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The combination of chitosan and molasses to improve nutritive value, chemical quality, and in vitro rumen degradability of pineapple peel silage

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Abstract. *Harahap RP, Rohayeti Y, Setiawan D, Herliatika A, Widiwati Y. 2024. The combination of chitosan and molasses to improve nutritive value, chemical quality, and in vitro rumen degradability of pineapple peel silage. Biodiversitas 25: 2800-2810.* This study aimed to evaluate and determine nutritive value, chemical silage quality, *in vitro* rumen fermentation profile, microbial population, digestibility, and methane (CH_4) emission of pineapple (*Ananas camosus* L.) peel silage with the addition of chitosan and molasses. The experiment was carried out using a 2×3 factorial design with 6 treatments, 4 replications, and different in vitro runs acting as blocks. In addition, the treatments comprised control (no additives), C3 (3 g of chitosan per 1000 g of fresh pineapple peel), C5 (5 g of chitosan per 1000 g of fresh pineapple peel), M20 (20 g of molasses per 1000 g of pineapple peel), C3M20 (3 g of chitosan and 20 g of molasses per 1000 g of pineapple peel), and C5M20 (5 g of chitosan and 20 g of molasses per 1000 g of pineapple peel). The descriptive analysis results for nutritive value showed that the 5 g chitosan group had the highest dry matter (DM) content in pineapple peel silage after 21 days. Chitosan was shown to improve organic matter (OM) and crude protein (CP) levels while reducing hemicellulose content. The administration of molasses significantly reduced weight loss and pH, enhancing preservation quality ($P<0.05$). The results also showed that chitosan reduced weight loss, particularly at 5 g/kg fresh pineapple peel ($P<0.05$). Rumen fermentation analysis revealed higher acetic acid levels in silage without molasses ($P<0.05$), while pH, ammonia (NH_3), propionic acid, butyric acid, and branched-chain volatile fatty acid (BCVFA) concentrations did not affect rumen fermentation ($P>0.05$). However, chitosan increased in vitro dry matter digestibility (IVDMD) and reduced total gas production ($P<0.05$). Higher levels also reduced microbial and protozoa populations in rumen. Based on the results, the use of both additives improved the quality of silage, where chitosan increased IVDMD and reduced gas production during rumen in vitro fermentation.

Keywords: Chitosan, molasses, pineapple peel, rumen fermentation, silage

INTRODUCTION

Pineapple (*Ananas camosus* L.) is an essential fruit, which thrives in Indonesia due to the conducive tropical climate. At present, Indonesia ranks as the fourth largest pineapple-producing country in the world, following the Philippines, Costa Rica, and Brazil, with an annual production of approximately 2,447.24 million tons (Rosmaina et al. 2022). Despite the potential, only 35% of the fruit is edible, while the remaining 65% consists of waste, including peel and pomace (Sukirah et al. 2023). The study indicates that pineapple peel, along with other parts like the crown and bud end, can be utilized as fiber sources, while the core and pomace can substitute or supplement concentrate feedstuffs due to their high sugar content and digestibility (Kiatti et al. 2023). Several studies have shown that improper disposal of peel silage contributes to environmental degradation and wastage of valuable resources (Gasmi Benahmed et al. 2021). Pineapple peel, as a significant by-product of pineapple processing, contains essential nutrients and bioactive compounds, including bromelain, which may offer therapeutic benefits such as digestive support and anti-parasitic effects (Mehraj et al. 2024).

Pineapple peel has been reported to be rich in insoluble fiber and holds the potential for developing low-calorie, fiber-enriched foods (Roda and Lambri 2019). Pineapple peel residue is a major by-product of pineapple processing, which contains valuable nutrients and bioactive compounds (Hu et al. 2019; Roda and Lambri 2019), making it a potential feedstock. Pineapple peel contains significant amounts of carbohydrates, dietary fiber, and sugars such as sucrose, glucose, and fructose, along with essential minerals like calcium and potassium (Mehraj et al. 2024). Additionally, research has identified the presence of bioactive compounds such as phenolic compounds, flavonoids, and bromelain protein in pineapple peel, which contribute to its antioxidant and antimicrobial properties (Moreira et al. 2023).

In addition, a previous study revealed that it can enhance animal feed intake without causing damage to health or metabolism due to the high palatability and nutrient content (Gowda et al. 2015; Aili Hamzah et al. 2021; Sukri et al. 2023). The potential of the by-product as feed for ruminants has also been extensively studied, revealing significant nutritional benefits and cost-effectiveness. Pineapple waste, including peel and stem by-product, serves as a low-cost feed source for cattle, positively influencing growth performance and ruminal fermentation (Hattakum et al. 2019). Studies on the use of agro-industrial by-product as dietary roughage have demonstrated the effectiveness of pineapple peel silage in fattening steers (Maneerat et al. 2015). Varying concentrations of the material have also been recommended for optimal feed utilization and nutrient digestibility in ruminants (Sukri et al. 2023).

In line with previous results, fermented pineapple peel residue is a promising alternative that can partially replace whole corn silage for goats (Yang et al. 2022). The use of pineapple waste silage as a roughage source also enhances nutrient intake, energy status, and growth performance in cattle (Kyawt et al. 2020). Studies on sheep-fed waste silage showed improved intake, digestibility, and microbial efficiency at different nutrition levels (Cordeiro et al. 2022). However, due to the moisture content, the perishability of these residues poses a challenge, indicating the need for effective preservation methods, such as silage to extend shelf life and ensure utility as high-quality feed.

