage (Chung et al., 2012; Adesogan et al., 2014). In view of its practical interest, it was therefore considered opportune to test the efficacy of exogenous enzymes derived from dried tomato pomace incubated with *A. niger*. *Leucaena leucocephala* is widely used in ruminant nutrition and is considered an excellent source of crude protein (22–25% CP in leaves) (De Angelis et al., 2021). Similar to other tropical forages, *Leucaena* is also rich in fibre components such as cellulose and xylan which negatively affect its degradability (Du et al., 2016). Thus, using native *Leucaena* as the main source of roughage in ruminant diets may not be favourable in ensuring animal production. Therefore, a home-made enzyme cocktail, derived from *A. niger*, was produced using dried tomato pomace as substrate. The primary aim of the current study was to evaluate the efficacy of the home-made enzyme cocktail on nutrient digestibility and rumen fermentation in goats and we hypothesized that the supplementation of the enzyme cocktail improves the nutrient digestibility and rumen fermentation of *Leucaena* silage in goats.

## 2. Materials and methods

### 2.1. Preparation of the home-made enzyme cocktail

A pure culture of *A. niger* was obtained from the National Centre for Genetic Engineering and Biotechnology (BIOTEC, Pathum Thani, Bangkok). This pure inoculum of *A. niger* was sub-cultured on potato dextrose agar (PDA) at 30 °C and stored at 4 °C. After 5 days, the spores were harvested by using sterile 0.01% Tween 80 (w/v) (Q RēC™, New Zealand) to obtain a suspension containing  $1 \times 10^7$  spores/ml. The enzyme cocktail was produced as described by Saithi et al. (2016). Briefly, the moisture content of dried tomato pomace was adjusted to about 50% and subsequently autoclaved. The autoclaved tomato pomace was cooled and mixed with 10% (w/w) of the *A. niger* spore suspension ( $1 \times 10^7$  spores/ml). Then, the *A. niger*-enriched tomato pomace was incubated at 30 °C for 72 h. Then, the substrate was dried at 50 °C for 3–5 days and subsequently milled to pass a 1 mm sieve (POLYMIX® PX-MFC 90 D, Kinematica AG, Luzern, Switzerland) before being used as an enzyme cocktail. The amylase and protease activities of the enzyme cocktail were measured according to modified methods of Nelson (1944) and Anson (1938). Amylase activity was expressed as the amount of amylases required to release 1  $\mu$ g of glucose in 1 min at 40 °C and a pH of 4.0. Protease activity was expressed as the amount of protease required to release 1  $\mu$ g of tyrosine in 1 min at 40 °C and a pH of 4.0. The activities of xylanase and cellulase of the enzyme cocktail were tested according to modified methods described by Bailey et al. (1992) and Mandels et al. (1976). The cellulase and xylanase activities were measured using cellulose and xylan, respectively. One unit of activity (crude enzyme/gram dry weight) was defined as the amount of cellulase required to hydrolyze cellulose into 1  $\mu$ g of reducing sugar and the amount of xylanase required to hydrolyze xylan into 1  $\mu$ g of xylose in 1 min. Both activities of cellulase and xylanase were determined at 40 °C and pH of 4. The cellulase and xylanase activities of the enzyme cocktail were found to be 46,144- and 34,299-units / g dry weight, respectively (Table 1). Total aflatoxins were determined according to AOAC (2005). The total aflatoxin content of the enzyme cocktail was found to be 1.42

**Table 1**

Moisture content, pH, aflatoxin content and enzyme activities<sup>1</sup> of the enzyme cocktail prepared from dried tomato pomace incubated with *Aspergillus niger* (n = 4).

Composition	Mean $\pm$ Standard Deviation
Moisture content (%)	6.57 $\pm$ 0.63
pH value	6.25 $\pm$ 0.08
Total aflatoxin ( $\mu$ g/kg dry weight)	1.42 $\pm$ 0.03
Amylase activity (Unit/ g dry weight)	55,658 $\pm$ 9836
Protease activity (Unit/ g dry weight)	3706 $\pm$ 1021
Cellulase activity (Unit/ g dry weight)	46,144 $\pm$ 13,079
Xylanase activity (Unit/ g dry weight)	34,299 $\pm$ 9085

mg/ kg dry weight, which was below the maximum acceptable limit (Mahato et al., 2019).

