

RESEARCH ARTICLE

Effects of dietary papaya peel inclusion on *in vitro* rumen fermentation, methane production, and degradability in sheep



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ABSTRACT

Background and Aim: Papaya peel (PP) is an agro-industrial by-product containing nutrients and bioactive compounds with potential for use as an alternative ruminant feed ingredient. However, information on the effects of graded dietary PP inclusion on rumen fermentation, methane production, and nutrient degradability remains limited. This study evaluated the effects of dietary PP inclusion on *in vitro* rumen fermentation characteristics, methane production, and degradability using sheep rumen fluid.

Materials and Methods: A completely randomized design comprising four dietary treatments with eight incubation units per treatment was used. PP was included at 0% (PP-0), 10% (PP-10), 20% (PP-20), or 40% (PP-40). Rumen fluid was collected from five Garut-cross male lambs (37.03 ± 1.05 kg), pooled, and mixed with McDougall buffer. Experimental diets were incubated for 48 h. Total gas and methane production, rumen pH, ammonia nitrogen (NH₃-N), volatile fatty acids, *in vitro* dry matter (DM) degradability, and *in vitro* organic matter degradability were determined. Data were analyzed using one-way analysis of variance followed by Tukey's test when treatment effects were significant.

Results: PP inclusion significantly increased gas production at 12, 18, and 24 h ($p \leq 0.006$), with the highest 24-h value observed in PP-20 (32.5 mL) compared with PP-0 (25.0 mL). NH₃-N increased from 3.88 mM in PP-0 to 8.65 mM in PP-40 ($p < 0.001$). Total volatile fatty acid concentration was highest in PP-20 (29.4 mM) compared with PP-0 (18.0 mM; $p = 0.010$), while acetate increased significantly ($p = 0.031$). PP inclusion did not significantly affect 48-h gas production, methane production, rumen pH, DM or organic matter degradability, propionate, butyrate, or valerate concentrations ($p > 0.05$).

Conclusion: Dietary PP, particularly at 20% inclusion, enhanced selected *in vitro* rumen fermentation responses without adversely affecting rumen pH or degradability; however, it did not significantly reduce methane production. Because the experiment used a single pooled rumen fluid inoculum batch, these findings should be interpreted as preliminary. Further *in vivo* studies are required to determine effects on nutrient utilization, animal performance, and methane emissions.

Keywords: agricultural by-product, feed degradability, methane production, PP, rumen fermentation, sheep nutrition, sustainable feed, volatile fatty acids.

INTRODUCTION

Sheep farming is an important livestock activity among farmers in Indonesia. The national sheep population reached 9.1 million in 2025, up 1.26% from the previous year [1]. Despite this growth, sheep production remains predominantly traditional and small-scale. Converting grazing land to infrastructure and residential areas has reduced the availability of conventional feed resources, potentially constraining sheep productivity. Increasing the proportion of concentrate in the diet may compensate for limited forage availability; however, this strategy is not always economically feasible for small-scale farmers because of the high cost of concentrate feeds. Furthermore, excessive concentrate feeding can decrease rumen pH and alter the microbial balance, thereby increasing the risk of ruminal acidosis and potentially compromising animal health and productivity. These constraints highlight the need to identify locally available, nutritionally valuable, and cost-effective alternative

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Received: 26-06-2026, **Accepted:** 06-10-2026, **Published online:** 24-10-2026

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How to cite: Ramdani D, Hernaman I, Juandita KN, Alhuur KR, Mburu LM, Rano NB. Effects of dietary papaya peel inclusion on *in vitro* rumen fermentation, methane production, and degradability in sheep. Vet World. 2026;19(10):4788–4797.

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feed resources for sheep production.

Papaya (*Carica papaya* L.) is a widely cultivated and economically important fruit crop in tropical and subtropical regions. Papaya processing and consumption generate peel as a by-product that could potentially be utilized as an alternative feed resource. Papaya peel (PP) contains 6.89–10.0% crude protein (CP), indicating its potential as a moderate protein source for sheep [2]. Beyond its nutritional value, PP contains several bioactive compounds, including cysteine proteinases, alkaloids, papain, chymopapain, papaya latex constituents, saponins, flavonoids, carpain, and tannins, that may influence rumen fermentation [2, 3]. Some plant bioactive compounds have been associated with antimethanogenic activity, including effects mediated through the suppression of rumen protozoa. However, their effects on methane production are not consistent and may vary according to compound characteristics, concentration, microbial adaptation, and compensatory mechanisms within the rumen microbial ecosystem [4]. Therefore, specific evaluation of PP's effects on fermentation efficiency and methane production is needed before establishing its potential use as a ruminant feed ingredient.

