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Research article

Investigation of the effect of different additives on the qualities, *in vitro* degradation, and rumen fermentation profile of indigo waste silage

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Abstract: Natural indigo dye production produces indigo waste as a by-product. Our purpose of this study was to examine the effects of calcium hydroxide ($Ca(OH)_2$), cellulase (CE), molasses (MO), and their combinations on the silage quality, *in vitro* degradability, and rumen fermentation parameters of indigo waste silage. A completely randomized design (CRD) was used for the experiment. Indigo waste was chopped and ensiled in a small-scale silo with no additive (control), $Ca(OH)_2$, MO, CE, $Ca(OH)_2$:MO, $Ca(OH)_2$:CE, MO:CE, and MO:CE:$Ca(OH)_2$. After 30 days of storage, the silages were tested for quality and chemical composition, as well as an *in vitro* fermentation. The ruminal fluid inoculum was collected from two beef cattle with a body weight (BW) of 200±10 kg, and the inoculum had been pre-heated before being transported to the laboratory. Silage with MO, CE, or their combination increased the amount of lactic acid ($p < 0.01$). The silage pH was lowest in MO:CE (4.5) and was highest in $Ca(OH)_2$:CE (10.6) in indigo waste ($p < 0.01$). In comparison to the control (19.5% CP), the CP content of all additives increased by 20.7% to 21.5% ($p = 0.02$). The addition of $Ca(OH)_2$:MO and $Ca(OH)_2$:CE resulted in a reduction of NDF content by 60.7% and 59.4%, respectively, in comparison to the control group (72.4%) ($p < 0.01$). Silage with additives had no effect on the cumulative gas production or gas kinetics, except that the constant rate of gas production for the insoluble fraction (c) was higher in MO ($p = 0.03$). *In vitro* dry matter degradability (IVDMD) was higher in CE and MO and highest in MO:CE-treated silage ($p < 0.01$). The *in vitro* organic matter degradability (IVOMD) increased in $Ca(OH)_2$:MO compared with the control ($p = 0.03$). The additives alone or in their two combinations in silage reduced the ruminal ammonia-nitrogen (NH_3-N) concentration (28.0 to 31.5 mg/dL) when compared to the control (32.7 mg/dL) ($p < 0.01$). In addition,

the highest total volatile fatty acid (VFA) level was found in the silage of the MO (92.9 mmol/L) compared with the control (71.3 mmol/l) ($p < 0.01$). The proportion of propionic acid and butyric acid increased ($p < 0.01$) whereas acetic acid decreased ($p < 0.01$) in the rumen of silage with MO and CE. In summary, the addition of MO and CE has the potential to be used in the silage of indigo waste.

Keywords: indigo waste; silage; calcium hydroxide; cellulase; molasses; rumen fermentation

1. Introduction

The plant *Indigofera tinctoria* L., often known as indigo, belongs to the legume family *Fabaceae* and is found in Africa and tropical Asia [1]. Indigo has been used to produce natural indigo dye for cotton textiles by traditional methods in small-scale industries in Thailand. Indigo plants are harvested for indigo dye production in the rainy season from August to October of each year in the northeast. The indigo biomass produced an average of 339 kg/ha [2] and indigo waste was separated from the water at 70–77% [3], or 237–261 kg/ha. The remaining indigo waste after the dye production procedure contains the stem and leaves [4]. The indigo waste consists of 19.8% CP, 46.6% NDF, 3,487.5 kcal/kg gross energy (GE), 5.4% condensed tannins, and 13.1% crude saponins [5]. In order to enhance the cost-effectiveness and environmental sustainability of indigo by-products, they might be used as a potential source of animal feed. Our previous studies found that the addition of dried indigo waste at 10% in a concentrate diet suitable for feeding to growing beef cattle while increasing levels of the dried indigo waste up to 30% resulted in a slightly lower feed intake, nutrient digestibility, rumen fermentation, and growth performance [5]. In addition, after the indigo dye production process, indigo waste quickly deteriorates after a few days, and sun-drying can be difficult during the rainy season.

Silage is the appropriate method for the controlled fermentation of crop residues with high moisture levels. It is now widely understood that the addition of silage additives improves silage quality, nutritive value, and degradability [6,7]. Chemical and biological treatments are usually utilized in silage additive studies, such as fermentable cellulase, sugar substrates, or $Ca(OH)_2$ [8,9]. The inclusion of cellulase may enhance the breakdown of cell walls, thereby facilitating the release of fermentable substrate that can support the growth of lactic acid bacteria (LAB) [10,11]. In theory, enzymes' ability to release more substrate for LAB growth could increase lactic acid production, which would improve the quality of fermentation in the silage [11,12]. Adding molasses can provide a source of readily fermentable sugars for the growth of LAB to produce lactic acid [13]. Previous studies have reported that the addition of molasses and cellulase or combination ensiling of by-products improved the quality of silage, its nutritive value*, in vitro* degradability, and rumen fermentation patterns [14–16]. In addition, alkaline agents, especially $Ca(OH)_2$ breaks down the ester bonds that connect lignin, hemicellulose, and cellulose, which improve the degradation and fermentation of silage [17]. Earlier studies found that treating the by-product with $Ca(OH)_2$ has a positive effect on chemical compositions, *in vitro* gas production, digestibility of nutrients, and rumen metabolites in ruminants [7,9,18]. However, the indigo waste treated with the additives as ruminant feed has not been studied.

We hypothesize that the additives enhance the quality of silage, degradation, and fermentation characteristics. Therefore, our purpose of the study was to examine the impact of $Ca(OH)_2$, molasses, cellulase, and their combinations on the silage quality, *in vitro* degradability, and rumen fermentation of indigo waste silage.