### 2.2. Experimental silage

Leaves and branch tips from *Leucaena* (*L. leucocephala*) were harvested from experimental plots at Rajamangala University of Technology Isan, Nakhon Ratchasima province, Thailand. The experimental silages were prepared from fresh *Leucaena* (414 g/kg dry matter (Table 2). *Leucaena* was chopped to 1–2 cm using a crop cutter, packed tightly in 100-kg plastic drums, and stored at ambient temperatures ranging from 27 to 30 °C for at least 30 days before feeding. The silage (~ 500 g fresh material) was sampled and subsequently chemically analyzed, and tested for in vitro gas production; the remainder of the silage was used during the feeding trial with the goats.

### 2.3. In vitro gas production

An in-vitro experiment was conducted prior to the goat feeding trial to confirm the potency of the home-made enzyme cocktail in increasing the nutrient digestibility of *Leucaena* silage. Rumen fluid (~ 300 ml) was obtained from two male adult crossbred Anglo Nubian x Native goats fed silage from Napier grass (71% of total dry matter intake, DMI) supplemented with commercial concentrate (29% of DMI, Table 2). The experimental substrates consisted of either *Leucaena* silage or *Leucaena* silage supplemented with 0.2, 0.4 or 0.8% (w/w) of the home-made enzyme cocktail. In addition to the aforementioned substrates, *Leucaena* silage was supplemented (0.2%, w/w) with a commercial cellulase preparate (Cellulase from *A. niger*, 1.2 U/mg, Sigma Lot #BCBM7947V). The latter treatment was considered as a positive control. In vitro gas production (GP) was measured as described by Menke and Steingass (Menke and Steingass, 1988). The rumen fluid samples were collected 1 hr after the morning feeding at 8:00 hr. After collection, the rumen fluid was filtered through four layers of cheesecloth, mixed with a buffer solution (1:2 v/v), and kept at 39 °C in a water bath under continuous flushing with CO<sub>2</sub>. The experimental substrates (0.2 g DM) were precisely weighed into 100 ml syringes. Each substrate was weighed into quadruplicate syringes. Syringes of blanks (rumen fluid without sample) were run in triplicate. Syringes were pre-warmed at 39 °C, and then 30 ml rumen fluid-buffer mixture was inhaled. Gas production was measured at different incubation times, i.e., after 1, 2, 4, 6, 8, 12, 18, 24, 36, 48, 60, 72, 84 and 96 h of fermentation. Total gas production was

**Table 2**

Chemical composition of fresh *Leucaena*, *leucaena* silage and concentrate.

Item	Fresh <i>Leucaena</i>	<i>Leucaena</i> silage	Concentrate
Dry matter (DM, g/kg)	414	391	914
Crude protein (g/kg DM)	170	170	133
Neutral detergent fibre (g/kg DM) <sup>a</sup>	496	487	310
Acid detergent fibre (g/kg DM) <sup>a</sup>	373	396	175

<sup>a</sup> Expressed exclusive of residual ash.

corrected for the amount of substrate organic matter (OM) incubated and gas released from blanks. Cumulative gas production data were fitted according to the following model (Ørskov and McDonald, 1979):

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

where:  $a$  = the gas production associated with the immediate soluble fraction (ml),  $b$  = the gas production associated with 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), at timepoint “ $t$ ”, and  $y$  = the gas produced at timepoint “ $t$ ”.

#### 2.4. In vivo experiment

The animal experiment was carried out in accordance with a protocol approved by the Ethical Committee of Rajamangala University of Technology Isan, Nakhon Ratchasima province, Thailand (U1-03319-2559). Eight male Anglo Nubian x Thai native crossbred goats with a body weight (BW)  $36 \text{ kg} \pm 3.8 \text{ kg}$  (mean  $\pm$  SD) were used. Prior to the experiment, goats were de-wormed by means of Ivermectin (IVOMEC F plus, Bangkok, Thailand) and injected with vitamin A (500,000 I.U.), vitamin D<sub>3</sub> (75,000 I.U.), and vitamin E (50 I.U) (Biotecnocem, Dallas, USA). The goats were individually housed in pens ( $148 \times 115 \times 120 \text{ cm}$ ) and fresh water was provided ad libitum. The trial had a double  $4 \times 4$  Latin square design. Each experimental period lasted 21 days, and samples were taken in the last week of each period. The goats were randomly assigned to each sequence of feeding of the four levels of enzyme cocktails, i.e. 0%, 0.2%, 0.4%, and 0.8% (w/w, dry matter) of Leucaena silage. Before each feeding, the enzyme mixture was supplemented to the silage while mixing. Then, all diets were kept for approximately 30 min before the start of feeding to increase enzyme efficacy. The Leucaena silages were provided ad libitum to the goats. In addition to the experimental silage, goats were also offered a concentrate at a level of 0.8% of body weight. The macro nutrient composition of the pelleted concentrate is shown in Table 2. The experimental rations were provided two times per day in equal portions at 08.00 and 16.00 h. The refused portions were weighed daily before the morning feeding. The goats were weighed before the morning feeding at the beginning and the end of each experimental period. The daily DM intake per unit of BW was calculated with the mean value of the initial BW and the final BW of each period.

