https://doi.org/10.17221/173/2023-CJAS

In vitro and *in sacco* evaluation of total mixed ration silage added different levels of chitosan

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Citation: Anggraeni A.S., Jayanegara A., Sofyan A., Laconi E.B., Kumalasari N.R., Gunadarma I.N., Herdian H., Fidriyanto R. (2024): *In vitro* and *in sacco* evaluation of total mixed ration silage added different levels of chitosan. Czech J. Anim. Sci., 69: 178–190.

Abstract: This experiment aimed to evaluate the effects of chitosan administration to total mixed ration (TMR) silage on ruminal fermentation characteristics and degradability by using both *in vitro* and *in sacco* methods. The completely randomized design (CRD) used in this study included five treatments and six replications. The treatments consisted of TMR silage with distilled water as control (SA), TMR with 1% acetic acid (SB), 0.5% chitosan in 1% acetic acid diluent (SC), 1% chitosan in 1% acetic acid diluent (SD), and 1.5% chitosan in 1% acetic acid diluent (SE). The variables observed were ruminal fermentation parameters, methane production and *in sacco* nutrient degradability. Results revealed that adding 1–1.5% chitosan (SD and SE treatments) reduced ($P < 0.05$) total protozoa, total gas production, methane production, the percentage of acetic acid, and the ratio of acetic acid to propionic acid, while it increased ($P < 0.05$) the proportion of propionic acid. The addition of chitosan (SE treatment) decreased ($P < 0.05$) the degradation of dry matter, but it increased ($P < 0.05$) the degradability of organic matter and crude protein, as determined by *in sacco* analysis.

Keywords: chitosan; *in vitro*; *in sacco*; rumen modifier; silage

All authors are grateful to the Indonesian ministry of Education, Culture, Research, and Technology for funding the research through the "Penelitian Hibah Doktor" program in, the year 2022. Ayu Septi Anggraeni thanks to DAAD-SEARCA for the scholarship.

https://doi.org/10.17221/173/2023-CJAS

In terms of its ability to trap heat, methane (CH_4) has a 28-times greater potential of causing global warming than carbon dioxide (Tapio et al. 2017). Agriculture has a substantial environmental impact owing to CH_4 emissions from ruminant livestock (Tapio et al. 2017). Recent studies on animal nutrition have targeted the control of rumen fermentation to decrease CH_4 emissions through manipulating the ruminal microbial ecosystem with feed additives, antibiotics, CH_4 inhibitors, defaunating agents and plant extracts that alter the microbial ecosystem in order to improve animal performance (Wencelova et al. 2014; Belanche et al. 2016; Harahap et al. 2020). Chitosan is a natural substance with antibacterial capabilities that can be used to modify the rumen microbial community (Kong et al. 2010; Harahap et al. 2020; Anggraeni et al. 2022). Two repeating units, namely D-glucosamine and *N*-acetyl-D-glucosamine, are linked by -(14)-linkages to form the linear polysaccharide chitosan. Classified as α, β, and γ chitin, it is a naturally occurring polymorph with three distinct microfibril orientations. They are characterized by intrinsic properties such as molecular weight, viscosity, and degree of deacetylation. Insects, crustaceans, molluscs, fungi, and some algae are common sources of chitosan; their structural exoskeletons contain chitosan, but the majority are derived from marine crustaceans (Jimenez-Ocampo et al. 2019).

One technique for forage preservation is silage. Inadequate ensiling can occasionally lower the dry matter (DM) content, allowing mould to grow as well as yeast, which produces ethanol in the produced silage. Several additives have been applied during silage production to reduce fermentative losses, improve the silage chemical composition and rate of digestibility, lower CH_4 emissions, and prevent the rot in the silage product. Among the silage additives, chitosan was demonstrated to improve the nutritional and fermentative quality of the product, as well as to decrease yeast and mould growth (Gandra et al. 2016; Seankamsorn et al. 2019). Chitosan reduced silage ethanol concentration and fungal activity, in which the ethanol generation in silage is promoted by fermentation of water-soluble carbohydrates catalyzed by yeasts (Gandra et al. 2016).

Chitosan has been shown to affect feed intake, fermentation, digestion, and enteric CH_4 generation as well as silage additives that enhance its quality (Harahap et al. 2020). The potential of chitosan to reduce CH_4 emissions has been demonstrated in numerous studies (Seankamsorn et al. 2019; Harahap et al. 2020). The potential application of chitosan to modulate ruminal fermentation and modify the feed efficiency of ruminant animals was suggested by Goiri et al. (2009). However, the degradability of DM and neutral detergent fibre (NDF) in high forage diets is negatively affected by chitosan *in vitro* compared with control diet (Wencelova et al. 2014). This effect is apparently related to the negative effects of chitosan on cellulolytic bacteria (Belanche et al. 2016). As chitosan is a harmless and biodegradable biopolymer, it affects the fermentation patterns of various ruminant diets.

Although a number of studies have evaluated the effect of chitosan as a rumen modifier as described above, there are only limited studies addressing its effect when added to TMR silage at graded levels. This study therefore aimed to evaluate how the level of natural chitosan as a rumen modifier affects *in vitro* rumen fermentation characteristics and nutrient degradability in TMR high-protein silage.

MATERIAL AND METHODS

Ethical clearance approval

All procedures were in accordance with the standard of ethical procedure from the Commission of Ethical Clearance for Pre-clinical Experiment, Integrated Laboratory for Research and Testing (LPPT), Universitas Gadjah Mada, Yogyakarta, Indonesia (Approval No.: 00004/04/LPPT/IV/2022).