Despite the potential nutritional and bioactive properties of PP, evidence regarding the response of rumen fermentation to graded levels of PP inclusion remains limited. In particular, information is limited on the inclusion of PP at 10%, 20%, and 40% on a dry matter (DM) basis in concentrate- and corn stover-based diets and on its effects on *in vitro* fermentation using rumen fluid from Garut-cross sheep. Moreover, concurrent evaluation of total gas production (tGP), methane (CH₄) production, nutrient degradability, ammonia nitrogen (NH₃-N), and individual volatile fatty acid (VFA) profiles is needed to characterize the fermentation response comprehensively. Establishing whether increasing PP inclusion modifies these parameters without adversely affecting rumen conditions or nutrient degradability is important for determining an appropriate inclusion level for subsequent investigation.

Therefore, this study hypothesized that dietary PP inclusion would modify *in vitro* rumen fermentation characteristics without adversely affecting nutrient degradability or rumen conditions. The study aimed to evaluate the effects of four dietary PP inclusion levels, namely, 0% (PP-0), 10% (PP-10), 20% (PP-20), and 40% (PP-40), on tGP and CH₄ productions, rumen pH, NH₃-N concentration, VFA profiles, and *in vitro* dry matter (DM) and organic matter (OM) degradability using rumen fluid from Garut-cross sheep. The findings were intended to identify a candidate PP inclusion level for further evaluation as an alternative feed ingredient for sheep.

MATERIALS AND METHODS

Ethical approval

The study protocol was reviewed and approved by the Research Ethics Commission of Universitas Padjadjaran (Approval No. 787/UN6.KEP/EC/2025). Although the experimental procedures were conducted *in vitro*, rumen fluid was collected orally from live donor lambs; therefore, animal handling and rumen fluid collection were performed in accordance with the applicable principles of the ARRIVE 2.0 guidelines. During rumen fluid collection, the donor lambs were handled appropriately without analgesia or physical restraint.

Study period and location

The study was conducted from March to May 2026. The *in vitro* incubation was performed for 48 h in a single experimental run at the Biotechnology Research and Testing Laboratory, Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia. Following incubation, rumen pH, CH₄ production, *in vitro* DM degradability (IVDMD), and *in vitro* organic matter degradability (IVOMD) were determined at the same laboratory. VFA profiles and NH₃-N concentration were analyzed at the Central Laboratory, Universitas Padjadjaran, Indonesia.

Study design

The experiment used a completely randomized design with four dietary PP inclusion levels (0% [PP-0], 10% [PP-10], 20% [PP-20], and 40% [PP-40]) and eight incubation units per treatment (n = 8). Each syringe represented an experimental incubation unit. All incubation units received inoculum prepared from a single pool of rumen fluid collected from five donor lambs. Therefore, the donor lambs provided inoculum sources rather than independent biological replicates, and statistical inference was limited to the pooled inoculum batch used in this experiment.

Diet preparation

PP (California variety) at approximately 50% ripeness, characterized by slight yellowing of the peel, were consistently obtained from a local farmer in Karang Pucung, Kebumen, Central Java, Indonesia. The PP was dried at 58°C for 48 h in a drying oven (DHG-9053A, Thermo Fisher Scientific, United States) and ground using a hammer

mill to pass through a 1-mm sieve.

The commercial concentrate (Starter Grade [SG-2]) was obtained from PT Dilar Lintas Raya (Tasikmalaya, Indonesia) and had an ingredient formulation similar to that reported by Juandita *et al.* [5]. It consisted of palm kernel meal (21%), cassava meal (15%), coffee husk (11%), cacao husk (6%), rice bran (10.5%), copra meal (9%), molasses (8.5%), dried cassava (8.45%), soybean meal (2%), distillers' dried grains with solubles (1%), rapeseed meal (1.5%), wheat pollard (0.5%), mineral and vitamin premix (Lagantor F1 Customix, Kalbe Animal Health; 2%), lime (2%), corn gluten feed (1%), sodium bicarbonate (0.35%), and salt (0.2%). Corn stover was ground using a hammer mill to pass through a 1-mm sieve.