#### 2.5. Collection of samples

At the end of each experimental period, rumen fluid samples were collected at 0, and 4 h after the morning feeding; the rumen fluid was immediately filtered through 2 layers of cheesecloth. The pH of the filtrates was measured with a glass electrode pH meter SI analytics GmbH, Mainz, Germany). The filtrate samples were acidified with 1 M H<sub>2</sub>SO<sub>4</sub>, centrifuged at 16,000g for 15 min, and the supernatant was stored at  $-20^\circ\text{C}$  before volatile fatty acids (VFAs) analysis.

Feed and feces samples were collected throughout the last 7 days of each period. Feces were collected quantitatively from each goat in the morning and afternoon, according to the total collection method. The daily feces production of each goat was mixed thoroughly, and 3% of the wet weight was stored at  $-20^\circ\text{C}$ . At the end of each collection period, the stored fractions of feces from each goat were combined, mixed thoroughly, and sampled. The samples were then dried (48 h at  $60^\circ\text{C}$ ), ground and stored in air-dry form and stored in sealed jars at  $32\text{--}35^\circ\text{C}$  until analysis.

#### 2.6. Chemical analysis

Volatile fatty acid in rumen fluid was determined by means of HPLC (column Novapak C<sub>18</sub>; column size  $3.9 \times 300 \text{ mm}$ ; Waters Corporation,

Milford, MA, USA) (Samuel et al., 1997). The ruminal methane (CH<sub>4</sub>) production (mmol/100 mol VFA) was estimated using the formula:  $(0.45 \times \text{Acetate}) - (0.275 \times \text{propionate}) + (0.4 \times \text{butyrate})$  (Moss et al. 2000).

The dry matter content of roughage, concentrates, and feces was determined after oven drying at  $60^\circ\text{C}$  for 48 h. The dried samples were milled to pass a 1.0-mm sieve (POLYMIX® PX-MFC 90 D, Kinematica AG, Luzern, Switzerland). The crude protein (CP) (N-Kjeldahl  $\times 6.25$ ) and ash were determined as described by AOAC (1995). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to the method of Van Soest et al. (1991) and were expressed exclusive of residual ash.

#### 2.7. Ruminal microbial community

The observation of the ruminal microbial community was done at the end of the first experimental period. Rumen fluid samples were collected from the rumen cannulated goats 4 h after the morning feeding. Rumen fluid ( $\sim 50 \text{ ml}$ ) from each goat was placed into a sterilized container and all samples were immediately stored at  $-20^\circ\text{C}$  until analysis. Prior to analysis, the samples were thawed until they could be homogenized in a blender. Bacterial total genome DNA was extracted from ruminal fluid samples using the TIANamp stool DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The purity and concentrations of extracted DNA were determined by 1% agarose gel electrophoresis and the use of a micro-spectrophotometer. Finally, all samples were sent to the First Base Laboratory (Selangor, Malaysia) for 16 S rRNA gene pyrosequencing. The V3-V4 region of the 16 S ribosomal RNA gene was amplified using Phusion®High-Fidelity PCR Master Mix (New England Biolabs, Massachusetts, USA). The mixed PCR products were purified with the GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using NEB Next Ultra™ DNA library Prep Kit for Illumina (NEB, USA). The amplicons were then sequenced using the Illumina HiSeq platform.