2. Materials and methods

2.1. Ethics approval of research

The animal experimentation procedure was approved by the Animal Care and Use Committee of the Rajamangala University of Technology Isan (approval number 46/2565). The present investigation has substantiated that all met.

2.2. Treatment and fermentation preparation

The indigo waste (leaf and stem) was obtained after the dye was extracted from the indigo fabric community enterprise group in Baan Nohnrua, Pannanikom, Sakon Nakhon, Thailand. Indigo waste was chopped to a size of 1–2 cm. We conducted this study using a completely randomized design (CRD). The indigo waste was treated with eight treatments: 1) no additive (control), 2) calcium hydroxide ($Ca(OH)_2$) at 2% fresh matter (FM), 3) molasses (MO) at 3% FM, 4) cellulase (CE) at 0.2 g/kg FM, 5) calcium hydroxide and molasses ($Ca(OH)_2$:MO), 6) calcium hydroxide and cellulase ($Ca(OH)_2$:CE), 7) molasses and cellulase (MO:CE), and 8) molasses, cellulase, and calcium hydroxide (MO:CE:$Ca(OH)_2$). The CE enzyme (10,000 U/g, Sinobios IMP. & Exp. Co., Ltd, Shanghai, China). The additives were sprayed onto the indigo waste and then thoroughly combined. The indigo waste mixtures containing 500 g were packed into vacuum bags (Hiryu KN type, Asahi Kasei Pax Corp., Tokyo, Japan) with 32 bags (4 bags per treatment, 8 treatments), then sealed using a vacuum sealer (SQ-303, Asahi Kasei Pax Corp.). The silages were stored at room temperature and were then opened after a fermentation period of 30 days for silage quality, chemical analysis, and an *in vitro* investigation.

2.3. Fermentation end products and chemical composition of silages

A sample of silage (10 g of FM) was mixed with 90 mL of sterile distilled water and stored overnight at 4 °C [19]. The pH value was then measured with a portable pH meter (FiveGo, Mettler-Toledo GmbH, Greifensee, Switzerland). Measured the concentrations of lactic acid, acetic acid, propionic acid, and butyric acid by adding 480 μL of periodic acid (100 mM) and 300 μL of formic acid (10%) to 720 μL of standard as well as sample [20]. The mixture was then transferred to a gas chromatography machine (Nexis GC-2030, Shimadzu Co., Kyoto, Japan). The samples were evaluated for estimating the quantity of dry matter (DM), ash, and CP [21]. The NDF and acid detergent fiber (ADF) [22] were determined using a fiber analyzer (ANKOM 200, ANKOM Technology, NY, USA).

2.4. Animals and preparation of rumen inoculum

To supply the rumen fluid, two crossbred (Brahman x Thai native) beef cattle with a body weight (BW) of 200 ± 10 kg were used. Concentrate was added at a rate of 0.5% BW as DM, and rice straw was provided to the animal *ad libitum*. The cattle were fed twice daily, at 7:00 a.m. and 3:00 p.m., for 14 days. On day 15, 1000 mL of rumen fluid was collected from each animal using a stomach tube connected with a vacuum pump before the animal's morning feeding. The first 100 mL of rumen fluid were discarded in order to prevent saliva contamination. Rumen fluid was filtered through four layers of cheesecloth, placed in thermos flasks that had been pre-heated, and then transported to the laboratory.

Samples from each indigo waste silage were obtained, subjected to oven drying at 60 °C for a duration of 72 h, and crushed after passing through a 1 mm sieve. Samples of 0.5 g of each silage were weighed into 50 mL serum bottles. For a total of 36 bottles, there were four replicates of each treatment, 32 sample bottles, and 4 blank bottles. The ruminal fluid obtained from each animal was combined with an artificial saliva solution in a ratio of 2:1 (mL/mL) at a temperature of 39 °C while maintaining a constant flow of CO_2 [23]. Each of the bottles contained 40 mL of rumen inoculum and was flushed with CO_2. Aluminum caps and rubber stoppers have been used to cover the bottles. They were incubated at 39 °C and shaken at 60 rpm (Stuart orbital incubator S1600, Staffordshire, UK).

2.5. In vitro gas production and fermentation characteristics

The volume of gas produced was measured using a 25 mL calibrated glass syringe with an air connection at 0, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours. Each experimental run at each sample time contained four bottles containing only rumen inoculums. The average gas production quantities from these bottles were used as blanks. The net gas volume was calculated by subtracting the blank values from each value. The total amount of gas produced was put into the model of Orskov and McDonald [24] as follows: $y = a + b\ [1 - e^{(-ct)}]$. In this context, let us define the following variables: a = the gas production from the immediately soluble fraction; b = the gas production from the insoluble fraction; c = the constant rate of gas production for the insoluble fraction; a + b = the potential extent of gas production; t = the incubation time; and y = the gas production at time "t".

Utilizing 32 bottles (4 bottles per treatment, 8 treatments), the IVDMD and IVOMD [25] were assessed after 24 hours of incubation. Using a second set of 32 bottles (4 bottles per treatment, 8 treatments), the pH, NH_3-N [26] (Kjeltech Auto 1030 Analyzer, Tecator, Hoganiis, Sweden), and VFA [27] of ruminal fluid were assessed.

2.6. Statistical analysis

The data was evaluated for variances using an ANOVA procedure in SAS software [28], which uses a CRD. The following model was used to examine the data: $Yij = \mu + \alpha i + \varepsilon ij$, where Yi is the observation, μ is the overall mean, αi is the treatment effect, and εij is the residual error. Using Duncan's new multiple-range test, the mean difference between treatments was evaluated at $p < 0.05$.