The basal diet consisted of corn stover and commercial concentrate at a 60:40 ratio. For the experimental diets, PP replaced the commercial concentrate at the assigned treatment level, the experimental diet is presented on a DM basis. The detailed formulations and nutrient compositions of the experimental diets are presented in Tables 1 and 2.

Animal handling and rumen fluid collection

Animal handling and rumen fluid collection were performed following the procedures described by Wang *et al.* [4] and Juandita *et al.* [5]. Five Garut-cross male lambs with a mean body weight of 37.03 ± 1.05 kg were used as rumen fluid donors. Before rumen fluid collection, the lambs were adapted to the basal diet for 1 month; the basal diet was provided twice daily in the morning (07.00 AM) and afternoon (04.00 PM). Rumen fluid was collected orally from each lamb in its individual pen before morning feeding.

All donor lambs received the same basal diet consisting of corn stover and commercial concentrate at a 60:40 ratio. The diet was formulated to provide a DM intake equivalent to approximately 3.5%–4.0% of body weight. Drinking water was provided *ad libitum*. Approximately 100 mL of rumen fluid was collected from each lamb before morning feeding, yielding approximately 500 mL in total. The rumen fluid collection was conducted orally using an orogastric tube, with the initial 25 mL of rumen fluid discarded to avoid salivary contamination. The rumen fluid from all five lambs was pooled in a 650-mL flask (Lion Star Brand, PT Cahaya Perdana Plastics, Indonesia) to obtain a homogeneous inoculum.

The pooled rumen fluid was filtered through four layers of muslin cloth under continuous CO₂ flushing into a 650-mL flask that had been prewarmed and maintained at 39°C. The flask was filled completely, then tightly sealed to maintain anaerobic conditions during transport to the laboratory.

In vitro incubation

The *in vitro* rumen incubation procedure followed Juandita *et al.* [5] and Ramdani *et al.* [6]. The filtered rumen fluid was mixed with McDougall buffer [7], which had been flushed with CO₂ and prewarmed to 39°C, at a buffer-to-rumen fluid ratio of 2:1. The pH of the buffered inoculum was adjusted to approximately 7.0 ± 0.2 by the dropwise addition of HCl. The pooled buffered inoculum was then distributed uniformly among the experimental syringes following the procedure described by Juandita *et al.* [5].

Approximately 200 ± 4 mg of each experimental diet was transferred into a 100-mL glass syringe (Fortuna Optima Poulten Graf, Wertheim, Germany). Subsequently, approximately 20 mL of buffered rumen inoculum was added to each syringe, and the syringes were incubated in a water bath at 39°C for 48 h. tGP was measured every 2 h during the first 24 h and subsequently at 4-h intervals until the end of the 48-h incubation period [5].

At the end of incubation, most of the warm water in the water bath was replaced with ice to terminate further fermentation. Subsequently, 20 mL of gas from each incubated syringe was transferred to a clean syringe fitted with a stopcock for direct CH₄ measurement using a calibrated GX-2012 Confined Space Gas Monitor (Riken Keiki, Japan). The gas monitor was calibrated according to the ISO/IEC 17025 standard at PT Adhigana Perkasa Mandiri (Jakarta, Indonesia). Methane production was expressed as a percentage and as mL/200 mg of incubated substrate following the methodology described by Medjekal *et al.* [8].

The contents of each syringe, including the inoculum and residue, were transferred to a preweighed 20-mL polyethylene tube (PT Jayamas Medica Industri, Indonesia). Rumen pH was measured using a calibrated pH meter (Eutech pH 700, Germany). The tubes were then centrifuged at 5,000 rpm for 10 min, and the resulting supernatants were collected for VFA and NH₃-N analyses and stored at –20°C until analysis [5, 9]. Residual particles were washed with water, collected, and oven-dried at 60°C to determine IVDMD; IVOMD was determined by ashing the oven-dried residue in a muffle furnace at 550°C for 5 h, as described by Khan and Chaudhry [10].

Ammonia nitrogen analysis

Ammonia nitrogen concentration was determined using the phenol-hypochlorite colorimetric method

coupled with spectrophotometric measurement, as described by Widyarini *et al.* [11]. The $\text{NH}_3\text{-N}$ standard curve comprised concentrations of 0.25, 0.5, 1, 3, 6, and 12 mM. Approximately 100 μL of sample supernatant was transferred to a test tube, then 500 μL of phenol reagent (50 g/L phenol and 0.25 g/L sodium nitroprusside) and 500 μL of NaOCl reagent (16.9 mL/L NaOCl and 25 g/L NaOH) were added. The mixture was thoroughly mixed and incubated in a water bath at 39°C for 20 min. Absorbance was subsequently measured at 630 nm using a UV–visible spectrophotometer (Lambda 35, PerkinElmer, Waltham, MA, USA), following Sagala *et al.* [9]. Ammonia nitrogen concentrations were calculated from the standard curve and expressed as mM.