#### 2.8. Sequencing data processing

Illumina-generated sequence reads were merged by using FLASH (Magoč and Salzberg, 2011). Quality reads were obtained after the ambiguous and homologous reads were screened by using QIIME (Version.1.7.0) (Caporaso et al., 2010). The UPARSE (version 7.0) (Edgar, 2013) were used to cluster the operational taxonomic units (OTUs) with 95% and 97% similarity cutoff. Chimeric sequences were removed by using the UCHIME algorithm (Edgar et al., 2011). The representative sequences were taxonomically classified using the QIIME-based wrapper of the Ribosomal Database Project (version 2.2) (Wang et al., 2007), a naïve Bayesian classifier, retained on the GreenGenes 16 S rRNA gene database with a 0.80 confidence threshold. The analysis result of species annotation was visually shown by KRONA (Ondov et al., 2011). Relative abundances of OTUs were generated, and the Chao1 (estimates the species abundance) (Chao, 1984) Observed Species (estimates the amount of unique OTUs found in each sample) and Shannon indexes were calculated. Rarefaction curves were generated based on these three metrics. Cluster analysis were preceded by principal component analysis (PCA) using the QIIME software.

#### 2.9. Statistical analysis

Gas production parameters were estimated using PROC NLIN in SAS/STAT (SAS Inst. Inc. Cary, NC). All data of in vitro gas production were analyzed according to a complete randomized design using General Linear Models (GLM) procedures (SAS, 2006). First, orthogonal contrast was performed to compare the average gas production parameters of commercial enzyme vs 0.4% cocktail enzyme treatments. Second, orthogonal polynomials were employed in order to determine the effect of increasing in the level of cocktail enzyme application on in vitro gas

production. The in vivo data were analyzed using GLM procedures using the following model:  $Y_{ijk} = \mu + M_i + A_j + P_k + \varepsilon_{ijk}$ , where  $Y_{ijk}$  is the response variable (feed intake, digestibility),  $\mu$  is the overall of the mean,  $M_i$  is the experimental treatment ( $i$  = inclusion level of the enzyme cocktail; 0%, 0.2%, 0.4%, and 0.8% w/w),  $A_j$  is the effect on the animal,  $j = 1, 2, 3, 4, 5, 6, 7, 8$ ,  $P_k$  is experimental period ( $k = 1, 2, 3, 4$ ), and  $\varepsilon_{ijk}$  is the residual error.

### 3. Results

#### 3.1. In vitro gas production

After 24 h of incubation (Table 3), cumulative GP was similar between treatments ( $P = 0.637$ ), but after 96 h of incubation, cumulative GP increased linearly with increasing levels of supplemental enzymes ( $P = 0.046$ ); however, increasing the supplemental enzyme cocktail from 0.4 to 0.8% did not further increase the GP. The GP associated with the soluble fraction (a) was not affected by the level of enzyme supplementation ( $P = 0.950$ ) while GP associated with the insoluble fraction (b) increased with increasing levels of supplemental enzymes ( $P = 0.039$ ). Both the degradation rate and potential gas production ( $a + b$ ) were not affected ( $P \geq 0.130$ ) by the level of enzyme supplementation. With the exception of the degradation rate of the insoluble fraction ( $P = 0.039$ ), the remaining in vitro gas production parameters were found to be similar between 0.2% supplemental cellulase and 0.4% supplemental enzyme cocktail ( $P = 0.112$ ).

#### 3.2. Feed intake and digestibility

Increasing levels of the supplemental enzyme cocktail caused a linear increase in silage DMI ( $P = 0.025$ ) and total DMI ( $P = 0.033$ ) (Table 4). The total intake of OM, NDF, and ADF increased linearly ( $P \leq 0.047$ ) with an increase in the levels of enzyme supplementation. In addition, the intake of CP tended ( $P = 0.067$ ) to increase with increasing levels of enzyme supplementation. In line with the latter results, the absolute amount of digested nutrients (g /day) also linearly increased with increasing proportions of the supplemental enzyme cocktail ( $P \leq 0.015$ ). The relative digestion of the nutrients (% of intake) was similarly affected by the supplemental enzymes ( $P \leq 0.033$ ).

#### 3.3. Rumen fermentation

The addition of the enzyme cocktail did not affect the pH, total VFA, and the proportions of acetic, propionic, and butyric acid in the rumen before morning feeding and 4 h thereafter (Table 5). The enzyme cocktail supplementation did not affect  $CH_4$  production.