3. Results

3.1. Fermentation quality and chemical composition of silage

The pH of silage was lower for MO and MO:CE, whereas it was higher when adding only $Ca(OH)_2$ to their combination when compared to the control ($p < 0.01$) (Table 1). The lactic acid content increased by 49.6, 44.9, and 72.2 g/kg DM for MO, CE, and MO:CE, respectively, when compared to the control (20.5 g/kg DM). The addition of $Ca(OH)_2$:MO and $Ca(OH)_2$:CE decreased the lactic acid content by 5.8 and 0.4 g/kg DM, respectively ($p < 0.01$). In addition, the acetic acid content of MO and MO:CE was lower than that of other treatments ($p < 0.01$). Adding $Ca(OH)_2$:MO and $Ca(OH)_2$:CE resulted in the lowest butyric acid concentration ($p < 0.01$). The CP content was increased in all additives (20.7–21.5% CP) than that of the control (19.5% CP) ($p = 0.02$) (Table 2).

The NDF content decreased when adding $Ca(OH)_2$:MO and $Ca(OH)_2$:CE (60.7 and 59.4%, respectively) compared with the control (72.4%) ($p < 0.01$). The ash was higher for silage treated with $Ca(OH)_2$, $Ca(OH)_2$:MO, and MO:CE:$Ca(OH)_2$, and highest for $Ca(OH)_2$:CE ($p < 0.01$).

Table 1. Effect of different additives of indigo (*Indigofera tinctoria* L.) waste silage on fermentation characteristics of silage after 30 d fermentation.

Additive at ensiling*	pH	(g/kg DM)			
		Lactic acid	Acetic acid	Propionic acid	Butyric acid
Control	5.5^{d}	20.5^{c}	43.9^{abc}	12.2^{a}	3.0^{c}
$Ca(OH)_2$	7.4^{c}	14.9^{c}	48.7^{ab}	2.3^{bc}	2.1^{c}
MO	5.0^{e}	49.6^{b}	24.5^{d}	1.7^{cd}	6.0^{b}
CE	5.1^{de}	44.9^{b}	37.9^{c}	3.3^{bc}	11.2^{a}
$Ca(OH)_2$:MO	8.9^{b}	5.8^{d}	53.5^{a}	0.2^{d}	0.3^{d}
$Ca(OH)_2$:CE	10.6^{a}	0.4^{d}	39.4^{bc}	0.2^{d}	0.1^{d}
MO:CE	4.5^{f}	72.2^{a}	24.6^{d}	0.5^{d}	3.1^{c}
MO:CE:$Ca(OH)_2$	7.4^{c}	21.0^{c}	44.8^{abc}	4.2^{b}	6.1^{b}
SEM	0.17	2.70	3.20	0.59	0.44
p-value	<0.01	<0.01	<0.01	<0.01	<0.01

Note: *Control: no additive; $Ca(OH)_2$: calcium hydroxide; MO: molasses; CE: cellulase (CE); $Ca(OH)_2$:MO: calcium hydroxide and molasses; $Ca(OH)_2$:CE: calcium hydroxide and cellulase; MO:CE: molasses and cellulase; MO:CE:$Ca(OH)_2$: molasses, cellulase and calcium hydroxide.

Table 2. Effect of different additives of indigo (*Indigofera tinctoria* L.) waste silage on chemical composition.

Additive at ensiling*	Chemical composition (%)					
	DM	OM	CP	NDF	ADF	Ash
Control	38.4	93.7^{a}	19.5^{b}	72.4^{a}	51.6	6.4^{d}
$Ca(OH)_2$	38.7	88.1^{b}	20.8^{a}	71.4^{a}	51.7	11.9^{c}
MO	35.3	93.5^{a}	20.8^{a}	70.3^{a}	49.8	6.5^{d}
CE	34.3	93.6^{a}	21.5^{a}	73.5^{a}	53.5	6.4^{d}
$Ca(OH)_2$:MO	38.8	83.4^{c}	21.2^{a}	60.7^{b}	45.1	16.5^{b}
$Ca(OH)_2$:CE	36.8	81.8^{d}	20.7^{a}	59.4^{b}	47.6	18.2^{a}
MO:CE	38.3	93.3^{a}	21.4^{a}	70.1^{a}	47.4	6.7^{d}
MO:CE:$Ca(OH)_2$	40.6	87.9^{b}	21.5^{a}	67.7^{a}	48.3	12.1^{c}
SEM	1.57	0.23	0.37	1.99	2.13	0.22
p-value	0.15	<0.01	0.02	<0.01	0.27	<0.01

Note: *Control: no additive; $Ca(OH)_2$: calcium hydroxide; MO: molasses; CE: cellulase (CE); $Ca(OH)_2$:MO: calcium hydroxide and molasses; $Ca(OH)_2$:CE: calcium hydroxide and cellulase; MO:CE: molasses and cellulase; MO:CE:$Ca(OH)_2$: molasses, cellulase and calcium hydroxide.

3.2. Gas kinetics, cumulative gas production, and in vitro degradability

All treatments did not change ($p > 0.05$) the cumulative gas at 96 h, the gas production from the immediately soluble fraction (a), the gas production from the insoluble fraction (b), or the potential extent of gas production (a + b) (Table 3). However, the constant rate of gas production for the insoluble fraction (c) was higher when MO was added ($p = 0.03$). The IVDMD was higher ($p < 0.01$) when MO, CE (33.7 and 32.4%, respectively), and the highest found in MO:CE (37.8%) in comparison to the control group (30.2%) (Table 4). The $Ca(OH)_2$:MO treatment showed a significantly higher IVOMD (51.3%) compared to the control treatment (39.5%) ($p = 0.03$).

Table 3. Effect of different additives of indigo (*Indigofera tinctoria* L.) waste silage on gas kinetics, cumulative gas production (96 h) and *in vitro* digestibility.