Silage made from fresh pineapple peel is a well-known method for preserving feed quality. This process comprises anaerobic fermentation, which produces acids with the ability to prevent the growth of harmful microorganisms (Guo et al. 2014). During the process, water-soluble carbohydrate (WSC) is converted into organic acids under anaerobic conditions, which lowers pH and facilitates long-term preservation of the forage (Rohmah et al. 2019). Feed additive has been reported to play an essential role in enhancing silage quality. For example, molasses and those combined with ecomass significantly improve quality by facilitating fermentation and enhancing nutrient retention (Olfaz et al. 2019). The most common additive in recent times is chitosan derived from chitin, which stands out for the ability to elevate silage quality. This material has antimicrobial properties, which improve chemical and microbiological stability as well as augment the nutritional value of feed (Del Valle et al. 2018). Chitosan can also restrict the growth of undesirable microorganisms in silage due to antimicrobial properties, leading to reduced fermentative losses and enhanced silage fermentation (Gandra et al. 2022). Therefore, this study aimed to determine silage quality, in vitro rumen fermentation profile, microbial population, and digestibility of pineapple peel silage with the addition of chitosan and molasses. Although the use of additive has been previously established, this current study pioneers the synergy between chitosan's antimicrobial benefits and molasses' fermentable sugars in pineapple silage.

MATERIALS AND METHODS

Ethical approval and experiment location

All procedures comprising fistulated Ongole crossbred cattle in this study were conducted under the approval of the Animal Care and Utilization Committee of the Report Center for Animal Husbandry, Indonesia. The procedures were carried out at the Report Center for Animal Husbandry in the Study Organization for Agriculture and Food, NRIA, located in Cibinong, Bogor, West Java, Indonesia.

Silage preparation

In this study, pineapple peel was used from a fruit market in West Java Province located in Bogor City, Indonesia. Pineapple peel samples were wilted for one day under laboratory conditions at 27°C. Wilted pineapple peel was chopped into 2-3 cm pieces and randomly separated into 6 groups. Chitosan (PT Nano Herbaltama Internasional) with particle sizes below 1 micrometer from shrimp shells had a degree of deacetylation of 87.5%, viscosity of 50 cps, pH 7.1, and solubility of 99%. Molasses were obtained from by-product of the sugar processing industry in West Java, Bogor, Indonesia.

The additives were applied as a weight basis to fresh pineapple peel samples for each chitosan and molasses level and were combined. Additives were not used in the first group and assessed as the control (CONT) group. The second group was added with 3 g chitosan in 1000 g fresh pineapple peel (C3), the third group was added with 5 g chitosan in 1000 g fresh pineapple peel (C5), fourth group was added with 20 g molasses in 1000 g fresh pineapple peel (M20), fifth group was added with 3 g chitosan and 20 g molasses in 1000 g fresh pineapple peel (C3M20), and sixth group was added with 5 g chitosan and 20 g molasses in 1000 g fresh pineapple peel (C5M20). A detailed description of these treatments was shown in Table 1.

Table 1. Description of treatments and additives used in the experiment

Materials	Treatments					
	Control	**C3**	**C5**	**M20**	**C3M20**	**C5M20**
Fresh pineapple peel (g)	1000	1000	1000	1000	1000	1000
Chitosan (g)	0	3	5	0	3	5
Molasses (g)	0	0	0	20	20	20

Note: C3: 3 g of chitosan per 1000 g of fresh pineapple peel, C5: 5 g of chitosan per 1000 g of fresh pineapple peel, M20: 20 g of molasses per 1000 g of pineapple peel, C3M20: 3 g of chitosan and 20 g of molasses per 1000 g of pineapple peel, C5M20: 5 g of chitosan and 20 g of molasses per 1000 g of pineapple peel

A total of 6 groups of silages were placed in 1 L HDPE mini-silos with randomization. Mini silos with valves were built to prevent gas escape, and each group received 4 replicates for a total of 24 silos. Silages used in this study were kept for 21 days at room temperature or 25°C. According to Anggraeni et al. (2023), mini-silos were weighed immediately following the opening to document weight loss. Before sampling, silages were opened, and the top 1/5 of each silage was thrown away, and these samples were splitted into 2 parts. The first part was filtered to yield the supernatant used to measure pH and ammonia (NH_3) after blending with distilled water (1:7 w/v) (Kondo et al. 2016). The second part was finely crushed to pass a 1 mm filter size after oven-drying for 24 hours at 60°C. This was then analyzed for nutritive value and in vitro rumen fermentation.

Nutritive value analysis

The proximate analysis was conducted following the AOAC (2023) guidelines, and dry matter (DM) was evaluated by subjecting the samples to a drying process in an oven at 105°C for 6 hours. Crude ash was measured by subjecting the sample to a furnace at 600℃ for 4 hours. Organic matter (OM) was the difference between silage sample's DM content and ash content (DM-crude ash). Crude protein (CP) was determined using the Micro Kjeldahl Apparatus to measure nitrogen (N) content. N content was multiplied by 6.25, assuming protein typically contained 16% N while the fat content was analyzed using the Soxhlet method. Crude fiber content was assessed by subjecting the sample to extraction in 2 liters of 0.255 N H_2SO_4 and 0.313 N NaOH, followed by an additional 40-minute extraction. However, the gross energy was measured using a bomb calorimeter, as described by Miller and Payne (1959), and these analyses were performed in duplicate.