#### 3.4. Ruminal microbial community

The profile of ruminal microbiota changed according to different

levels of enzyme cocktail addition. All the samples had coverage values greater than 0.99 (Table 6), indicating that the sequencing depth was sufficient to reveal the entire bacterial diversity of the samples. The Shannon index, which reflects microbial community diversity, did not differ across enzyme cocktail treatments. However, when 0.2% of the cocktail enzymes was supplemented to the silage, the treatment had a numerically lower Shannon index value. The Simpson index, which measures microbial community diversity, did not differ across treatments. The Chao and Ace indices, which reflect the richness of microbial communities or the abundance of species, were highest in the absence of cocktail enzyme.

The Shannon estimates of diversity indices ranged from 6.98 to 7.48 and the Simpson indices ranged from 0.95 to 0.98. The Chao1 indices, which ranged from 1342 to 1553, indicated the abundance of species in each treatment. The lowest Chao1 index trend of bacterial richness was observed when silage was supplemented with 0.8% of the enzyme cocktail.

Four hours after feeding, Bacteroidetes, Proteobacteria, Firmicutes, and Euryarchaeota were the dominant phyla in the ruminal microbiota (Fig. 1, panel a). Fig. 1, panel b shows the percentage of bacterial community based on genus-level classification, with *Prevotella* 1 (30.6–43.45%) being the predominant genera in all treatments. *Ruminobacter* (1.18–10.45%), *Rikenellaceae*\_RC9\_gut\_group (4.94–9.26%), *Prevotellaceae*\_UCG-001 (3.66–5.30%), *Succinivibrionaceae*\_UCG-002 (1.71–4.27%), *Christensenellaceae*\_R-7\_group (0.83–2.29%), *Methanobrevibacter* (0.25–2.25%), *Prevotellaceae*\_UCG-003 (1.64–2.56%), *Quinella* (0.35–1.64%), *Pseudomonas* (0.01–1.36%) and others (35.58–42.70%) were detected in all treatments.

### 4. Discussion

#### 4.1. In vitro digestibility

In the current experiment, the addition of fibrolytic enzymes had no effect on the GP at 24 h. This lack of impact is probably related to the short incubation time of the silage (Eun, Beauchemin, 2008) because the enzymes were added to the dried forage just before the ruminal fluid was added to the incubation. In line with this notion, 96 h after the incubation of enzyme-treated silage, the addition of at least 0.4% of the home-made enzyme cocktail resulted in an increased gas production. Apparently, a minimum dose of 0.4% of homemade enzyme is required to effectively degrade the fibre fraction of silage to render hexoses available for fermentation. In line with the enzyme composition of the home-made enzyme cocktail, the supplemental enzymes degraded the insoluble fraction of the substrate but not the soluble fraction.

In the current study, the degradation rate of the insoluble fraction with 0.4% enzyme cocktail versus the commercial enzyme was higher, which is most likely explained by the fact that the homemade enzyme cocktail contained not only cellulase but also xylanase. Indeed, next to cellulase, xylanase is also instrumental to degrade polymers such as

**Table 3**  
Effect of enzyme cocktail addition to Leucaena silage on in vitro total gas production (GP).

	Cellulase*	Enzyme cocktail (%)				SEM	P-value		
	0.2	0	0.2	0.4	0.8		Cell vs 0.4%	L	Q
Total GP (ml/g OM)									
24 h	110.8	98.1	89.6	95.1	99.3	2.2	0.250	0.637	0.168
96 h	208.2	177.8	175.8	191.9	192.4	3.0	0.333	0.046	0.846
Gas kinetics									
a	94.8	86.9	79.6	95.4	82.1	2.9	0.173	0.950	0.618
b	222.4	197.1	206.0	208.9	212.9	2.5	0.195	0.039	0.641
c	0.032	0.035	0.032	0.030	0.033	0.0	0.039	0.328	0.083
a+b	317.1	283.9	285.6	304.2	295.1	3.7	0.112	0.130	0.208

\* Commercial cellulase enzyme. Enzyme cocktail derived from *Aspergillus niger* (0 = no enzyme additive, 0.2%, 0.4%, and 0.8% of dry matter). L, linear; Q, Quadratic; a, the gas production associated with the immediate soluble fraction (ml); b, the gas production associated with the insoluble fraction (ml); c, the gas production rate constant for the insoluble fraction b (ml/h); a+b, the potential gas production (ml). SEM, standard error of the mean.



**Table 4**

Feed intake, fecal excretion and apparent fecal digestibility of selected macronutrients after the feeding of the experimental rations.