Individual VFA analysis

Individual VFA concentrations were determined following the method described by Juandita *et al.* [5], with slight modifications. Rumen fluid supernatants were mixed with 200 g/L metaphosphoric acid (Merck KGaA, Germany) at a metaphosphoric acid-to-supernatant ratio of 1:4 before analysis.

Individual VFAs were determined using gas chromatography–mass spectrometry (GC–MS) with an Agilent 7890A system equipped with an Agilent 5977B mass-selective detector and a DB-FFAP column (Agilent 123-3262; 60 m $\times$ 0.325 mm internal diameter; 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min. Particle-free samples were injected using a split injection mode and a 3-min delay following Sagala *et al.* [9]. The injector temperature was maintained at 160°C. The oven temperature was initially held at 80°C for 1 min, then increased at 15°C/min to 115°C and held for 3 min; it was then increased at 3°C/min to 130°C and finally at 15°C/min to 230°C, where it was held for 3 min. The split ratio was 15:1, following Juandita *et al.* [5].

Individual VFAs were identified and quantified by comparing sample peak areas with those of a certified VFA standard mixture (Supelco CRM46975, USA) containing 10.00 mM each of acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids.

Chemical analysis

Samples of PP, commercial concentrate, and corn stover were periodically collected from feed-storage bins for chemical analysis. Representative samples were collected from the top, middle, and bottom portions of each storage container. Samples were oven-dried at 58°C for 48 h, ground using a disk mill, and passed through a 1-mm sieve before analysis.

DM (method 934.01), ash (method 942.05), CP (method 984.13), and ether extract (EE; method 920.39) were determined according to AOAC International procedures. Neutral detergent fiber (NDFom; determined without decalin and α -amylase) and acid detergent fiber (ADFom) were determined following Van Soest *et al.* [12]. Metabolizable energy (ME) was estimated using the Menke and Steingass equation, following the procedure described by Ramdani *et al.* [13].

Total tannin (TT) concentrations in corn stover, commercial concentrate, and PP were determined using the Folin–Ciocalteu method with tannic acid as the reference standard, following Ramdani *et al.* [13]. Briefly, 200 mg of dried and ground sample was extracted with 10 mL of 70% aqueous acetone (v/v) in an ultrasonic water bath for two 10-min periods separated by a 5-min interval. The extract was centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant was collected.

For total phenol determination, 0.02 mL of extract was mixed with 0.48 mL of distilled water, 0.25 mL of 1 N Folin–Ciocalteu reagent, and 1.25 mL of 20% sodium carbonate. After vortexing and incubation for 40 min, absorbance was measured at 725 nm using a UV spectrophotometer (UV-1800, Shimadzu, Japan). Tannic acid was used to generate the calibration curve, and the results were expressed as g/kg DM tannic acid equivalents.

For determination of non-tannin phenols, tannins were removed from the extract using polyvinylpyrrolidone (PVPP). The extract was mixed with PVPP and acidified distilled water (pH 3), incubated at 4°C for 15 min, and centrifuged at 3,000 rpm for 10 min at 4°C. The resulting supernatant was analyzed for non-tannin phenols using the same Folin–Ciocalteu procedure. TT was calculated as the difference between total phenols and non-tannin phenols and expressed as g/kg DM tannic acid equivalents. This procedure quantified TT but did not differentiate between condensed and hydrolyzable tannins. All nutrient concentrations were expressed on a DM basis, except ME (MJ/kg DM) and DM content (% fresh sample).

Statistical analysis

The experimental design comprised four PP inclusion levels with eight incubation units per treatment ($n = 8$). Each syringe was considered an experimental incubation unit. Because all syringes received inoculum prepared from a single pooled rumen fluid batch, the five donor lambs were sources of inoculum and were not independent

biological replicates. Consequently, statistical inference was restricted to the pooled inoculum batch used in this experiment.