Materials

The chitosan used in this study was extracted from swimming crab shells using a green chemistry extraction method based on Liu et al. (2012) and Anwar et al. (2017). The crab shells were washed, air-dried, boiled in water at 80 °C for 15 min, soaked in 5% phosphoric acid (H_3PO_4) for 15 min, oven-dried for 48 h at 50 °C, and ground to pass through a 2 mm screen. The sample powder was treated with a 1 : 5 solution of 2 *N* hydrochloric acid (HCl) at 70 °C for 2 hours. Following the demineralization step, the sample powder was rinsed with distilled water until neutrality was achieved. Deproteinization was accomplished using alkaline

https://doi.org/10.17221/173/2023-CJAS

Table 1. Formula and chemical composition of the feed ingredients used in the high-protein total mixed ration silage (% DM of TMR)

Feed ingredient	Proportion (%)	Mstr	DM	CP	CF	EE	Ca	P	TDN	NFE	Ash	WSC
King grass	7.50	0.900	6.60	0.840	2.22	0.140	0.040	0.030	5.04	3.12	1.18	0.610
Ground corn	67.5	2.700	58.0	6.55	2.90	4.66	0.030	0.420	47.2	51.2	2.23	15.9
Soybean meal	24.0	0.960	20.6	10.7	1.06	0.260	0.070	0.140	17.3	10.6	1.39	1.44
Salt	0.500	–	–	–	–	–	0.000	–	–	–	–	–
Mineral mix	0.500	–	–	–	–	–	0.250	0.120	–	–	–	–
Total	100	4.56	85.3	18.1	6.18	5.06	0.400	0.720	69.5	64.8	4.80	17.9

Each treatment added 1.2 litres of water to 12 kg of as feed material to achieve a moisture content of ± 37%
Ca = calcium; CF = crude fibre; CP = crude protein; DM = dry matter; EE = ether extract; Mstr = moisture; NFE = nitrogen-free extract; P = phosphorus; TDN = total digestible nutrient; TMR = total mixed ration; WSC = water soluble carbohydrate

treatment with a 2 M sodium hydroxide (NaOH) (1 : 2.5) solution at 85 °C for 16 h, followed by washing with distilled water until neutral pH was reached (Kaya et al. 2014). The sonication method was used to deacetylate chitin, which was suspended in a 60% NaOH aqueous solution (1 : 5). The solution was heated in an ultrasonic bath at 60 °C for 30 minutes. The product was then filtered, washed with distilled water until neutral pH, and allowed to dry (Anwar et al. 2017).

Chitosan was added to the TMR silage formulation (Table 1) at the concentration of chitosan dissolved in 1% acetic acid. Water was added to feed materials to obtain a moisture content of 75%. Thoroughly blended components were packaged in a 2.0 l/silo plastic mini silo (± 1.5 kg) and incubated for 49 days (Muck et al. 2018). This study used a completely randomized design (CRD) with five treatments and five replications: control/SA (TMR without chitosan), SB (TMR with 0% chitosan, diluted in 1% acetic acid), SC (TMR with 0.5% chitosan diluted in 1% acetic acid), SD (1% chitosan diluted in 1% acetic acid), and SE (1.5% chitosan diluted in 1% acetic acid).

In vitro ruminal fermentation

The *in vitro* gas production technique was utilised to measure *in vitro* fermentability. Two glass syringes (100 ml) with a rumen buffer (blank), two syringes for the standard (Pangola grass), and five syringes for each treatment, totalling 25 syringes for treatments were used in the experiment. All syringes were placed in an incubator at random, and the samples and blank were kept at 39 °C for 48 hours. At 0, 1, 2, 4, 8, 10, 12, 18, 24, 36, and 48 h incubation times, the total amount of produced gas was observed (Sofyan et al. 2017). Cumulative total gas production was fitted according to the Gompertz model (Machado et al. 2014):

$$y = ae^{-be-ct} \quad (1)$$

where:
y – cumulative total gas production (ml);
a – maximum gas production (ml/g);
b – lag period before exponential gas production starts (h);
c – specific gas production rate (ml/h) at time t (h);
e – Euler's constant (2.7183).

To measure CH_4, at 8th and 24th h into incubation a gas sample was collected during the *in vitro* gas production process. Methane from gas production was analysed using gas chromatography (Shimadzu GC14B, Shimadzu, Germany) completed with a Porapak N Column (50 °C) (Porapak N Column, Shimadzu, Germany) with helium (He) carrier gas at a flow rate of 60 ml/min and a flame ionization detector (150 °C), as described by Sofyan et al. (2017).

The gas was let out after 48 h, and the fluid in the syringe was taken out to test the *in vitro* dry matter degradability. Following the method previously reported by Sofyan et al. (2017), the *in vitro* degradability was calculated as follows:

$$\text{IVDMD} = \frac{[\text{Dmf} - (\text{DMr} - \text{DMb})]}{\text{DMf}} \quad (2)$$

https://doi.org/10.17221/173/2023-CJAS

$$IVOMD = \frac{[OMf - (OMr - OMb)]}{Omf} \quad (3)$$

where:

IVDMD – *in vitro* dry matter degradability;
DMf – dry matter of the feed;
DMb – dry matter of the blank;
DMr – dry matter of the residue;
IVOMD – *in vitro* organic matter degradability;
OMf – organic matter of the feed;
OMb – organic matter of the blank;
OMr – organic matter of the residue.

Based on Friggens et al. (1998), volatile fatty acids (VFA) were determined using GC-MS (Shimadzu QP2010, Germany) with a MEGA-WAX MS column (025-025-30) from MEGA S.r.l. (Italy). The split mode, 50 splits, 3 ml/min of purge flow, and 250 °C injector temperature were used for the test. The column temperature was set at 100 °C and kept there for 9 minutes. The temperature was then raised to 200 °C at a rate of 10 °C/min and kept there for 10 minutes.