Additive at ensiling*	Gas kinetics[#]				Gas (96 h)
	a	b	c	a + b	(ml/0.5 g DM substrate)
Control	−2.7	79.8	0.03^{bc}	77.0	73.6
$Ca(OH)_2$	−0.2	72.4	0.03^{bc}	72.2	67.5
MO	−1.5	75.3	0.05^{a}	73.8	71.2
CE	−2.2	82.9	0.04^{abc}	80.6	76.7
$Ca(OH)_2$:MO	−1.3	82.4	0.04^{ab}	81.2	79.2
$Ca(OH)_2$:CE	−1.0	75.5	0.04^{abc}	74.5	73.5
MO:CE	0.1	67.6	0.03^{bc}	67.6	65.9
MO:CE:$Ca(OH)_2$	−1.0	68.2	0.02^{c}	67.2	61.5
SEM	0.77	5.75	0.004	5.25	5.15
p−value	0.33	0.52	0.03	0.55	0.46

Note: *Control: no additive; $Ca(OH)_2$: calcium hydroxide; MO: molasses; CE: cellulase (CE); $Ca(OH)_2$:MO: calcium hydroxide and molasses; $Ca(OH)_2$:CE: calcium hydroxide and cellulase; MO:CE: molasses and cellulase; MO:CE:$Ca(OH)_2$: molasses, cellulase and calcium hydroxide. [#]a: the gas production from the immediately soluble fraction; b: the gas production from the insoluble fraction; c: the gas production rate constant for the insoluble fraction (b); a + b: the potential extent of gas production.

3.3. In vitro rumen fermentation characteristics

The ruminal pH was similar among treatments ($p > 0.01$) (Table 4). When adding $Ca(OH)_2$, CE, $Ca(OH)_2$:CE, or MO:CE, the ruminal NH_3-N concentration was lower (28.0, 29.2, 26.9, and 28.6 mg/dL, respectively). The lowest concentration was found in $Ca(OH)_2$:MO (24.5 mg/dL), compared to the control (32.7 mg/dL) ($p < 0.01$). The total VFA was increased for MO (92.9 mmol/l) when compared to the control (71.3 mmol/l) ($p < 0.01$) (Table 5). The proportions of propionic acid (C3) and butyric acid (C4) were increased, while acetic acid (C2) and C2:C3 were decreased using MO and CE treated indigo waste silage ($p < 0.01$).

Table 4. Effect of different additives of indigo (*Indigofera tinctoria* L.) waste on *in vitro* degradability, ruminal pH and NH_3-N.

Additive at ensiling*	Degradability[#] (%)		pH	NH_3-N (mg/dL)
	IVDMD	IVOMD		
Control	30.2^{bc}	39.5^{b}	6.5	32.7^{b}
$Ca(OH)_2$	26.8^{c}	43.9^{ab}	6.5	28.0^{cd}
MO	33.7^{ab}	45.2^{ab}	6.3	31.5^{b}
CE	32.4^{b}	40.4^{b}	6.4	29.2^{c}
$Ca(OH)_2$:MO	31.4^{bc}	51.2^{a}	6.5	24.5^{e}
$Ca(OH)_2$:CE	29.4^{bc}	49.1^{ab}	6.5	26.9^{d}
MO:CE	37.8^{a}	44.5^{ab}	6.4	28.6^{cd}
MO:CE:$Ca(OH)_2$	28.4^{bc}	40.2^{b}	6.5	35.6^{a}
SEM	1.70	2.92	0.02	0.69
p-value	<0.01	0.03	<0.01	<0.01

Note: *Control: no additive; $Ca(OH)_2$: calcium hydroxide; MO: molasses; CE: cellulase (CE); $Ca(OH)_2$:MO: calcium hydroxide and molasses; $Ca(OH)_2$:CE: calcium hydroxide and cellulase; MO:CE: molasses and cellulase; MO:CE:$Ca(OH)_2$: molasses, cellulase and calcium hydroxide. [#]IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability.

Table 5. Effect of different additives of indigo (*Indigofera tinctoria* L.) waste on VFA production.

Additive at ensiling*	Total VFA (mmol/L)	VFA[#] (mol/100 mol)						C2:C3
		C2	C3	C4	i-C4	C5	i-C5	
Control	71.3^{bcd}	68.0^{a}	18.4^{bc}	10.0^{bc}	1.0^{a}	1.0^{b}	1.6^{b}	3.7^{bc}
$Ca(OH)_2$	79.6^{ab}	72.4^{a}	16.5^{cd}	8.3^{c}	0.8^{b}	0.8^{c}	1.2^{c}	4.4^{ab}
MO	92.9^{a}	63.0^{b}	21.0^{a}	12.2^{a}	1.0^{a}	1.2^{ab}	1.6^{b}	3.0^{d}
CE	53.1^{d}	63.0^{b}	20.0^{ab}	12.8^{a}	1.1^{a}	1.2^{a}	1.9^{a}	3.2^{cd}
$Ca(OH)_2$:MO	60.3^{cd}	69.9^{a}	18.0^{bc}	9.6^{bc}	0.7^{b}	0.7^{c}	1.2^{c}	3.8^{b}
$Ca(OH)_2$:CE	59.5^{cd}	71.5^{a}	17.4^{c}	8.7^{bc}	0.7^{b}	0.6^{c}	1.1^{c}	4.1^{ab}
MO:CE	76.1^{abc}	69.9^{a}	17.1^{c}	10.2^{bc}	0.8^{b}	0.8^{c}	1.1^{c}	4.2^{ab}
MO:CE:$Ca(OH)_2$	60.0^{cd}	71.8^{a}	15.0^{d}	10.4^{b}	0.8^{b}	0.7^{c}	1.3^{c}	4.8^{a}
SEM	5.52	1.34	0.65	0.60	0.04	0.06	0.08	0.21
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: *Control: no additive; $Ca(OH)_2$: calcium hydroxide; MO: molasses; CE: cellulase (CE); $Ca(OH)_2$:MO: calcium hydroxide and molasses; $Ca(OH)_2$:CE: calcium hydroxide and cellulase; MO:CE: molasses and cellulase; MO:CE:$Ca(OH)_2$: molasses, cellulase and calcium hydroxide. [#]C2: acetic acid; C3: propionic acid; C4: butyric acid; i-C4: iso-butyric acid; C5: valeric acid; i-C5: iso-valeric acid; C2:C3: acetic acid to propionic acid ratio.