Data were analyzed using the General Linear Model procedure in IBM SPSS Statistics version 26.0 (IBM Corp., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate treatment effects, with statistical significance established at $p \leq 0.05$. When a significant treatment effect was detected, means were compared using Tukey's test. The normality of model residuals was assessed using the Shapiro–Wilk test, and all evaluated variables satisfied the normality assumption ($p > 0.05$).

RESULTS

The nutrient compositions of the feed ingredients and experimental diets are presented in Tables 1 and 2, respectively. PP contained 16.2% CP on a DM basis, and dietary CP increased slightly with increasing PP inclusion, from 11.6% in PP-0 to 12.2% in PP-40.

Table 1: Proximate and chemical composition of feed ingredients.

Nutrient composition	Corn stover	Commercial concentrate	Papaya peel
DM (% fresh sample)	31.4	84.3	9.21
ME (MJ/kg DM)	3.93	6.53	9.15
Ash (% DM)	8.80	10.2	8.97
CP (% DM)	9.56	14.7	16.2
EE (% DM)	2.78	6.21	2.47
ADFom (% DM)	38.5	32.3	42.5
NDFom (% DM)	66.1	48.1	31.3
TT (% DM)	2.27	1.75	18.1

DM = Dry matter, ME = metabolizable energy, CP = crude protein, EE = ether extract, ADFom = acid detergent fiber expressed exclusive of residual ash, NDFom = neutral detergent fiber expressed exclusive of residual ash, TT = total tannins.

Table 2: Formulation and nutrient composition of the experimental diets.

Feed ingredient/nutrient	PP-0	PP-10	PP-20	PP-40
Feed ingredients				
Corn stover (% DM)	60	60	60	60
Commercial concentrate (% DM)	40	30	20	0
Papaya peel (% DM)	0	10	20	40
Nutrient composition				
DM (% fresh sample)	52.5	45.0	37.5	22.5
ME (MJ/kg DM)	4.97	5.23	5.49	6.02
Ash (% DM)	9.40	9.26	9.13	8.87
CP (% DM)	11.6	11.7	11.9	12.2
EE (% DM)	4.15	3.78	3.40	2.66
ADFom (% DM)	36.0	37.0	38.0	40.1
NDFom (% DM)	58.9	57.2	55.5	52.1
TT (% DM)	2.06	3.69	5.33	8.60

PP-0 = 0% PP, PP-10 = 10% PP, PP-20 = 20% PP, PP-40 = 40% PP, PP = papaya peel, DM = dry matter, ME = metabolizable energy, CP = crude protein, EE = ether extract, ADFom = acid detergent fiber expressed exclusive of residual ash, NDFom = neutral detergent fiber expressed exclusive of residual ash, TT = total tannins. Nutrient composition values were calculated from the nutrient composition of the individual feed ingredients presented in Table 1 according to their respective inclusion levels in each experimental diet.

PP inclusion significantly affected tGP during the early incubation period (12–24 h) (Table 3). At 12 h, tGP increased from 15.5 mL in PP-0 to 20.3 and 21.2 mL in PP-20 and PP-40, respectively ($p < 0.001$). Similar treatment effects were observed at 18 h ($p = 0.002$) and 24 h ($p = 0.006$), with PP-20 and PP-40 showing higher tGP than PP-0. At 48 h, however, tGP did not differ among treatments ($p = 0.173$). Although CH₄ production was numerically lower in the PP-supplemented treatments, the differences were not statistically significant ($p > 0.05$).

Rumen pH did not differ among treatments ($p = 0.218$), with values ranging from 6.41 to 6.80 (Table 3). Similarly, PP inclusion did not significantly affect IVDMD ($p = 0.360$) or IVOMD ($p = 0.300$). In contrast, NH₃-N concentration differed significantly among treatments ($p < 0.001$), increasing from 3.88 mM in PP-0 to 6.49, 8.22, and 8.65 mM in PP-10, PP-20, and PP-40, respectively.

Total VFA concentration was significantly affected by PP inclusion ($p = 0.010$), with PP-20 and PP-40 showing higher concentrations than PP-0, while with PP-10 showing an intermediate value between PP-0 and the higher concentrations observed in PP-20 and PP-40. Acetate concentration also differed significantly among treatments ($p = 0.031$), with the highest value observed in PP-40 (20.2 mM) and the lowest in PP-0 (12.3 mM). No significant

treatment effects were observed for propionate ($p = 0.076$), isobutyrate ($p = 0.206$), butyrate ($p = 0.103$), isovalerate ($p = 0.733$), valerate ($p = 0.085$), or the acetate-to-propionate ratio ($p = 0.084$).