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were assessed using the method described by Van Soest et al. (1991). In summary, 1 g of the sample was placed into a 600 mL beaker, and 100 mL of neutral detergent solution (NDS) was added. The mixture was then heated to boiling for 60 minutes, and the residue was isolated from the supernatant through filtration using a container and identified as NDF. The determination of ADF was similar to NDF determination, with the only difference of using acid detergent solution (ADS) rather than NDS. Additionally, NDF and ADF values were reported after eliminating any residual ash. Hemicellulose could be determined by calculating the difference between NDF and ADF content (NDF-ADF), and these analyses were also performed in duplicate.

Chemical silage quality determination

Weight loss was obtained by weighing pineapple peel silage after the opening of silo (kg) and before the ensiling (kg) described by Anggraeni et al. (2023). WSC was obtained by soaking 40 g of fresh silage sample in 400 mL of distilled water (Ridla and Uchida 1998). Meanwhile, pH and NH_3 concentration of the supernatant obtained from the ensiling method were measured. pH value was determined with a Jenway (Model 3505) pH meter, which was calibrated to pH 7 and 4. Ammonia (NH_3) concentration was determined using the Conway microdiffusion method (Conway 1962). This experimental procedure was conducted in 4 replicates, with sample placement in the experiment following a 2×3 factorial completely randomized design.

In vitro rumen fermentation and digestibility

Rumen fluid for this experiment was collected just before morning feeding from rumen-fistulated Ongole crossbred cattle animal and was fed a diet of native grass and commercial concentrate with a 50:50 ratio. The groups of treatments were subjected to *in vitro* incubation using buffered rumen fluid, adhering to the protocol established by Theodorou et al. (1994). Before the utilization, rumen content was strained through 4 gauze layers. In each experiment, 0.75 g of the sample was placed into a 125 mL serum bottle, to which 75 mL of rumen fluid and buffer mixture (1:4 v/v) was added. The bottles were flushed with CO_2 for 30 seconds to create an anaerobic environment, then sealed with butyl rubber stoppers and aluminum crimp seals to start incubating, and were incubated in a water bath maintained at 39°C for 48 hours. Gas production was measured at intervals of 2, 4, 6, 8, 12, 24, 36, and 48 hours post-incubation using a needle-equipped syringe, and manual shaking was performed during each gas measurement. Methane (CH_4) levels in the gas samples were determined by following the methods described by Fievez et al. (2005).

A different group of bottles containing similar samples underwent a 24-hour incubation to assess rumen fermentation and digestibility parameters. After incubation, each bottle's solid and liquid components were divided using a centrifuge. Additionally, the clear liquid, or supernatant, was analyzed to measure pH, the molar proportions of volatile fatty acid (VFA), and NH_3 concentrations. The molar proportions of VFA at the end of the 24-hour fermentation were evaluated using a Chrompack CP9002 gas chromatograph with a flame-ionized detector and a WCOT Fused Silica capillary column in an oven programmed to rise from 60°C to 115°C, using nitrogen as the carrier gas. pH measurements were performed using a Jenway Model 3505 pH meter, calibrated at pH 7 and 4, and NH_3 concentrations were determined using the microdiffusion method (Conway 1962). The solid residue was subjected to a further 24-hour incubation in 75 mL of 0.2 N pepsin-HCl solution. Following this additional incubation, the remaining solids were vacuum filtered using filter paper, then oven-dried at 105°C for 24 hours, and finally calcined in a furnace at 500°C for 4 hours to determine DM and OM weights. *In vitro* dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD) were calculated by comparing the initial weights of DM and OM before the incubation with their respective weights following the 2-stage in vitro process (Tilley and Terry 1963). This experimental procedure was conducted in 4 replicates, with sample placement in the experiment following a 2×3 factorial randomized block design.

Protozoal and bacterial counts

At 24 hours incubation, 1 mL aliquots were taken from each fermentation tube to analyze protozoal and bacterial populations. The contents of fermentation tube were correctly mixed, and 1 mL of the sample was mixed with 1 mL methyl green formaldehyde saline solution containing 35% formaldehyde, distilled water, methyl green, and NaCl (Ogimoto and Imai 1981). The stained sample was kept at room temperature, and the protozoal population was counted directly using a Counting chamber (0.1 mm) with a microscope (40×). This was calculated using the formula:

$$\text{Protozoal population} = \frac{1 \times 1000 \times C \times DF}{0.1 \times 0.065 \times 16 \times 5}$$

Where:
C : protozoal number
DF : dilution factor (=2)

The bacterial population was estimated using the serial dilution technique at 24 hours of incubation, and 0.05 mL rumen sample was transferred into 4.95 mL of dilution medium. The dilutions conducted were 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}, using a brain heart infusion (BHI) medium, as described by Gérard-Champod et al. (2009). The total number of bacterial colony-forming units (CFU) was calculated using the following formula:

$$\text{Bacterial population} = \frac{\text{colony unit}}{0.05 \times 10^{x} \times 0.1}$$

Where: *x* means dilution factor.