4. Discussion

4.1. Fermentation quality and chemical composition of silage

Lactic acid is the most efficient acid for lowering silage pH and preserving forage quality [29]. With the addition of MO and CE, lactic acid levels increased, and the maximum levels were found in

MO:CE, resulting in a decrease in pH. Similarly, So *et al.* [8] reported that sugarcane bagasse fermenting with MO, CE, or their combination increased lactic acid and dropped the pH. The addition of molasses can offer a high quantity of soluble carbohydrate substrate for lactic acid bacteria (LAB) to produce lactic acid, thereby increasing the silage quality [13]. On the theory that exogenous enzymes can degrade cell walls and release soluble carbohydrates, lactic acid bacteria can then convert further into lactic acid [11]. In the present study, adding only $Ca(OH)_2$ or a combination with molasses and cellulase lowered lactic acid while increasing the pH of silage. This result is consistent with Cook *et al.* [30], who showed that $Ca(OH)_2$-treated corn silage reduced lactic acid and had a higher pH. These could explain the fact that the alkaline agents increased the pH, resulting in a lower lactic acid concentration when fermented with forage. Moreover, butyric acid was lowest (<0.3 g/kg DM) when adding $Ca(OH)_2$:MO and $Ca(OH)_2$:CE. Butyric acid shows clostridial metabolic activity, which results in substantial DM loss and insufficient energy recovery [31,32]. These results indicated that adding lime with molasses or cellulase prevented the growth of clostridia and improved the quality and palatability of silage.

All silage additives increased the CP content in the indigo waste silage. It is possible that MO, CE, or a combination stimulates fermentation to produce lactic acid, which speeds up the pH drop and inhibits CP degradation and harmful microorganisms [33]. In addition, previous studies suggested that $Ca(OH)_2$-treated corn silage had a higher CP content when compared to untreated silage [34]. Some *Clostridium* species are capable of converting lactic acid to butyric acid and are also highly proteolytic. In the present study, adding $Ca(OH)_2$ and its combination led to the lowest amount of butyric acid. This result might have inhibited clostridial growth and decreased the silage's proteolytic activity, which improved the CP content. The NDF content decreased when adding $Ca(OH)_2$ with MO or CE to silage. The elimination of lignin and a portion of hemicellulose is referred to as alkali treatment [35]. MO is an easily accessible fermenting stimulant that supplies energy to microbes that hydrolyze plant cell walls [36], and CE may degrade the cellulose components effectively [37]. The lower NDF could be explained by the synergistic impact of $Ca(OH)_2$ with MO or CE. Furthermore, the addition of $Ca(OH)_2$ alone or combined with CE and MO improved the ash content of indigo waste silage. The increasing levels of ash found in alkali-treated forages demonstrated the additive's influence on adapting the mineral content of forages [38].

4.2. Gas kinetics, cumulative gas production, and in vitro degradability

The addition of MO enhances the constant rate of gas production for the insoluble fraction (c). This result was in agreement with So *et al.* [14], who reported that the addition of MO to sugarcane bagasse silage increases the c value *in vitro* gas production technique. It may be due to MO providing soluble carbohydrates for the growth of the bacterial population and also increasing quantities of fermentable substrates in the rumen. When comparing MO and CE to the control group, the IVDMD was higher, with the highest value observed in MO:CE in the silage. Similarly, So *et al.* [14] found that IVDMD was higher in silage treated with MO and CE together than in the control silage. At the time of ensiling, the CE had broken down the plant cell walls in the silage [15]. This makes it easier for microorganisms in the rumen to ferment the silage and improves degradation in the rumen [39,40]. Moreover, high-soluble carbohydrates are obtained by adding MO. These results indicated that the synergistic effect of MO:CE showed the highest efficiency of rumen degradation. The $Ca(OH)_2$:MO-treated silage had a higher IVOMD. It could be plausible that the increase in protein content and a

decrease in fiber level in $Ca(OH)_2$:MO, while also providing more soluble carbohydrates, enhanced the ability of rumen microbes to efficiently degrade nutrients. Previous studies showed that $Ca(OH)_2$ and MO increase the IVOMD of sugarcane bagasse and corn stover [7,41].

4.3. In vitro rumen fermentation characteristics

Not all the additives in an indigo waste silage alter the rumen pH. These results agree with Cherdthong *et al.* [42], who report that using MO or CE-treated rice straws has no effect on the rumen pH of beef cattle. However, So *et al.* [14] found that sugarcane bagasse fermented with molasses showed to lower *in vitro* rumen pH. The rumen pH in the present study for all additives ranges from 6.3–6.5, and the optimal pH range was 6.0–7.0 for the growth and activity of rumen microorganisms [43,44]. The NH_3-N concentration in $Ca(OH)_2$ and CE alone or in two additive combinations was lower than that of the control. The additives in indigo waste silage increase CP content. This makes it plausible that rumen microorganisms breaking down protein into NH_3-N, which is linked to the supply of energy from soluble carbohydrates, could improve microbial protein synthesis and lower NH_3-N. However, the addition of MO:CE:$Ca(OH)_2$ in silage resulted in a higher ruminal NH_3-N concentration than the control. The three additive combinations showed a lower constant rate of gas production for the insoluble fraction (c). This is possible due to rumen microbes lower degradation of nutrients, particularly carbohydrates, while protein is readily available to produce NH_3-N, which then lowers microbial growth and also causes ruminal NH_3-N accumulation. The influence of additives in silage on ruminal NH_3-N may be confounding in our study. Satter and Slyter [45] showed that *in vitro* NH_3-N concentrations of 20–50 mg/dL were optimal for rumen microbial growth. In the current study, the ruminal NH_3-N concentrations (24.5–35.6 mg/dL) were within the normal range.