The Lowry method was used to dissolve the proteins (Lowry et al. 1951), which was based on the principle of gradually altering centrifugation from Makkar et al. (1982) for the microbial protein synthesis (MPS) assay. A haemocytometer with methyl green formalin solution (MFS) was used to count the number of protozoa populations in rumen fluid (Sofyan et al. 2017). The number of protozoa per ml (N) was calculated as N × 10^4 × dilution factor. The pH of the rumen fluid was measured using a pH meter (Eutech PC 700 Thermo Scientific) standardized with buffer solutions of pH 4 and pH 7. The concentration of ammonia (NH_3) was determined using the Conway microdiffusion method (Nocek et al. 1987), using a Conway plate, Na_2CO_3 solution, boric acid solution, and 0.005 N H_2SO_4 for titration. Ammonia concentration was calculated using the following formula: Ammonia concentration (mM/l) = ml H_2SO_4 (result of titration) × N H_2SO_4 × 1 000.

In sacco degradation

In sacco feed degradation in the rumen was performed according to Orskov and McDonald (1979). The experimental animals were fed 80% of grass (*Pennisetum purpuphoides* hybrid) and 20% of concentrate twice a day. Five grams of TMR silage samples were incubated in a 6 × 11 cm nylon bag with 45 µm porosity that was made manually (SA, SB, SC, SD, and SE treatments). Triplicate sample bags were incubated in two fistulated animals for 0, 6, 12, 24, 48, and 72 h, generating six samples at each interval. All bags (6, 12, 24, 48, and 72 h of incubation) (Moloney and Flynn 1992) were put into the rumen simultaneously before morning feeding and removed gradually according to the incubation time (Dong et al. 2017).

Thirty samples were collected at each incubation interval. The nylon bags were removed from the rumen, rinsed with running water to remove any feed particles that had adhered to the outside of the bag, washed in a washing machine (Samsung WT75) with continuously running water for 10 min, rinsed, air-dried, and then oven-dried at 55 °C for 48 hours. In particular, samples at 0 h were not placed in the rumen and were rinsed with running water for 10 min before being washed in the same way as the bag was incubated in the rumen and then handled as if incubated in the rumen The remaining samples were weighed and tested for dry matter (DM), organic matter (OM), and crude protein (CP). Dry matter testing and organic matter testing were performed in 110 °C ovens and 550 °C furnaces, respectively (Amodu et al. 2014). The Kjeldahl technique was used to analyse crude protein.

Before calculating the effective degradability, the actual degradation of the samples was determined using the Neway Excel Program v6.

The profiles of DM, OM, and CP degradation (DMD, OMD, CPD) were computed using the nonlinear equation of Orskov and McDonald (1979) as follows:

$$P = a + b\left(1 - e^{-cxt}\right) \quad (4)$$

where:

P – actual degradation of the nutrient components under investigation during incubation time (%);
a – intercept of the degradation curve at time zero (%), it describes a fraction of soluble nutrients that are rapidly degraded in the rumen;
b – insoluble fraction of nutrients but potentially degraded (%);
c – rate constant of degradation from fraction "*b*" (%.h^{-1});
t – incubation time (h).

https://doi.org/10.17221/173/2023-CJAS

Based on the constants of obtained degradation, the value of potential and effective degradability of each sample nutrient was calculated using the following equation:

$$PD = a + b \quad (5)$$

$$ED = a + (b \times c)/(k + c) \quad (6)$$

where:
PD – potential degradability of nutrients (%);
ED – effective degradability of nutrients (%);
a, *b*, *c* – the same as in Equation 4;
k – passage rate constant of a small particle of the feed leaving the rumen due to the degradation activity of the rumen microbes. In this study, the passage rate constant was 6% per hour.

Experimental design and data analysis

This study used a completely randomized design (CRD) with five treatments and five replications: control/SA (TMR without chitosan), SB (TMR with 0% chitosan, diluted in 1% acetic acid), SC (TMR with 0.5% chitosan diluted in 1% acetic acid), SD (1% chitosan diluted in 1% acetic acid), and SE (1.5% chitosan diluted in 1% acetic acid). The used linear model is as follows:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \quad (7)$$

where:
i – treatment;
j – replication;
i, j – 1, 2, 3,..., n;
Y_{ij} – observations in the i^{th} treatment in the j^{th} replication;
μ – general average;
τ_i – effect of treatment i;
ε_{ij} – experimental error for the i^{th} treatment in the j^{th} replication.

All data were examined using analysis of variance (ANOVA). If there were discernible differences between treatments, the LSD test was performed by employing the CoSTAT statistical software. Hierarchical cluster analysis (HCA) and heatmap visualization were used to analyse the relationships between various parameters in the *in vitro* assay. The R Console software (R-4.3.1 for Windows) was used to conduct the HCA and heatmap (R Core Team 2023).