Statistical analysis

Descriptive analysis was employed to examine nutritive value, and the chemical silage quality was assessed using a 2×3 factorial completely randomized design. In addition, parameters related to in vitro rumen fermentation and microbial populations were evaluated using a 2×3 factorial randomized block design, with different *in vitro* runs acting as blocks. The first factor was without molasses and with 20 g molasses/kg fresh pineapple peel. The second factor was that without chitosan, this was added to the pineapple peel at 3g/kg and chitosan at 5 g/kg. This experimental procedure was conducted in 4 replicates, and the analysis of variance (ANOVA) was performed on the collected data. When ANOVA showed significant differences for a specific parameter at P<0.05, Duncan's multiple range test was employed to compare the means of different treatments. Statistical analysis was carried out using SAS (Statistical Analysis System) on demand for academics (SAS 2004).

RESULTS AND DISCUSSION

Nutritive value

The nutritive analysis of pineapple peel material used for silage, as shown in Table 2, reveals a dry matter (DM) content of 15.49%. Crude ash constitutes 12.43%, and organic matter comprises 87.57% of the DM. The crude protein content is relatively low at 4.22%, and the ether extract is minimal at 0.88% of the DM. The pineapple peel is notably high in crude fiber, which makes up 17.88% of the DM. Additionally, the neutral detergent fiber (NDF) content is 52.47%, and the acid detergent fiber (ADF) content is 27.76%, indicating a significant fibrous component. The hemicellulose content, derived from the difference between NDF and ADF, is calculated at 24.72% of the DM. The water-soluble carbohydrate (WSC) content is substantial, accounting for 80.78% of the DM. These results suggest that pineapple peel material is rich in fiber and carbohydrates, with moderate levels of ash and low protein content, making it a viable candidate for use in silage production. Specifically, the study on pineapple by-products highlights that the peel serves as a significant source of fiber, which is essential for ruminant diets, while also contributing to the overall carbohydrate content necessary for energy provision (Kiatti et al. 2023).

Nutritive value of pineapple peel silage was evaluated after a 21-day incubation period using different additives, including chitosan and molasses, as shown in Table 3. DM content exhibited variability across treatment groups, with the highest proportion (11.66%) detected in the 5 g chitosan group. Studies had shown that chitosan incorporation increased DM content of silage (Gandra et al. 2016; Del Valle et al. 2020; Guo et al. 2022; Meirelles-Júnior et al. 2024). The increase in DM content was attributable to improved DM recovery. For instance, studies on soybean whole-plant silage treated with chitosan and lactic acid bacteria had also reported enhanced DM recovery, underscoring chitosan capability to augment DM profile of silage (Gandra et al. 2018). Furthermore, the rise in DM recovery was associated with decreased fermentative losses and degradation (Del Valle et al. 2018).

Variations in the levels of crude ash, OM, CP, ether extract (EE), ADF, and hemicellulose in DM were relatively minor, suggesting that the additives had a limited effect on these components. Studies by Del Valle et al. (2018) had shown that chitosan could enhance OM content in sugarcane silages. According to Meirelles-Júnior et al. (2024), it was reported that chitosan could increase CP levels in silages while reducing hemicellulose content. Guo et al. (2022) noted that adding chitosan increased levels of non-protein-N and ammonia nitrogen (NH_3-N), and albeit with a decrease in true protein content in sugarcane top silage. Chitosan, a naturally occurring polycationic linear polysaccharide derived from chitin, incorporated nitrogen in primary aliphatic amine groups, facilitating typical amine reactions (Kim 2018). Furthermore, Sırakaya and Büyükkılıç Beyzi (2022) observed that chitosan addition increased crude ash and NDF levels in alfalfa silages, but it led to a decrease in EE.

Table 2. Nutritive value of pineapple peel material used for silage

Composition	Content
Dry matter (%)	15.49
Crude ash (% DM)	12.43
Organic matter (% DM)	87.57
Crude protein (% DM)	4.22
Ether extract (% DM)	0.88
Crude fiber (% DM)	17.88
NDF (% DM)	52.47
ADF (% DM)	27.76
Hemicellulose (% DM)	24.72
WSC (%)	80.78

Note: DM: dry matter, NDF: neutral detergent fiber, ADF: acid detergent fiber, WSC: water-soluble carbohydrate

Crude fiber and NDF levels demonstrated an upward trend with increasing concentrations of chitosan. NDF analysis method excluded starches, sugars, free amino acids, and other water-soluble constituents, isolating components like hemicellulose, cellulose, and acid detergent lignin (ADL), as described by Nielsen et al. (2019). The resistance of cellulose and hemicellulose to dissolution in neutral detergents was primarily due to their crystalline structure and strong intermolecular hydrogen bonds. Chitosan, recognized for the biocompatibility, exhibited limited solubility in neutral and alkaline environments, which restricted some of the applications (Cheung et al. 2015). In alkaline solutions, chitosan solubility further decreased, leading to the formation of large aggregates, thereby enhancing the adsorption properties (Feng et al. 2017). The presence of components, such as lignin and cellulose in ADF also contributed to the overall composition of NDF (Ramya et al. 2021). Increases in NDF and ADF were typically associated with higher crude fiber contents in forage and feed materials, as supported by various studies examining their nutritional composition and quality (Ottoni et al. 2021).

The addition of molasses to silage significantly boosted WSC content, leveraging the rich composition of fermentable sugars, such as sucrose and glucose. These sugars served as an economical source for lactic acid bacteria (LAB), offsetting potential sugar deficit in the forage, as noted by Zhao et al. (2019). Chitosan treatment also appeared to enhance WSC concentration in silage. This increase could be attributed to chitosan beneficial impacts on silage fermentation and preservation, where it was observed to minimize fermentative losses and improve nutritive value and degradation of silage (Del Valle et al. 2018).