Items	Dose of enzyme cocktail				SEM	P - values dose effect	
	0	0.2	0.4	0.8		L	Q
Intake (g DM/d)							
Silage	1174	1176	1220	1213	7.36	0.025	0.767
Concentrate	310	307	307	307	0.33	0.258	0.100
Total DM intake, g/d	1484	1483	1527	1520	7.44	0.033	0.827
Feces DM, g/d	558	576	515	513	6.59	0.005	0.806
DM digested g/d	926	907	1012	1007	9.19	< 0.001	0.379
% of intake	62.5	61.2	66.2	66.2	0.38	< 0.001	0.664
Total OM intake, g/d	1360	1359	1400	1393	6.81	0.047	0.391
Feces OM, g/d	498	504	464	457	9.72	0.065	0.853
OM digested g/d	862	855	936	937	9.45	0.015	0.530
% of intake	63.4	63.0	67.0	67.0	0.37	0.033	0.827
Total CP intake, g/d	225	228	234	232	1.35	0.067	0.171
Feces CP, g/d	95	98	89	88	1.49	0.066	0.995
CP digested g/d	131	131	145	144	1.66	0.004	0.266
% of intake	58.0	57.3	61.8	61.8	0.42	0.008	0.647
Total NDF intake, g/d	669	668	690	687	3.64	0.044	0.385
Feces NDF, g/d	296	291	264	275	3.76	0.028	0.078
NDF digested g/d	372	378	426	412	4.72	0.002	0.043
% of intake	55.6	56.5	61.7	60.2	0.55	0.003	0.216
Total ADF intake, g/d	520	519	538	535	3.11	0.042	0.441
Feces ADF, g/d	196	197	181	189	3.01	0.299	0.299
ADF digested g/d	324	322	357	346	2.96	0.003	0.072
% of intake	62.2	62.0	66.1	64.6	0.47	0.021	0.111

Dose of enzyme cocktail as percent of DM forage substrate (0 = no enzyme additive, 0.2%, 0.4%, and 0.8% of dry matter).

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; SEM, standard error of the mean.

**Table 5**Effect of the enzyme cocktail on ruminal pH, volatile fatty acid (VFA) concentration, and methane (CH<sub>4</sub>) production before and 4 h after feeding the experimental rations.

Items	Dose of enzyme cocktail				SEM	P-value dose effect	
	0	0.2	0.4	0.8		L	Q
Before feeding							
pH	6.9	7.0	6.9	6.9	0.02	0.293	0.873
Total VFA (mmol/l)	116.7	105	125.6	113.1	3.90	0.896	0.665
Acetic acid (AA) (mol/100 ml)	59.1	53.5	58.5	56.0	1.10	0.679	0.664
Propionic acid (PA) (mol/100 ml)	34.7	30.2	33.9	32.3	0.54	0.506	0.367
Butyric acid (BA) (mol/100 ml)	3.65	3.7	3.65	3.48	0.18	0.693	0.816
AA/PA ratio	2.0	2.2	2.0	2.0	0.04	0.721	0.354
CH <sub>4</sub> production (mmol/l)	18.3	17.2	17.1	17.7	0.44	0.740	0.304
4 h after feeding							
pH	6.5	6.6	6.5	6.6	0.03	0.817	0.656
Total VFA (mmol/l)	152.7	128.3	153.3	132.8	4.70	0.351	0.954
Acetic acid (AA) (mol/100 ml)	59.5	60.7	62.8	55.3	1.24	0.213	0.106
Propionic acid (PA) (mol/100 ml)	34.1	37.1	34.1	35.4	0.90	0.918	0.811
Butyric acid (BA) (mol/100 ml)	3.0	2.3	3.0	3.6	0.21	0.150	0.258
AA/PA ratio	2.0	1.9	2.1	1.8	0.07	0.395	0.321
CH <sub>4</sub> production (mmol/l)	16.8	17.4	17.1	16.0	0.47	0.462	0.472

Dose of enzyme cocktail as percent of DM forage substrate (0 = no enzyme additive, 0.2%, 0.4%, and 0.8% of dry matter). SEM, standard error of the mean.

xylan into simple monosaccharide and xylooligosaccharides (Bhardwaj et al., 2019).