RESULTS

In vitro ruminal fermentation characteristics

The effect of adding chitosan to TMR silage on *in vitro* gas production kinetics is presented in Figure 1. The addition of 1.5% chitosan to TMR silage reduced the total gas production for 48 h ($P < 0.05$). The ru-

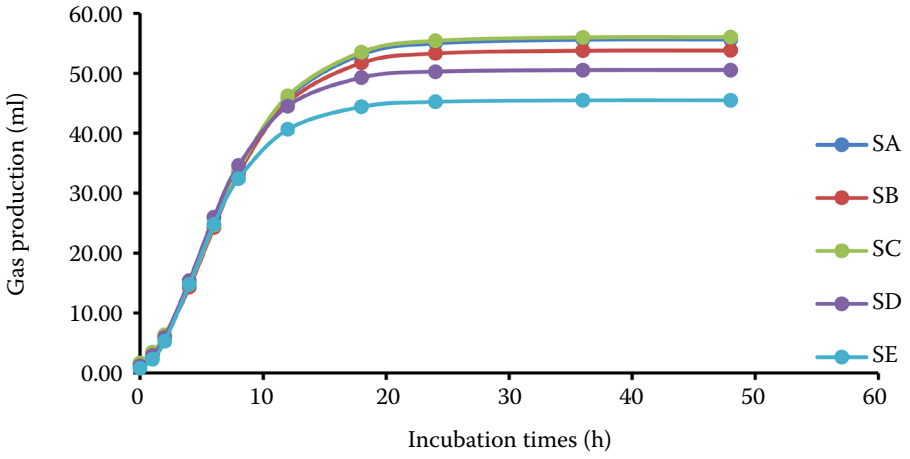

Figure 1. *In vitro* gas production kinetics of TMR silage with the addition of chitosan at different levels
Different superscripts indicate significant differences ($P < 0.05$)
SA = TMR silage with aquades control, chitosan 0%; SB = TMR silage with 1% acetic acid control; SC = TMR silage with 0.5% chitosan diluted in 1% acetic acid; SD = TMR silage with 1% chitosan diluted in 1% acetic acid; SE = TMR silage with 1.5% chitosan diluted in 1% acetic acid

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Table 2. *In vitro* ruminal fermentation characteristics of TMR silage with the addition of chitosan

Variable	Treatment					SEM	*P*-value
	SA	SB	SC	SD	SE		
Gas production kinetic parameters							
a (ml/g)	55.6	53.8	56.0	50.5	45.5	1.41	0.168
b (h)	3.62	3.71	3.56	4.04	4.22	0.121	0.450
c (ml/h)	0.247	0.257	0.2445	0.305	0.335	0.013	0.001
a + *b* (ml)	59.3	57.5	59.6	54.6	49.7	1.30	0.153
Cummulative gas 48 h (ml)	55.6^{a}	53.8^{ab}	56.0^{a}	50.5^{b}	45.5^{c}	0.489	0.001
NH_3 (mM/l)	10.3	9.77	11.0	9.28	9.98	0.326	0.588
Microbial protein synthesis (mg/ml)	5.12	5.34	4.46	5.70	5.18	0.186	0.089
Protozoa (log/ml)	5.38^{a}	5.28^{abc}	5.26^{bc}	5.25^{c}	5.37^{ab}	0.019	0.038
CH_4 (%)	10.7^{ab}	10.8^{a}	10.6^{ab}	7.34^{c}	8.62^{bc}	0.462	0.009
pH	6.54	6.49	6.45	6.49	6.49	0.026	0.888
IVDMD (%)	69.8	71.8	68.1	71.1	72.9	0.802	0.364
IVOMD (%)	78.1	80.3	77.3	80.0	78.4	0.960	0.863
VFA							
Acetic acid (%)	54.3^{b}	56.4^{a}	54.2^{b}	53.4^{b}	53.7^{b}	0.309	0.001
Propionic acid (%)	29.7^{c}	30.0^{bc}	31.8^{a}	30.6^{bc}	31.0^{ab}	0.239	0.019
Total VFA (mM/l)	53.2^{a}	52.1^{a}	52.0^{a}	40.8^{b}	37.8^{b}	1.818	0.000
A : P	1.82^{ab}	1.88^{a}	1.71^{c}	1.74^{bc}	1.73^{c}	0.020	0.007

Different superscripts within the same column in the treatment average indicate significant differences ($P < 0.05$)
a = the maximum gas production (ml/g); A/P = the ratio of acetate to propionate; *b* = the lag period before exponential gas production starts (h); *c* = the specific gas production rate (ml/h) at time t (h); IVOMD = *in vitro* organic matter digestibility, acetic acid, propionic acid; SA = TMR silage with aquades control, chitosan 0%; SB = TMR silage with 1% acetic acid control; SC = TMR silage with 0.5% chitosan diluted in 1% acetic acid; SD = TMR silage with 1% chitosan diluted in 1% acetic acid; SE = TMR silage with 1.5% chitosan diluted in 1% acetic acid); SEM = standard error of the mean; TMR = total mixed ration; VFA = volatile fatty acids