Quality of pineapple peel silage

Table 4 showed that silage treated with 20 g molasses consistently showed lower weight loss compared to 0 g (without molasses addition) treatment across all chitosan levels (P<0.05), with mean weight losses of 4.33±0.37% and 4.39±0.37%, respectively. Moreover, the incorporation of chitosan into silage formulation resulted in a significant reduction in weight loss (P<0.05), with the minimal loss observed at the highest chitosan concentration (5 g), which was 4.04±0.03%. According to Anggraeni et al. (2023) similarly found that administering 1.5% chitosan in TMR silages tended to decrease weight loss compared to other treatments (0.05<P<0.1). Moreover, Harahap et al. (2023) also reported that chitosan reduced DM loss in silage. This result was further supported by other studies showing that chitosan addition improved DM recovery in silage (Gandra et al. 2016; Del Valle et al. 2018; Gandra et al. 2018; Guo et al. 2022). Chitosan reduced weight loss in silage primarily because of antifungal and antimicrobial properties, particularly during the aerobic phase of ensilage.

Table 3. Nutritive value of pineapple peel silage for 21 days incubation treated with chitosan, molasses, or a combination of both

Composition	Without molasses			With 20 g molasses		
	0 g CHI	3 g CHI	5 g CHI	0 g CHI	3 g CHI	5 g CHI
Dry matter (%)	9.61	9.31	11.66	10.50	9.63	10.53
Crude ash (% DM)	9.98	10.07	10.94	10.02	10.10	12.43
Organic matter (% DM)	90.02	89.93	89.06	89.98	89.90	87.57
Crude protein (% DM)	6.41	6.00	5.91	6.72	6.45	6.51
Ether extract (% DM)	2.19	2.24	2.20	2.11	2.18	2.14
Crude fiber (% DM)	29.55	29.71	29.99	28.21	28.83	31.75
NDF (% DM)	56.04	57.47	57.62	56.78	57.33	57.57
ADF (% DM)	36.91	37.10	36.55	37.12	35.22	30.94
Hemicellulose (% DM)	19.13	20.37	21.07	19.66	22.11	26.63
WSC (%)	21.82	24.89	30.28	24.10	26.19	28.01

Notes: CHI: Chitosan; DM: Dry matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; WSC: Water-soluble carbohydrate

Table 4. Effects of various levels of molasses and chitosan on the quality of pineapple peel silage for a 21-day incubation period

Items	Molasses levels (g)	Chitosan levels (g)			Mean	SEM	*P-value*		
		0	3	5			M	C	M*C
Weight loss (%)	0	4.89	4.23	4.06	4.39^{y}	0.074	<0.001	<0.001	0.562
	20	4.82	4.17	4.01	4.33^{x}				
	Mean	4.86^{C}	4.20^{B}	4.04^{A}					
pH	0	3.93	3.94	3.95	3.94^{y}	0.007	<0.001	0.779	0.574
	20	3.88	3.88	3.89	3.88^{x}				
	Mean	3.91	3.91	3.91					
NH_3 (g/L)	0	100.94	106.25	108.38	107.67	3.016	0.220	0.192	0.575
	20	113.69	114.75	124.31	115.10				
	Mean	103.59	116.34	114.22					

Note: A-C: Different superscript letters indicated significant differences for chitosan levels; x-y: Different superscript letters indicated significant differences in molasses levels. NH_3: Ammonia; SEM: Standard error mean

Antimicrobial activity of chitosan was attributed to the primary amine groups, which interacted with the negatively charged components of microbial cell membranes, disrupting the structure and function (Xing et al. 2016). This disruption led to the leakage of intracellular components, ultimately inhibiting the growth of fungi and bacteria (Hashemi et al. 2022). This inhibited the growth of yeast and molds that caused spoilage, reduced ethanol production, and led to a more stable fermentation profile with increased acetic acid, which had antifungal properties (Del Valle et al. 2018; Gandra et al. 2022). Chitosan also minimized liquid and gas losses, further conserving DM and maintaining the nutritional value of feed (Del Valle et al. 2020).

Regarding pH, the addition of 20 g molasses led to a slight reduction compared to 0 g (without molasses addition) treatment ($P<0.05$), with the lowest recorded pH of 3.88±0.02 across chitosan treatments. However, pH values were not significantly altered by different chitosan concentrations, suggesting that chitosan alone did not affect the acidity of silage ($P>0.05$). Previous studies also reported that the addition of chitosan to silage preservation did not affect pH of silage (Gandra et al. 2018; Del Valle et al. 2020; de Morais 2021; Guo et al. 2022; Del Valle et al. 2022; Meirelles-Júnior et al. 2024). Chitosan role was mainly in shaping the microbial environment to favor these beneficial microbes and inhibit the harmful ones, not in altering pH directly (Anggraeni et al. 2023). However, molasses could help chitosan in reducing pH of pineapple peel silage. Moreover, the presence of molasses provided fermentable substrates that enhanced lactic acid fermentation, leading to a rapid decrease in pH levels (Ke et al. 2023).