#### 4.2. Feed intake and digestibility

In the current study, the addition of an enzyme cocktail to the silage resulted in an increased digestibility of NDF. The increase in NDF digestibility can be explained by the action of the enzymes which can break down hemicellulose-cellulose, making cellulose more accessible. Furthermore, the enzyme cocktail also improved the digestibility of the ADF fraction of the diet. The observed improvement of fibre digestion are consistent with previous studies showing that supplemental fibrolytic enzymes, rich in xylanase and cellulase, to dairy cow diets

increased NDF digestibility (Refat et al., 2018; Salem et al., 2013; Peters et al., 2015). In addition, a greater NDF digestibility results in a more rapid disappearance of NDF from the rumen, thereby reducing the physical fill of the rumen, and therefore allowing a greater voluntary feed intake (Allen and Oba, 1996). This notion is in line with the current observation that the supplementation of silage with enzyme cocktail resulted in a greater DMI of *Leucaena* silage. This finding is consistent with results reported by Kondratovich et al. (2019), who also found that supplemental fibrolytic enzymes increased DMI and in steers.

The observed improvement of the CP digestibility is in line with the fact that the home-made enzyme cocktail also contains protease. Moreover, xylanases and cellulases act in concert with proteases to attack nitrogen-containing compounds in cell walls (Beauchemin and

**Table 6**

Alpha diversity of ruminal microbial community of goats fed with the experimental rations.

Items	Dose of enzyme cocktail			
	0	0.2	0.4	0.8
Observed_species	1427	1268	1318	1218
Shannon	7.35	6.98	7.48	7.16
Simpson	0.97	0.95	0.98	0.97
Chao1	1553	1372	1380	1347
ACE	1571	1396	1425	1347
Good_coverage	0.994	0.995	0.996	0.995

Dose of enzyme cocktail as percent of DM forage substrate (0 = no enzyme additive, 0.2%, 0.4%, and 0.8% of dry matter).

Holtshausen, 2010; Arce-Cervantes et al., 2013), thereby further explaining the improved CP digestibility of the enzyme-treated silages. For obvious reasons, the observed increase in the digestibility of NDF, ADF, and CP also explains the increase in the digestibility of OM and DM.

Increasing the enzyme cocktail supplementation up to 0.4% enhanced fibre digestibility in goats that were fed enzyme-treated Leucaena silage. However, increasing the supplemental home-made enzyme cocktail to 0.8% did not lead to further improvements in nutrient digestibility. This observation is corroborated by Morgavi et al. (2004), who reported that low levels of exogenous enzymes stimulate the adhesion of rumen microbes to substrate, while high levels of exogenous enzymes may compete with the enzymes produced by the rumen population for binding sites on feed. It thus appears that a high dose of the

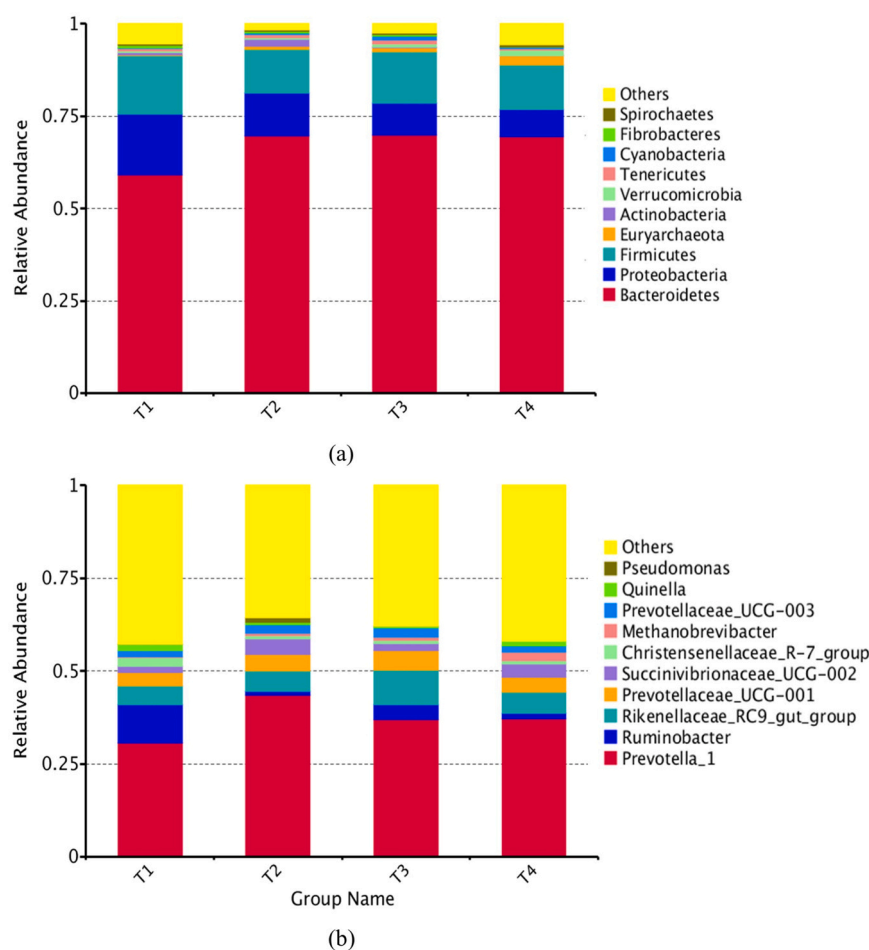
enzyme cocktail is not warranted to further improve fibre digestion in the rumen environment.