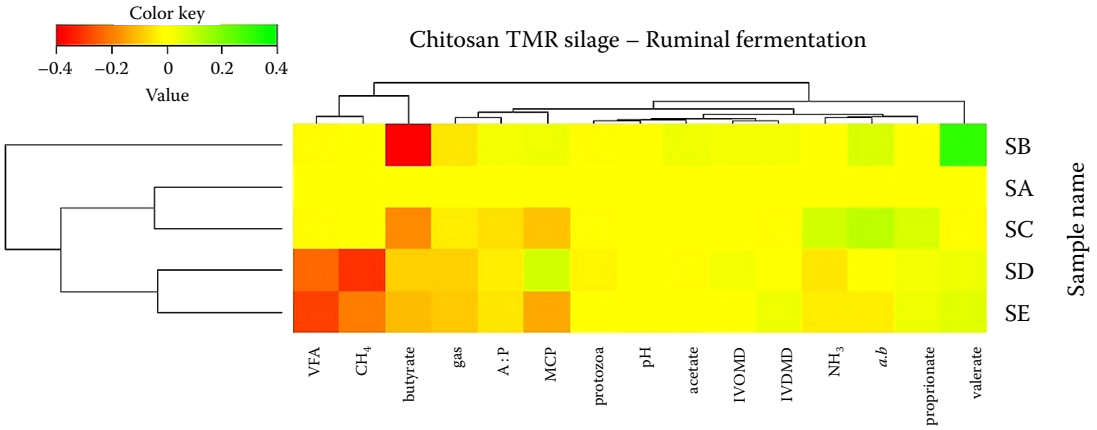

Figure 2. The heatmap of the relationship between treatments and *in vitro* rumen fermentation characteristics of TMR silage with the inclusion of chitosan
Colours reflect a trend of treatment effects from a decreasing trend (red) to an increasing trend (green)
a.b = potential gas production (*a* + *b*); IVDMD = *in vitro* dry matter digestibility; IVOMD = *in vitro* organic matter digestibility; A : P = the ratio of acetic acid to propionic acid; MCP = microbial cell protein; SA = TMR silage with aquades control, chitosan 0%; SB = TMR silage with 1% acetic acid control; SC = TMR silage with 0.5% chitosan diluted in 1% acetic acid; SD = TMR silage with 1% chitosan diluted in 1% acetic acid; SE = TMR silage with 1.5% chitosan diluted in 1% acetic acid; SEM = standard error of the mean

https://doi.org/10.17221/173/2023-CJAS

Table 3. *In sacco* actual degradation (%) of TMR silage with the addition of chitosan

Parameter (%)	Treatment	Incubation time (h)						Average treatment	SEM	*P*-value
		0	6	12	24	48	72			
Actual DMD	SA	27.0	45.4	56.9	70.3	79.9	82.2	60.3^{ab}	–	–
	SB	26.8	45.7	57.6	71.1	80.9	83.1	60.9^{a}	–	–
	SC	23.5	45.6	59.0	73.1	80.1	84.0	60.9^{a}	–	–
	SD	27.8	43.5	53.4	69.5	79.4	81.4	59.2^{bc}	–	–
	SE	25.7	44.1	55.2	67.8	77.0	80.6	58.4^{c}	–	–
	Average	26.2^{f}	44.8^{e}	56.4^{d}	70.3^{c}	79.5^{b}	82.3^{a}	–	1.65	0.010
	SEM	–	–	–	–	–	–	1.65	–	–
	P	–	–	–	–	–	–	0.010	–	–
Actual OMD	SA	25.6	47.9	62.7	79.5	92.8	95.4	67.3^{ab}	–	–
	SB	24.0	42.9	60.4	77.6	91.7	96.5	65.5^{bc}	–	–
	SC	22.4	47.0	58.4	75.6	89.6	95.2	64.7^{c}	–	–
	SD	34.6	50.7	59.8	78.7	92.0	95.9	68.6^{a}	–	–
	SE	27.5	48.2	63.4	80.3	91.1	94.0	67.4^{ab}	–	–
	Average	26.8^{f}	47.3^{e}	60.9^{d}	78.3^{c}	91.5^{b}	95.4^{a}	–	2.03	0.008
	SEM	–	–	–	–	–	–	2.03	–	–
	P	–	–	–	–	–	–	0.008	–	–
Actual CPD	SA	49.2	64.9	73.3	85.3	95.7	97.7	77.7^{a}	–	–
	SB	47.6	64.7	74.0	85.3	93.1	98.0	77.1^{a}	–	–
	SC	45.8	57.2	69.2	86.2	90.8	96.3	74.3^{b}	–	–
	SD	53.4	65.6	73.5	83.2	92.6	97.2	77.6^{a}	–	–
	SE	55.6	66.9	72.1	85.6	91.2	96.0	77.9^{a}	–	–
	Average	50.3^{f}	63.9^{e}	72.4^{d}	85.1^{c}	92.7^{b}	97.0^{a}	–	1.78	0.021
	SEM	–	–	–	–	–	–	1.78	–	–
	P	–	–	–	–	–	–	0.021	–	–

$^{a–f}$Different superscripts within the same column in the treatment average indicate significant differences ($P < 0.05$)
CPD = crude protein degradation; DMD = dry matter degradation; OMD = organic matter degradation; SA = TMR silage with aquades control; chitosan 0%; SB = TMR silage with 1% acetic acid control; SC = TMR silage with 0.5% chitosan diluted in 1% acetic acid; SD = TMR silage with 1% chitosan diluted in 1% acetic acid; SE = TMR silage with 1.5% chitosan diluted in 1% acetic acid; Time inc = incubation time; TMR = total mixed ration

men fermentation characteristics are presented in Table 2. There were no differences in kinetic gas production parameters (*a*, *b*, *c*, *a* + *b*), NH_3, microbial protein synthesis (MPS), *in vitro* dry matter degradability (IVDMD), *in vitro* organic matter degradability (IVOMD), or ruminal pH for all treatments. There was a decrease in total gas production at 48 h, number of protozoa, CH_4 production, acetic acid (A) proportion, total VFA, and acetic acid to propionic acid (A : P) ($P < 0.05$), but an increase in propionic acid (P) in SD treatment ($P < 0.05$). The SD treatment significantly decreased ($P < 0.05$) the total protozoa and CH_4 production compared to the other treatments. The SD treatment slightly reduced ($P < 0.05$) NH_3 production compared to the other treatments. The addition of chitosan to silage significantly decreased ($P < 0.05$) the percentage of acetic acid, increased ($P < 0.05$) the percentage of propionic acid, and decreased ($P < 0.05$) the A to P ratio.