Although higher additions of chitosan led to an observable increase in NH_3 concentrations, this trend did not reach statistical significance ($P>0.05$), suggesting that NH_3 levels remained relatively low across the different chitosan treatments. Previous studies similarly found that the addition of chitosan did not significantly alter NH_3 content in silage (Del Valle et al. 2018, Del Valle et al. 2020; de Morais et al. 2021). However, Del Valle et al. (2020) reported a linear increase in NH_3-N concentration in sugarcane silage with increasing levels of chitosan. This increase could potentially be attributed to chitosan impact on protein degradation or the influence on microbial communities during the ensiling process. Inhibiting undesirable bacteria could alter fermentation dynamics, affecting the production and accumulation of NH_3-N, by-product of protein degradation.

Rumen fermentation profile and microbial population in the in vitro batch culture study of pineapple peel silage

Analysis of fermentation profile in Table 5 revealed that both molasses and chitosan levels had no impact on pH of rumen fluid ($P>0.05$). However, the inclusion of chitosan was associated with an increase in ruminal pH in beef heifers offered a grass-silage-based diet reported by Kirwan et al. (2021). pH values observed in this study were in the normal range, specifically between 6.68 and 6.73. The optimal pH range in rumen was generally considered to be between 6.0 and 7.0 (Nayohan et al. 2022). Deviations from this optimal pH range could impact rumen microbial life, nutrient utilization, and overall metabolic functions (Ningsih 2023).

Ammonia (NH_3) concentrations indicated no significant differences across treatments ($P>0.05$), although there was a trend towards increased NH_3 levels with higher chitosan additions. This aligned with results by Harahap et al. (2023), which reported that adding chitosan to elephant grass and *Indigofera* legume did not significantly affect NH_3 levels in rumen fermentation in vitro. A meta-analysis of in vivo studies, which involved the addition of chitosan to feed, demonstrated that dietary chitosan did not influence NH_3 concentrations in rumen (Harahap et al. 2022). However, chitosan inclusion interacted with dietary CP levels, leading to increased rumen NH_3 concentrations in high-protein diets but not in low-protein diets in beef heifers offered a grass silage-based diet (Kirwan et al. 2021). Beier and Bertilsson (2013) also suggested that the increased NH_3 levels in dietary chitosan could be attributed to the transformation of amine groups ($R\text{-}NH_2$) into NH_3, as chitosan, a nitrogenous compound, was susceptible to degradation by rumen microbes.

Volatile fatty acids (VFA) molar proportion profiles showed variations across treatments. The concentration of acetic acid (C_2) was notably higher in silage without molasses ($P<0.05$). Studies had shown that molasses supplementation could shift the fermentation pattern towards increased lactic acid fermentation, resulting in lower acetic acid levels and higher lactic acid concentrations (Fang et al. 2022). Furthermore, the addition of molasses to silage was associated with a decrease in the acetic acid to propionic acid ratio, indicating a shift towards propionate production and a reduction in acetic acid levels (Babaeinasab et al. 2015). Propionic acid (C_3) had a trend towards a slight increase in concentrations as chitosan levels increased, but no significant differences across treatments ($P>0.05$). Moreover, the ratios of acetic to propionic acid (C_2:C_3) trend towards decreased ratios observed with higher chitosan levels, however statistically there were no significant differences across treatments ($P>0.05$). Previous meta-analyses of in vitro rumen studies reported that the addition of chitosan increased the proportion of C_3, but decreased the proportion of C_2 and the total concentration of VFA (Harahap et al. 2020). This could interact with rumen microbiota, leading to changes in fermentation pathways and promoting the production of propionic acid over acetic acid (Jiménez-Ocampo et al. 2019). Belanche et al. (2015) reported that chitosan caused essential changes in the structure of rumen bacterial community, meaning, decreasing the number of *Firmicutes* and *Fibrobacter* but increasing *Bacteroidetes* and *Proteobacteria*, which included most amylolytic bacteria. There were no significant differences across treatments of the n-butyric acid (nC_4) and n-valeric acid (nC_5) ($P>0.05$). Similarly, branched-chain volatile fatty acid (BCVFA) such as isobutyric acid (iso-C_4) and isovaleric acid (iso-C_5) significant differences across treatments ($P>0.05$). According to Kirwan et al. (2021), the inclusion of chitosan in the diets of beef heifers fed a grass silage-based diet did not affect the levels of n-butyric acid (nC_4), isobutyric acid (iso-C_4), and isovaleric acid (iso-C_5) in rumen fermentation parameters. The alteration in fermentation products in rumen could be due to the degradation of chitosan, with the remaining carbon skeleton utilized by specific bacteria (Belanche et al. 2015). The inefficient eating and chewing efficiency associated with chitosan inclusion could have potentially impacted the production of VFA as a result (Haraki et al. 2018).

In terms of the microbial population, there was a significant difference in the bacterial counts, with higher populations observed in no-molasses treatments ($P<0.05$). The reduction in bacterial populations in rumen fermentation process, as observed with molasses supplementation, could be linked to the alterations in bacterial activity due to the intake of highly digestible carbohydrates. According to Petri et al. (2012), the introduction of molasses could lead to a decrease in fibrinolytic bacteria, which were crucial for fiber degradation, while simultaneously promoting the proliferation of amylolytic and lactic acid-utilizing bacteria.