### 4.3. Rumen Fermentation

In the current study, rumen pH values were found to in the optimum range (pH 6.5–7.0) to digest fibre (Zicarelli et al., 2011). The current results on rumen pH are in line with results reported by Colombatto et al. (2003) who showed that the capacity of fibrolytic enzymes to digest NDF was greater when pH values ranged from 6.0–6.6 instead of 5.4–6.0. The greater fibre digestion reported by Weimer (2022) was largely due to an increase in hemicellulose degradation. The addition of exogenous enzyme improved fibre digestion, which may lead to a greater molar proportion of acetate and lower molar proportions of propionate (Yang et al., 1999). However, the addition of 0.8% enzyme 4 h after feeding revealed a tendency toward low AA and low PA concentrations. It was already mentioned that supplemental, exogenous enzymes may compete with the endogenous enzymes in the rumen (Morgavi et al., 2004) which may explain the minimal effect of enzyme addition on VFA in this study.

### 4.4. Rumen microbes

Bacteroidetes, Firmicutes, Proteobacteria and Euryarchaeota were found to be the dominant phyla of the rumen microbiome of the goats. This finding is consistent with data reported by Li et al. (2021) in HNB goats (2 months old). In our study, *Prevotella\_1* was found to be the dominant genus which is corroborated by earlier findings in goat, deer, and bovine (Li et al., 2021; Han et al., 2015; Mao et al., 2015). In the



**Fig. 1.** Percent of relative abundance of microbial community on phylum (a) and genus (b) level. Dose of enzyme cocktail as percent of DM forage substrate (T1 = 0, T2 = 0.2, T3 = 0.4, T4 = 0.8% of dry matter).

current study, the abundance of *Prevotella*, was high in the goat fed the enzyme cocktail. This observation is not easy to explain but it might be related to the greater availability of insoluble fibre when an enzyme supplemented diet is fed. This notion is corroborated by Chen et al. (2019) who found that insoluble fibre increased the relative abundance of Bacteroidetes, including *Prevotella*. It is commonly accepted that the insoluble fraction slowly degrades, subsequently causing a slow release of glucose. This might stimulate the growth of *Prevotella* and *Prevotella*, which can utilize a wide variety of polysaccharides and are thought to be important contributors to xylan degradation in the rumen (Takizawa et al., 2022). These mode of actions might explain the observed increase in fibre digestibility when the goats were fed the silage treated with enzyme cocktail. However, the number of animals used in this study was limited, thus more research is warranted to substantiate the current findings.

## 5. Conclusion

The results of the current study indicate that a home-made enzyme cocktail derived from dried tomato pomace incubated with *A. niger* could improve the nutrient digestibility in goats fed the enzyme-treated *Leucaena* silage. Thus, the addition of 0.4% of the current enzyme cocktail was found to be effective in enhancing the fibre- and protein digestibility in *Leucaena* silage-based rations. Further research is needed to investigate the long-term effects of cocktail enzymes on growth performance and ruminant production.

## CRediT authorship contribution statement

**Smerjai Bureenok:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Nittaya Pitiwittayakul:** Visualization, Investigation. **Benya Saenmahayak:** Visualization, Investigation. **Sukanya Saithi:** Investigation. **Chalermporn Yuangklang:** Conceptualization, Supervision. **Yimin Cai:** Supervision. **Jan Thomas Schonewille:** Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare no conflicts of interest.

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