Table 3: Gas production and *in vitro* rumen fermentation characteristics at different levels of papaya peel inclusion ($n = 8$).

Parameter	PP-0	PP-10	PP-20	PP-40	SEM	p-value
Gas production						
tGP at 12 h (mL)	15.5 ^a	18.8 ^{ab}	20.3 ^b	21.2 ^b	0.799	<0.001
tGP at 18 h (mL)	21.0 ^a	24.9 ^{ab}	26.7 ^b	27.8 ^b	1.004	0.002
tGP at 24 h (mL)	25.0 ^a	29.2 ^{ab}	32.5 ^b	32.4 ^b	1.268	0.006
tGP at 48 h (mL)	32.9	38.0	40.5	38.9	2.124	0.173
CH ₄ (%)	14.4	12.0	12.8	11.4	1.320	0.468
CH ₄ (mL/200 mg)	5.65	4.75	4.64	4.79	0.790	0.897
Rumen fermentation characteristics						
pH	6.41	6.47	6.80	6.45	0.318	0.218
NH ₃ -N (mM)	3.88 ^a	6.49 ^b	8.22 ^b	8.65 ^b	0.626	<0.001
IVDMD (%)	40.6	37.6	31.0	37.4	4.428	0.360
IVOMD (%)	58.6	63.0	70.2	62.6	4.022	0.300
Total VFA (mM)	18.0 ^a	22.5 ^{ab}	29.4 ^b	27.8 ^b	2.350	0.010
Acetate (mM)	12.3 ^a	15.1 ^{ab}	19.1 ^{ab}	20.2 ^b	1.914	0.031
Propionate (mM)	3.92	4.04	5.86	4.97	0.577	0.076
Isobutyrate (mM)	0.82	0.58	2.07	0.84	0.406	0.206
Butyrate (mM)	0.30	1.06	1.88	0.89	0.511	0.103
Isovalerate (mM)	0.26	0.39	0.40	0.44	0.123	0.733
Valerate (mM)	0.29	1.31	0.41	0.39	0.232	0.085
A	3.24	3.81	3.46	4.07	0.432	0.084

PP-0 = 0% PP, PP-10 = 10% PP, PP-20 = 20% PP, PP-40 = 40% PP, PP = papaya peel, tGP = total gas production, CH₄ = methane, NH₃-N = ammonia nitrogen, IVDMD = *in vitro* dry matter degradability, IVOMD = *in vitro* organic matter degradability, VFA = volatile fatty acids, A = acetate-to-propionate ratio, SEM = standard error of the mean. Values within a row with different superscript letters (^a, ^b) differ significantly ($p < 0.05$).

DISCUSSION

Effects of PP on gas production and fermentation

The present study demonstrated that PP inclusion altered the temporal pattern of *in vitro* gas production, with significant increases during the early incubation period (12–24 h), whereas tGP at 48 h did not differ among treatments. This pattern suggests that PP inclusion affected the rate or timing of fermentation during early incubation rather than the final extent of gas production. Gas production is commonly used to indicate substrate fermentation because it reflects microbial degradation and utilization of feed components [14]. Wang *et al.* [5] similarly identified gas production as an indicator of feed fermentability and rumen microbial activity.

The observed early increase in gas production may partly reflect differences in the nutrient composition of the PP-containing diets. PP had a relatively higher CP concentration than corn stover and commercial concentrate, and increasing PP inclusion modestly increased dietary CP. Previous studies have also identified PP as a potential CP-containing feed resource [15]. The availability and fermentability of dietary nutrients can influence microbial fermentation and subsequent gas production [16]. In addition, PP contains bioactive compounds, including tannins, that may interact with rumen microorganisms and fermentation processes. However, tannin effects depend on concentration and characteristics, and the current experimental design cannot establish their specific contribution to the increased early gas production observed in the present study [17]. Importantly, the absence of treatment differences in tGP after 48 h indicates that the early response did not translate into a significant difference in final cumulative gas production.

Methane production

PP inclusion did not significantly affect CH₄ production, whether expressed as a percentage or as mL/200 mg of substrate. Although the PP-containing treatments showed numerically lower CH₄ values than PP-0, these differences were not statistically significant and therefore do not provide evidence of a methane-mitigating effect under the conditions of this experiment. The CH₄ concentrations observed in this study were generally comparable to values reported in previous *in vitro* rumen fermentation studies [5, 14].