In addition, there was a decrease in microbial population with higher chitosan levels addition ($P<0.05$). Chitosan, a hydrophilic biopolymer obtained through N-deacetylation of chitin, exhibited antimicrobial properties against a wide range of microorganisms, including protozoa, Gram-positive and Gram-negative bacteria, fungi, and yeasts (Sánchez-Machado et al. 2021). Belanche et al. (2015) reported that the incorporation of chitosan at a 5% DM concentration led to a notable decrease in the relative abundance of *Anaerovibrio* spp. and *Butyrivibrio* spp. in rumen simulation technique system. This outcome was quantified using Next Generation Sequencing methods, highlighting the impact of chitosan on specific microbial populations in rumen. This result suggested that chitosan could influence microbial dynamics and potentially affect ruminal fermentation processes.

Table 5. Effects of various levels of molasses and chitosan on rumen fermentation profile and microbial population in rumen *in vitro* batch culture study using pineapple peel silage

Items	Molasses levels (g)	Chitosan levels (g)			Mean	SEM	*P-value*		
		0	3	5			M	C	M*C
pH	0	6.73	6.72	6.68	6.71	0.014	0.604	0.920	0.096
	20	6.70	6.70	6.73	6.72				
	Mean	6.72	6.71	6.72					
NH_3 (g/L)	0	298.56	334.69	334.69	322.65	17.090	0.518	0.362	0.967
	20	291.13	314.50	321.94	309.19				
	Mean	294.84	324.59	328.31					
C_2 (g/mL)	0	2.47	2.64	2.86	2.66^y	0.264	0.047	0.369	0.793
	20	1.91	1.74	2.43	2.02^x				
	Mean	2.19	2.19	2.64					
C_3 (g/mL)	0	2.27	2.34	2.56	2.39	0.279	0.508	0.618	0.770
	20	2.07	2.40	2.26	2.25				
	Mean	2.17	2.37	2.41					
iso-C_4 (g/mL)	0	1.11	1.01	1.15	1.09	0.211	0.587	0.356	0.226
	20	0.92	1.12	1.12	1.05				
	Mean	1.01	1.07	1.14					
nC_4 (g/mL)	0	0.67	0.75	0.81	0.74	0.044	0.455	0.869	0.599
	20	0.71	0.68	0.66	0.68				
	Mean	0.69	0.72	0.74					
iso-C_5 (g/mL)	0	0.30	0.32	0.35	0.33	0.033	0.365	0.576	0.818
	20	0.29	0.32	0.30	0.30				
	Mean	0.30	0.32	0.33					
nC_5 (g/mL)	0	0.26	0.28	0.31	0.29	0.024	0.657	0.962	0.376
	20	0.30	0.28	0.23	0.27				
	Mean	0.28	0.28	0.27					
C_2:C_3	0	1.26	1.19	1.11	1.18	0.061	0.289	0.816	0.525
	20	1.07	0.98	1.16	1.07				
	Mean	1.16	1.08	1.13					
Bacteria (log CFU mL^{-1})	0	10.11	10.02	10.07	10.07^y	0.020	<0.01	<0.05	0.17
	20	9.95	9.92	9.87	9.91^x				
	Mean	10.03^B	9.97^A	9.97^A					
Protozoa (log cell mL^{-1})	0	6.62	6.58	6.58	6.59^x	0.016	<0.01	0.20	0.75
	20	6.73	6.71	6.67	6.70^y				
	Mean	6.67	6.64	6.62					

Note: A-C: Different superscript letters indicated significant differences for chitosan levels; x-y: Different superscript letters indicated significant differences in molasses levels; NH_3: Ammonia; C_2: Acetic acid; C_3: Propionic acid; iso-C_4: Isobutyric acid; nC_4: n-butyric acid; iso-C_5: Isovaleric acid; nC_5: n-valeric acid; and SEM: Standard error mean

Protozoal counts were slightly higher in the 20 g/kg molasses treatments than in no molasses addition ($P<0.05$), indicating possible interactions between dietary components and microbial dynamics. A report by Ravelo et al. (2022) suggested that including molasses in the diet could increase the protozoa population, leading to a decrease in the concentration of total VFA. Furthermore, Osman et al. (2020) noted that ruminal protozoa could contribute to the decrease in VFA concentration in animals fed molasses.

Rumen degradation of dry matter digestibility, organic matter digestibility, total gas, and methane production in rumen in vitro batch culture

The results, as shown in Table 6, indicated that chitosan positively influenced DM digestibility. Specifically, IVDMD increased significantly with the addition of chitosan ($P<0.05$), showing the highest digestibility at 5 g/kg chitosan (79.00±6.36% for 0 g/kg molasses and 77.34±6.60% for 20 g/kg molasses). However, there were no significant differences in treatment addition with IVOMD ($P>0.05$). Chitosan, with the positively charged $-NH^{2+}$ groups, could electrostatically interact with the negatively charged carboxyl groups of amino acids in the feed, as described by Chiang et al. (2009). This binding could protect proteins from rapid microbial degradation in rumen, allowing more protein to reach the lower gut for digestion and absorption. Such a mechanism indirectly improved nitrogen utilization and could potentially enhance DM digestibility. The favorable interaction between chitosan positive charge and deprotonated carboxyl groups of proteins could extend to mineral binding due to similar charge interactions (Kasimova et al. 2011).