The relationship between all treatments and the *in vitro* rumen fermentation characteristics is illustrated in Figure 2. Total mixed ration (TMR) silage with 0.5% chitosan (SC) was within the same cluster as the control (SA), whereas the TMR silage with 1% (SD) and 1.5% (SE) chitosan shared the same cluster. The four treatments had a closer relationship than the SB treatment, where the SB treatment was segregated in a separate cluster. As indicated in the

https://doi.org/10.17221/173/2023-CJAS

Table 4. *In sacco* degradability characteristics and effective degradability of TMR silage with the addition of chitosan

Parameter	Treatment					SEM	*P*-value
	SA	SB	SC	SD	SE		
DMD							
a	27.0^{a}	26.8^{a}	23.5^{b}	27.8^{a}	25.7^{ab}	0.485	0.032
b	54.8	58.4	60.5	57.9	53.2	0.931	0.077
c	0.062	0.053	0.074	0.046	0.075	0.004	0.062
a + *b*	83.9	86.2	85.0	83.2	81.7	0.595	0.155
ED	56.0	56.5	56.8	53.9	54.2	0.618	0.464
OMD							
a	25.6^{b}	23.9^{b}	22.4^{b}	33.4^{a}	27.5^{ab}	1.21	0.025
b	69.7^{ab}	75.8^{a}	73.8^{a}	62.1^{c}	66.7^{bc}	1.34	0.002
c	0.065	0.045	0.054	0.054	0.067	0.003	0.111
a + *b*	83.9	86.2	84.9	83.2	81.7	0.479	0.155
ED	61.3	56.7	60.6	62.6	61.6	0.926	0.313
CPD							
a	30.6^{a}	18.1^{bc}	14.5^{c}	27.1^{ab}	19.5^{bc}	1.79	0.014
b	66.9	77.0	79.7	68.5	74.1	1.64	0.052
c	0.069	0.069	0.069	0.059	0.069	0.003	0.708
a + *b*	97.6^{a}	95.2^{b}	94.2^{b}	95.6^{ab}	93.6^{b}	0.403	0.008
ED	65.8^{a}	59.0^{b}	56.9^{b}	61.2^{ab}	59.0^{b}	0.990	0.042

$^{a–c}$Different superscripts within the same row in each parameter indicate significant differences ($P < 0.05$)

a = the maximum gas production (ml/g); *a* + *b* = potential degradation; *b* = the lag period before exponential gas production starts (h); *c* = the specific gas production rate (ml/h) at time t (h); CPD = crude protein degradation; DMD = dry matter degradation; ED = effective degradation; OMD = organic matter degradation; SA = TMR silage with aquades control, chitosan 0%; SB = TMR silage with 1% acetic acid control; SC = TMR silage with 0.5% chitosan diluted in 1% acetic acid; SD = TMR silage with 1% chitosan diluted in 1% acetic acid; SE = TMR silage with 1.5% chitosan diluted in 1% acetic acid); TMR = total mixed ration

heatmap visualization, the SD treatment resulted in the lowest CH_4 production, followed by the SE treatment. The SD treatment tended to increase MPS, IVDMD, IVOMD, propionic acid and valeric acid levels. Nevertheless, all levels of the chitosan addition to silage tended to decrease butyric acid proportion, whereas SB treatment resulted in the lowest proportion among all treatments.

In sacco nutrient degradation

In sacco actual degradation for organic matter, dry matter, and crude protein of each treatment is presented in Table 3. Chitosan supplementation reduced ($P < 0.05$) the actual DMD and increased ($P < 0.05$) the actual OMD and CPD for SD and SE treatments (Table 3). Table 4 shows the values of the fractions from each sample, i.e., organic matter, dry matter, and crude protein fractions of "*a*", "*b*", "*c*", and "*a* + *b*". There were discernible differences ($P < 0.05$) for "*a*" fraction in dry matter OMD, and CPD in SD treatment. There were significant differences in SD treatment ($P < 0.05$) for the "*b*" fraction in OMD, but no discernible difference for DMD and CPD.

However, no discernible difference in the "*c*" fraction was observed for DMD, OMD, and CPD between all treatments. There was no discernible difference in the "*a* + *b*" for DMD and OMD for all treatments but a discernible difference ($P < 0.05$) for CPD in SE treatment. There were noticeable differences in ED found in CPD ($P < 0.05$) for SA and SD treatments, while there were no discernible differences between treatments for DMD and OMD for all treatments.

https://doi.org/10.17221/173/2023-CJAS

DISCUSSION

The inclusion of chitosan, especially 1.5%, in TMR silage reduced gas production (Figure 1 and Table 2). The reduction in gas production due to chitosan inclusion was in accordance with the findings of Anggraeni et al. (2022) that the addition of chitosan in the form of 1.5% commercial chitosan decreases total gas production. This is possible because chitosan has antimicrobial properties, so it decreases the rumen microbial population which reduces feed digestibility and gas production (Goiri et al. 2010; Harahap et al. 2020; Anggraeni et al. 2022). The breakdown and fermentation of the substrate by rumen microorganisms produce total gas (Harahap et al. 2020).