The enhanced production of VFA in the rumen is associated with the digestibility of nutrients and the rumen microbial population [46]. The higher rumen microbial population could enhance rumen VFA production [47]. The addition of MO to silage increases the total VFA concentration in the rumen. MO has high levels of soluble carbohydrates with higher fermentable forage, which may also increase the energy available for rumen microbial carbohydrate metabolisms, and may enhance VFA production. Ensiling indigo waste changes the VFA profile. Silages with MO and CE increase the proportions of propionic acid, while a decrease in acetic acid results in a lower C2:C3. A partial explanation for this phenomenon could be attributed to the high level of lactic acid in silages. MO in silage had a high population of lactic acid bacteria [15], resulting in a high lactic acid content, as found in our study. When feeding animals, rumen microorganisms rapidly ferment lactic acid to produce propionic acid. Adding cellulase enzyme to silage increases the water-soluble carbohydrate (WSC) by hydrolyzing the cellulose to glucose [48]. High glucose levels stimulate the growth of lactic acid bacteria (LAB), ultimately resulting in increased lactic acid production during ensiling, which is also converted to propionic acid in the rumen of ruminants [43]. In contrast, So *et al.* [14] reported that propionic acid in the rumen did not change when adding MO or CE to sugarcane bagasse silage *in vitro*. Moreover, adding MO and CE enhanced butyric acid and reduced acetic acid production in the present study. There exists a positive association between the production of butyrate and the levels of sugar and fermentable plant cell wall in forage [49]. This is possible due to silage with MO and CE, which may produce more WSC and higher degradability of fiber, resulting in higher butyric acid in the rumen. The process of converting two molecules of acetyl-CoA to butyrate additionally includes two steps that include the incorporation of hydrogen (H_2) [50]. This reduces the influence of the lower

H_2 in the rumen and also inhibits methane production [51]. The alteration in rumen fermentation, characterized by rising propionic acid levels and reduced acetic acid levels, has been documented to have advantageous effects on ruminant productivity [52,53].

5. Conclusions

Adding MO, CE, or their combination increased the lactic acid concentration and reduced the silage pH. The inclusion of $Ca(OH)_2$ or their combination higher silage pH, and lowest acetic acid and butyric acid. Silage with all additives increases the CP content of silage, while a lower NDF content was found when adding $Ca(OH)_2$ with MO or CE. Silages treated with MO, CE, and their combinations improved the IVDMD, while $Ca(OH)_2$:MO increase IVOMD of indigo waste. Ensiled indigo waste by MO and CE showed lower acetic acid and higher propionic acid in the rumen. Based on our results, the addition of MO and CE for ensiled 30 days is recommended for indigo waste silage. Further assessments of the inclusion of additives in indigo waste silage will be conducted in an *in vivo* study.

Use of AI tools declaration

The authors declare that they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflicts of interest.

References

1. Senasri N, Sriyasak P, Suwanpakdee S, et al. (2022) Toxicity of indigo dye-contaminated water on silver barbs (*Barbonymus gonionotus*) and pathology in the gills. *EnvironmentAsia* 15: 106–115. https://doi.org/10.14456/ea.2022.52
2. Srisamran J, Wimolsujarit B, Supuma Y, et al. (2015) Research and development on indigo in sakonnakhon province. Available from: https://www.doa.go.th/research/attachment.php?aid=2190.
3. Pattanaik L, Padhi SK, Hariprasad P, et al. (2020) Life cycle cost analysis of natural indigo dye production from *Indigofera tinctoria* L. plant biomass: A case study of India. *Clean Technol Environ Policy* 22: 1639–1654. https://doi.org/10.1007/s10098-020-01914-y
4. Pattanaik L, Naik SN, Hariprasad P (2019) Valorization of waste *Indigofera tinctoria* L. biomass generated from indigo dye extraction process-potential towards biofuels and compost. *Biomass Convers Biorefin* 9: 445–457. https://doi.org/10.1007/s13399-018-0354-2