In terms of gas production, Figure 1 showed a significant reduction in total gas volume with increasing chitosan levels ($P<0.05$). Chitosan had antimicrobial activity, particularly against certain types of bacteria, which could lead to a shift in the populations of microbes in rumen (Goiri et al. 2009). By inhibiting or altering the balance of microbial species, chitosan could change the fermentation pattern. This could lead to reductions in the production of gases such as CH_4 and carbon dioxide, which were typical by-products of microbial fermentation. Jayanegara et al. (2018) demonstrated that carbohydrates were the primary nutrients contributing to total gas production in rumen, resulting from the microbial degradation and fermentation of substrates, with a significantly higher impact than proteins.

Methane (CH_4) concentrations indicated no significant differences across treatments ($P>0.05$), although there was a trend towards decreased CH_4 with higher chitosan additions (Figure 2). However, Harahap et al. (2023) reported that the addition of chitosan to silages made from elephant grass and Indigofera legume resulted in a reduction in CH_4 production. Wencelová et al. (2014) found that the addition of chitosan led to a reduction in the protozoan population, particularly *Entodinium* spp. Since many methanogens existed in symbiosis with protozoa and benefited from these faunas, a decrease in protozoan populations was likely to also reduce methanogen numbers and their methanogenic activity, as suggested by Jayanegara et al. (2017). Chitosan could influence the proportions of VFA produced during fermentation, decreasing the acetate-to-propionate ratio (Goiri et al. 2009). Acetate production was typically associated with more hydrogen and CH_4 formation, while propionate production did not yield CH_4.

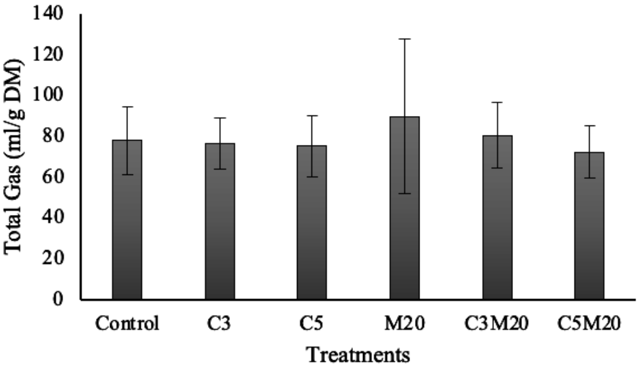

Figure 1. In vitro total gas production (mL/g DM) of pineapple peel silage with different levels of molasses and chitosan addition. Control, C3, pineapple peel + 3 g chitosan, C5, pineapple peel + 5 g chitosan; M20, pineapple peel + 20 g molasses; C3M20, pineapple peel + 3 g chitosan + 20 g molasses, C5M20, pineapple peel + 5 g chitosan + 20 g molasses. p: molasses levels=0.261, chitosan levels=0.041, molasses×chitosan=0.391. The error bar indicated the standard error for each treatment

Table 6. Effects of various levels of molasses and chitosan on DM digestibility and OM digestibility in rumen *in vitro* batch culture study using pineapple peel silage

Items	Molasses levels (g)	Chitosan levels (g)			Mean	SEM	P-value		
		0	3	5			M	C	M*C
IVDMD (%DM)	0	74.28	78.05	79.00	77.11	1.143	0.390	0.026	0.509
	20	75.05	76.38	77.34	76.26				
	Mean	74.67^A	77.22^B	78.17^B					
IVOMD (%DM)	0	81.47	83.77	80.95	82.06	1.013	0.416	0.188	0.100
	20	79.67	81.32	83.04	81.34				
	Mean	80.57	82.54	81.99					

Notes: A-C: Different superscript letters indicate significant differences for chitosan levels; NH_3: Ammonia; DM: Dry matter; IVDMD: In vitro dry matter digestibility; IVOMD: In vitro organic matter digestibility, SEM: standard error mean

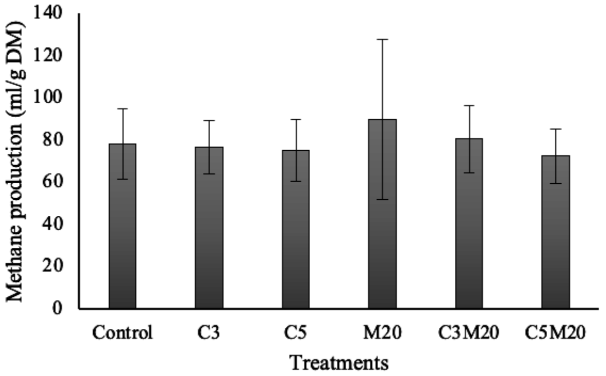

Figure 2. In vitro CH_4 production (mL/g DM) of pineapple peel silage with different levels of molasses and chitosan addition. Control, C3, pineapple peel + 3g chitosan, C5, pineapple peel + 5g chitosan, M20, pineapple peel + 20g molasses; C3M20, pineapple peel + 3g chitosan + 20g molasses, C5M20, pineapple peel + 5g chitosan + 20g molasses. p: molasses levels=0.370, chitosan levels=0.243, molasses×chitosan=0.460. The error bar indicated the standard error for each treatment

In conclusion, the use of molasses led to a decrease in pH and enhanced silage preservation quality. Both chitosan and molasses minimized weight loss during ensiling. Furthermore, chitosan alone increased IVDMD, particularly notable at a 5 g/kg fresh feed concentration, effectively reducing total gas production, but also showed potential in defaunation protozoa in rumen. There was also a potential reduction in CH_4 emissions with chitosan, although additional reports were necessary to confirm this effect.

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