The number of protozoa was lower because of the addition of chitosan to TMR silage. This result is similar to that of Belanche et al. (2016), who found that the inclusion of 2 g/l insoluble chitosan lowered the activity of protozoa by up to 56%, while 1 g/l soluble chitosan and 5% chitosan of DM reduced CH_4 emissions. Methane production decreased by 10% to 42% as a result of methanogens or metabolic pathways of CH_4 synthesis being blocked by chitosan electrostatic interaction and cell membrane instability (Jimenez-Ocampo et al. 2019). Furthermore, chitosan may reduce the principal microbial species responsible for the generation of CH_4 and methanogenic archaea (Harahap et al. 2020). The decrease in the population of protozoa, particularly *Entodinium* spp., is another potential explanation for the reduced methanogenesis caused by the addition of chitosan (Wencelova et al. 2014). A certain number of methanogens coexist with protozoa and benefit from the fauna, so a decrease in the protozoa population will also decrease its methanogenic activity (Harahap et al. 2020). The antibacterial properties of chitosan affect the permeability of bacterial methanogens, thereby damaging intracellular cells and resulting in damage to bacterial methanogen cells (Belanche et al. 2016; Harahap et al. 2020; Anggraeni et al. 2022). Another opinion states that the acetic acid fermentation pattern is affected by the addition of chitosan as a hydrogen producer. Since hydrogen is a substrate for the synthesis of CH_4, a decrease in hydrogen will result in a reduction in CH_4 production. Overall, there are several mechanisms by which chitosan supplementation reduces CH_4 emissions. Chitosan alters the bacterial community and promotes the growth of propionic acid-producing bacteria (Belanche et al. 2016), certain chitosan characteristics, such as a high degree of deacetylation, may change the cell wall permeability of methanogens (Harahap et al. 2020). Chitosan is positively charged and may interact with negatively charged methanogens, resulting in the leakage of cytosolic proteins and other intracellular components (Zanferari et al. 2018).

There was no significant difference in the effect of chitosan supplementation on ruminal pH (Dias et al. 2020); the same condition was found in this study. With an average pH value of 6.49 for all treatments, this pH is in the normal range suggested by Kong et al. (2010), and the pKa of the molecules is 6.3–6.5. Because of its effects on the bacterial population, the products of rumen fermentation, and the normal physiological function of the rumen, the rumen pH is crucial to its performance and stability. In the current investigation, it was shown that a pH value of 6.49 allowed for the highest activity, microbial growth, ruminal fermentation, and NDF digestibility.

Chitosan supplementation had no significant effect on NH_3 production in the rumen, but the addition of 1% chitosan slightly reduced NH_3 production compared with the other treatments (Table 2 and Figure 2). This is most likely related to a mechanism of protection from ruminal degradation; under rumen pH conditions, the positively charged $-NH_2^+$ groups of chitosan could potentially interact electrostatically with the negatively charged carboxyl groups in amino acids, potentially leading to NH_3 reduction (Harahap et al. 2020). A decrease in the level of NH_3 is either an enhancement in microbial protein synthesis or a decrease in amino acid breakdown due to the microbial characteristics of chitosan (Jacauna et al. 2021).

There was no significant effect of chitosan addition on IVDMD or IVOMD in this study. This result is consistent with the findings of Seankamsorn et al. (2019). Without impairing the feed digestion, chitosan shifts fermentation to a more energy-efficient pathway (Seankamsorn et al. 2019). In contrast, some studies have found that chitosan significantly decreased IVDMD (Jacauna et al. 2021; Wencelova et al. 2014). Additionally, Goiri et al. (2009) hypothesized that the composition of the basic diet, particularly the proportion of forage to concentrate, may have a significant impact on the efficacy of chitosan. Furthermore, as shown in the heatmap

https://doi.org/10.17221/173/2023-CJAS

in Figure 2, the use of chitosan reduced CH_4 production and, at the same time, tended to increase IVDMD and IVOMD. It is so because chitosan changes the microbial community that promotes the growth of propionic acid-producing bacteria (Belanche et al. 2016). Owing to the reduction in CH_4 production, feed digestibility will be more optimal. Chitosan reduces the protozoa population, decreases the predation intensity of protozoa on bacteria, and in turn, elevates the total bacteria population that is greatly responsible for nutrient degradation and fermentation (Harahap et al. 2020).

The inclusion of chitosan in silage resulted in a significant reduction in the proportion of acetic acid, an increase in the proportion of propionic acid, and a decrease in the A/P ratio. These findings are consistent with those of previous studies conducted by Goiri et al. (2009), Zanferari et al. (2018), and Jimenez-Ocampo et al. (2019), who demonstrated that the addition of chitosan led to an elevated proportion of propionic acid. It was further confirmed by the heatmap (Figure 2) that there was a tendency to increase the propionic acid to valeric acid ratio and decrease the acetic acid to butyric acid ratio. The products of feed fermentation in ruminants are NH_3, CH_4, carbon dioxide, and VFA, predominantly acetic acid, propionic acid, and butyric acid, but also branched-chain VFA and lactate to a lesser extent (Parnian-Khajehdizaj et al. 2023). As a feed additive, chitosan can reduce CH_4 production, enhance propionic acid production, decrease the acetic acid/propionic acid ratio, and boost livestock performance and nutrient use efficiency (Anggraeni et al. 2022). It is so because chitosan can affect the synthesis of H_2 and can help the transition of carbohydrate fermentation from acetic acid to propionic acid production, resulting in a decrease in acetic acid and an increase in propionic acid (Seankamsorn et al. 2019). According to Harahap et al. (2020), the increased propionic acid proportion caused by the inclusion of chitosan was also associated with a decrease in protozoa. According to a previous study, defaunation of protozoa resulted in higher molar levels of propionic acid and lower levels of butyric acid and acetic acid in the rumen. The modification of the individual VFA profiles may be explained by the antibacterial action of chitosan. Chitosan inhibits cellulolytic bacteria, including the genera *Fibrobacter*, *Butyrivibrio*, and *Ruminococcus*, which reduces the amount of acetic acid because acetic acid is largely produced by fibre fermentation (Harahap et al. 2020). On the other hand, chitosan supplementation tended to elevate the valeric acid and reduce the butyric acid proportion of SD and SE treatments, as shown on the heatmap in Figure 2. This result was in accordance with the findings of Goiri et al. (2009) and Goiri et al. (2010) that the chitosan addition increased feed efficiency in *in vitro* and *in vivo* assays. Acetic acid, propionic acid, and butyric acid are three VFA that have been related to animal feed efficiency. An increase in the level of valeric acid is due to lower feed degradation, particularly with a reduction in fibre degradation (Goiri et al. 2009). Chitosan appears to act in the rumen similarly like the grain (starch) is integrated into the diet by changing the fermentation, especially the molar ratios of the VFA pattern, to more propionic acid-oriented (Jimenez-Ocampo et al. 2019).