5. Gunun N, Kaewpila C, Khota W, et al. (2023) The effect of indigo (*Indigofera tinctoria* L.) waste on growth performance, digestibility, rumen fermentation, hematology and immune response in growing beef cattle. *Animals* 13: 84. https://doi.org/10.3390/ani13010084
6. Kaewpila C, Khota W, Gunun P, et al. (2020) Strategic addition of different additives to improve silage fermentation, aerobic stability and *in vitro* digestibility of Napier grasses at late maturity stage. *Agriculture* 10: 262. https://doi.org/10.3390/agriculture10070262
7. Gunun N, Gunun P, Wanapat M, et al. (2017) Improving the quality of sugarcane bagasse by urea and calcium hydroxide on gas production, degradability and rumen fermentation characteristics. *J Anim Plant Sci* 27: 1758–1765.
8. So S, Cherdthong A, Wanapat M (2020) Improving sugarcane bagasse quality as ruminant feed with *Lactobacillus*, cellulase, and molasses. *J Anim Sci Technol* 62: 648. https://doi.org/10.5187/jast.2020.62.5.648
9. Gunun N, Wanapat M, Gunun P, et al. (2016) Effect of treating sugarcane bagasse with urea and calcium hydroxide on feed intake, digestibility, and rumen fermentation in beef cattle. *Trop Anim Health Prod* 48: 1123–1128. https://doi.org/10.1007/s11250-016-1061-2
10. Zhang YC, Wang XK, Li DX, et al. (2020) Impact of wilting and additives on fermentation quality and carbohydrate composition of mulberry silage. *Asian-Australas J Anim Sci* 33: 254–263. https://doi.org/10.5713/ajas.18.0925
11. Irawan A, Sofyan A, Ridwan R, et al. (2021) Effects of different lactic acid bacteria groups and fibrolytic enzymes as additives on silage quality: A meta-analysis. *Bioresour Technol Reports* 14: 100654. https://doi.org/10.1016/j.biteb.2021.100654
12. Lynch JP, Baah J, Beauchemin KA. (2015) Conservation, fiber digestibility, and nutritive value of corn harvested at 2 cutting heights and ensiled with fibrolytic enzymes, either alone or with a ferulic acid esterase-producing inoculant. *J Dairy Sci* 98: 1214–1224. https://doi.org/10.3168/jds.2014-8768
13. Chen L, Guo G, Yuan X, et al. (2016) Effects of applying molasses, lactic acid bacteria and propionic acid on fermentation quality, aerobic stability and *in vitro* gas production of total mixed ration silage prepared with oat-common vetch intercrop on the Tibetan Plateau. *J Sci Food Agric* 96: 1678–1685. https://doi.org/10.1002/jsfa.7271
14. So S, Cherdthong A, Wanapat M, et al. (2020) Fermented sugarcane bagasse with *Lactobacillus* combined with cellulase and molasses promotes *in vitro* gas kinetics, degradability, and ruminal fermentation patterns compared to rice straw. *Anim Biotechnol* 33: 116–127. https://doi.org/10.1080/10495398.2020.1781146
15. Kaewpila C, Khota W, Gunun P, et al. (2022) Characterization of green manure sunn hemp crop silage prepared with additives: aerobic instability, nitrogen value, and *in vitro* rumen methane production. *Fermentation* 8: 104. https://doi.org/10.3390/fermentation8030104
16. Khota W, Panyakaew P, Kesorn P, et al. (2023) *In vitro* rumen fermentation of coconut, sugar palm, and durian peel silages, prepared with selected additives. *Fermentation* 9: 567. https://doi.org/10.3390/fermentation9060567
17. Wanapat M, Polyorach S, Boonnop K, et al. (2009) Effects of treating rice straw with urea or urea and calcium hydroxide upon intake, digestibility, rumen fermentation and milk yield of dairy cows. *Livest Sci* 125: 238–243.
18. Trach N, Mo M, Dan C (2001) Effects of treatment of rice straw with lime and/or urea on its chemical composition, *in-vitro* gas production and *in-sacco* degradation characteristics. *Livest Res Rural Dev* 13: 5–12.

19. Cai Y, Benno Y, Ogawa M, et al. (1999) Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. *J Dairy Sci* 82: 520–526. https://doi.org/10.3168/jds.S0022-0302(99)75263-X
20. Darwin, WipaCharles, Cord-Ruwisch R (2018) Concurrent lactic and volatile fatty acid analysis of microbial fermentation samples by gas chromatography with heat pre-treatment. *J Chromatogr Sci* 56: 1–5. https://doi.org/10.1093/chromsci/bmx086
21. AOAC (2016) *Official Method of Analysis,* 20th Ed., Arlington, VA, USA: Association of Official Analytical Chemists.
22. Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74: 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
23. Menke KH, Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. *Anim Res Dev* 28: 7–55.
24. Ørskov ER, McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J Agric Sci* 92: 499–503. https://doi.org/10.1017/S0021859600063048
25. Tilley J, Terry R (1963) A two-stage technique for the *in vitro* digestion of forage crops. *Grass Forage Sci* 18: 104–111. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x
26. AOAC (1995) *Official methods of analysis,* 16th Ed., Arlington, VA, USA: Association of Official Analytical Chemists.
27. Cai Y (2004) *Analysis method for silage Japanese Society of Grassland Science Field and Laboratory Methods for Grassland Science,* Tokyo: Tosho Printing Co. Ltd., Tokyo, 279–282.
28. Statistical Analysis System (SAS) (2013) User's guide: Statistic (Version 9), 4th Ed., Cary, NC, USA: SAS Institute Inc.
29. Álvarez S, Méndez P, Martí-Fernández A (2015) Fermentative and nutritive quality of banana by-product silage for goats. *J Appl Anim Res* 43: 396–401. http://dx.doi.org/10.1080/09712119.2014.978782
30. Cook DE, Bender RW, Shinners KJ, et al. (2016) The effects of calcium hydroxide—treated whole-plant and fractionated corn silage on intake, digestion, and lactation performance in dairy cows. *J Dairy Sci* 99: 5385–5393. http://dx.doi.org/10.3168/jds.2015-10402
31. Pahlow G, Muck RE, Driehuis F, et al. (2003) Microbiology of ensiling. In: Buxton DR, Muck DR, Harrison JH, *Silage Science and Technology,* Madison, WI: American Society of Agronomy, 31–93.
32. Kung Jr L, Shaver RD, Grant RJ, et al. (2018) Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J Dairy Sci* 101: 4020–4033. https://doi.org/10.3168/jds.2017-13909
33. Xia C, Liang Y, Bai S, et al. (2018) Effects of harvest time and added molasses on nutritional content, ensiling characteristics and *in vitro* degradation of whole crop wheat. *Asian-Australas J Anim Sci* 31: 354–362. https://doi.org/10.5713/ajas.17.0542
34. Dahlke GR, Euken RM (2013) Calcium oxide and calcium hydroxide treatment of corn silage. Iowa State University Animal Industry Report 2013.
35. Zhang Z (2021) Chapter 5—Waste pretreatment technologies for hydrogen production. In: Zhang Q, He C, Ren J, et al. (Eds.), *Waste to Renewable Biohydrogen: Volume 1: Advances in Theory and Experiments,* Academic Press, 109–122. https://doi.org/10.1016/B978-0-12-821659-0.00004-6