The actual values of nutrient degradation increased as the incubation period in the rumen lengthened. This occurs because the longer the feed particles are retained in the rumen, the greater the chance that bacteria may degrade nutrients, increasing the value of feed elements subjected to degradation (Jalilvand et al. 2008). Furthermore, one of the key factors affecting the degradation of feed ingredients is the length of feed residence in the rumen. Supplementation of 1–1.5% chitosan reduces actual DMD while increasing actual OMD and CPD. This result is in accordance with the studies by Wencelova et al. (2014), Harahap et al. (2020), and Jacauna et al. (2021) that the addition of chitosan to the diet decreases actual DMD and increases actual OMD and CPD. Because of the chitosan addition, the actual DMD in SE treatment decreased, which showed that SE treatments utilize slowly degrading materials as an energy and protein source in the rumen. The reduction in DMD observed in SE treatment may be attributed to the higher NDF and ADF content associated with this treatment. By reducing the capacity of bacteria to adhere to the feed and digest it readily, the high cell wall composition of the materials is achieved (Kirwan et al. 2022). Furthermore, the decrease in actual DMD is the result of the negative effect of chitosan, which decreases rumen microbes, especially cellulolytic bacteria that lead to a decrease in feed degradability (Wencelova et al. 2014). Chitosan supplementation increases organic matter and protein digestibility (Harahap et al. 2020).

https://doi.org/10.17221/173/2023-CJAS

Enhanced CP digestibility may be caused by alterations in fermentation (Paiva et al. 2016). The addition of black soldier fly (BSF) larvae which serve as a source of chitin increased protein degradation (Mulianda et al. 2021). An increase in nutrient digestibility is related to how the addition of chitosan changes the structure of the microbial community. Chitosan lowers the number of protozoa and the degree of protozoan predation on bacteria, which increases the overall bacterial population which is crucial for nutrient breakdown and fermentation. Despite having broad-spectrum antimicrobial properties, protozoa appear to be more susceptible to chitosan than to other microbes (Harahap et al. 2020). Proteolysis and deamination are caused by ruminal bacteria that are liable to chitosan activity (Dias et al. 2017).

The inclusion of chitosan in TMR silage gives various effects on the "*a*" fraction in organic matter degradation, dry matter degradation, and crude protein degradation. The significant difference in "*a*" fraction between DMD and OMD might be due to different amounts of tiny particles present in each sample. There are two types of fine feed particle sizes according to Jalilvand et al. (2008) and Kirwan et al. (2022) that influence the value of the "*a*" fraction. Specifically, fine particles that leak from the bag and can ferment quickly; fine particles that are lost during washing but do not ferment, yet continue to make up "*a*" percent in the calculation of the degradation constant. Although all samples were ground using the same procedure, the produced small particles differed depending on the sample due to variations in the physicochemical structure. The washing process is estimated as a proportion of a non-linear equation calculation procedure used in deterioration calculations. As a result, the value of the produced fraction "*a*" varies (Kirwan et al. 2022).

In addition, the inclusion of chitosan in TMR silage has various effects on *in sacco* degradability. The lowest "*b*" fraction of OMD and CPD was in SD treatment, furthermore SE treatment tends to lower the "*b*" fraction of DMD. This might be correlated with the high NDF-ADF content in SD and SE treatments, which led to a decrease in the "*b*" fraction. The high cell wall composition of the materials caused a decrease in the ability of bacteria to attach to the feed and digest it quickly (Kirwan et al. 2022). The maximum degradation of all treatments on DM and CPD did not show an optimal value (Table 4). After the maximum rate of degradation is reached, the size of the potentially degradable and non-degradable fractions would no longer change. The characteristics of the substrate would then be the only factor considered after that (Dias et al. 2020). Higher protein content in TMR silage treated with chitosan influences higher crude protein digestibility among other treatments. This result is in accordance with the result from Phesatcha et al. (2021) that higher protein levels in diets increase crude protein digestibility. Increasing the use of chitosan enhances CP content because chitosan performance as a protein protection agent reduces CP degradation during the ensiling process. The increased nutrient digestibility is speculated to be associated with the effect of chitosan on the structure of the microbial community (Harahap et al. 2020).

CONCLUSION

The addition of chitosan can modify several parameters of rumen fermentability, in which the addition of 1–1.5% chitosan reduces total protozoa, total gas production, and CH_4 production, as well as decreases the acetic acid to propionic acid ratio. Furthermore, the addition of chitosan decreases dry matter degradation but increases crude protein and organic matter degradation. Based on this research, the addition of 1–1.5% chitosan is the optimal level to improve the rumen fermentability and feed degradation in the rumen based on *in vitro* and *in sacco* assays.

Acknowledgment

The authors thank you for IPB University and BRIN for the support and facilities for the authors.

Conflict of interest

The authors declare no conflict of interest.

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Received: December 16, 2023
Accepted: May 13, 2024
Published online: May 30, 2024