36. Lunsin R, Duanyai S, Pilajun R, et al. (2018) Effect of urea-and molasses-treated sugarcane bagasse on nutrient composition and *in vitro* rumen fermentation in dairy cows. *Agric Nat Resour* 52: 622–627. https://doi.org/10.1016/j.anres.2018.11.010
37. Xu J, Zhang K, Lin Y, et al. (2022) Effect of cellulase and lactic acid bacteria on the fermentation quality, carbohydrate conversion, and microbial community of ensiling oat with different moisture contents. *Front Microbiol* 13: 1013258. https://doi.org/10.3389/fmicb.2022.1013258
38. Santos MC, Nussio LG, Mourão GB, et al. (2009) Nutritive value of sugarcane silage treated with chemical additives. *Sci Agric (Piracicaba, Braz)* 66: 159–163. https://doi.org/10.1590/S0103-90162009000200003
39. Li M, Zhou H, Zi X, et al. (2017) Silage fermentation and ruminal degradation of stylo prepared with lactic acid bacteria and cellulase. *Anim Sci J* 88: 1531–1537. https://doi.org/10.1111/asj.12795
40. Mu L, Wang Q, Wang Y, et al. (2023) Effects of cellulase and xylanase on fermentative profile, bacterial diversity, and *in vitro* degradation of mixed silage of agro-residue and alfalfa. *Chem Biol Technol Agric* 10: 40. https://doi.org/10.1186/s40538-023-00409-4
41. Sutaryono YA, Putra RA, Mardiansyah M, et al. (2023) Mixed *Leucaena* and molasses can increase the nutritional quality and rumen degradation of corn stover silage. *J Adv Vet Anim Res* 10: 118.
42. Cherdthong A, Suntara C, Khota W, Wanapat M. 2021. Feed utilization and rumen fermentation characteristics of Thai-indigenous beef cattle fed ensiled rice straw with *Lactobacillus casei* TH14, molasses, and cellulase enzymes. *Livest Sci* 245: 104405. https://doi.org/10.1016/j.livsci.2021.104405
43. Carrillo-Díaz MI, Miranda-Romero LA, Chávez-Aguilar, G, et al. (2022) Improvement of ruminal neutral detergent fiber degradability by obtaining and using exogenous fibrolytic enzymes from white-rot fungi. *Animals* 12: 843. https://doi.org/10.3390/ ani12070843
44. Gunun N, Wanapat M, Kaewpila, C, et al. (2023) Effect of heat processing of rubber seed kernel on *in vitro* rumen biohydrogenation of fatty acids and fermentation. *Fermentation* 9: 143. https://doi.org/10.3390/fermentation9020143
45. Satter LD, Slyter LL. (1974) Effect of ammonia concentration of rumen microbial protein production *in vitro*. *Br J Nutr* 32: 199–208. https://doi.org/10.1079/BJN19740073
46. Guo G, Shen C, Liu Q, et al. (2020) The effect of lactic acid bacteria inoculums on *in vitro* rumen fermentation, methane production, ruminal cellulolytic bacteria populations and cellulase activities of corn stover silage. *J Integr Agric* 19: 838–847. https://doi.org/10.1016/S2095-3119(19)62707-3
47. Suntara S, Cherdthong A, Uriyapongson S, et al. (2020) Comparison effects of ruminal crabtree-negative yeasts and crabtree-positive yeasts for improving ensiled rice straw quality and ruminal digestion using *in vitro* gas production. *J Fungi* 6: 109. https://doi.org/10.3390/jof6030109.
48. Wang Q, Wang R, Wang C, et al. (2022) Effects of cellulase and *Lactobacillus plantarum* on fermentation quality, chemical composition, and microbial community of mixed silage of whole-plant corn and peanut vines. *Appl Biochem Biotechnol* 194: 2465–2480. https://doi.org/10.1007/s12010-022-03821-y
49. Morvay Y, Bannink A, France J, et al. (2011) Evaluation of models to predict the stoichiometry of volatile fatty acid profiles in rumen fluid of lactating Holstein cows. *J Dairy Sci* 94: 3063–3080. https://doi.org/10.3168/jds.2010-3995

50. Ungerfeld EM (2020) Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front Microbiol* 11: 589. https://doi.org/10.3389/fmicb.2020. 00589
51. Børsting CF, Brask M, Hellwing, ALF, et al. (2020) Enteric methane emission and digestion in dairy cows fed wheat or molasses. *J Dairy Sci* 103: 1448–1462. https://doi.org/10.3168/jds.2019-16655
52. Gunun P, Wanapat M, Anantasook N (2013) Rumen fermentation and performance of lactating dairy cows affected by physical forms and urea treatment of rice straw. *Asian-Australas J Anim Sci* 26: 1295–1303. https://doi.org/10.5713/ajas.2013.13094
53. So S, Wanapat M, Cherdthong A (2021) Effect of sugarcane bagasse as industrial by-products treated with *Lactobacillus casei* TH14, cellulase and molasses on feed utilization, ruminal ecology and milk production of mid-lactating Holstein Friesian cows. *J Sci Food Agric* 101: 4481–4489. https://doi.org/10.1002/jsfa.11087

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