The lack of a significant effect on CH₄ production is noteworthy because PP contains tannins and other bioactive compounds associated with antimethanogenic activity. However, the effects of tannins on methanogenesis can vary with concentration, chemical characteristics, availability, and interactions with rumen microorganisms. Phenolic compounds, including tannins, may influence hydrogen availability and microbial

populations involved in methanogenesis [5]. However, this study did not directly evaluate these mechanisms, so the unchanged CH₄ production cannot be attributed to a specific microbial or biochemical mechanism.

Previous studies investigating fruit byproducts have similarly reported variable effects on ruminal fermentation and CH₄ production. Jafari *et al.* [16] reported that tropical fruit peels did not adversely affect rumen fermentation and showed potential to modify CH₄ production. The present findings indicate that PP could be incorporated at the evaluated levels without increasing CH₄ production under the experimental conditions; however, they do not demonstrate a significant CH₄ mitigation effect. Further investigation is required to determine whether PP composition, inclusion level, or other dietary conditions influence its potential effects on methanogenesis.

Rumen pH, NH₃-N, and nutrient degradability

Rumen pH is an important factor to regulate the nutrient degradation in the rumen [18]. Rumen pH remained unaffected by PP inclusion and ranged from 6.41 to 6.80 across treatments. This range is consistent with values reported in previous studies [8, 9]. Rumen pH is determined by the balance among fermentation acid production, buffering capacity, and substrate characteristics and is an important determinant of microbial fermentation and nutrient degradation [8]. The absence of significant differences in pH indicates that PP inclusion at the evaluated levels did not substantially alter the acid–base conditions of the buffered rumen fermentation system.

In contrast to pH, NH₃-N concentration increased significantly with PP inclusion. This response may reflect differences in the nitrogenous components of the experimental diets, as dietary CP increased modestly with PP inclusion. Previous research has associated increased NH₃-N concentrations with the availability and ruminal degradation of dietary nitrogen-containing compounds [19]. However, the present experiment did not directly measure protein degradability, microbial protein synthesis, or nitrogen utilization; therefore, these data cannot determine the mechanism responsible for the increased NH₃-N concentration.

Despite the increase in NH₃-N, IVDMD and IVOMD did not differ significantly among treatments. Previous studies have shown that increased ruminal NH₃-N does not necessarily correspond to improved substrate degradability [20–22]. Once NH₃-N availability is sufficient to support microbial activity, additional NH₃-N may not necessarily enhance degradation [20–23]. These findings align with this pattern because PP inclusion increased NH₃-N concentration without significantly altering IVDMD or IVOMD. Nevertheless, because we did not measure microbial biomass and nitrogen incorporation, we cannot determine whether rumen microorganisms effectively utilized the additional NH₃-N.

VFA profile

Total VFA concentration increased significantly with PP inclusion, with PP-20 and PP-40 showing higher concentrations than PP-0. Acetate concentration also changed significantly, whereas propionate, isobutyrate, butyrate, isovalerate, valerate, and the acetate-to-propionate ratio did not differ significantly among treatments. These findings indicate that the fermentation response to PP was characterized primarily by changes in total VFA and acetate production rather than broad alterations across all individual VFAs.

VFAs are major end products of ruminal microbial fermentation and represent an important energy source for ruminants [15]. The increase in total VFA concentration, together with the higher early gas production, indicates greater fermentation activity in some PP-containing treatments under the experimental conditions. Previous research suggests that rumen microorganisms can use nutrients in fruit byproducts as fermentable substrates [16]. However, because the present study did not characterize the rumen microbial community or directly quantify substrate-specific fermentation pathways, the microbial mechanisms underlying the observed changes in total VFA and acetate cannot be established.

Overall interpretation and study limitations

Collectively, the findings indicate that PP inclusion modified selected *in vitro* fermentation responses, particularly early gas production, NH₃-N, total VFA, and acetate concentrations, without significantly altering final 48-h tGP, CH₄ production, rumen pH, IVDMD, or IVOMD. Among the evaluated treatments, PP-20 produced the highest total VFA concentration while maintaining pH, degradability, and CH₄ production comparable to the other treatments. These findings support PP-20 as a candidate inclusion level for further investigation rather than as a confirmed optimal dietary level.

The interpretation of these findings should consider the experimental design. Rumen fluid from five donor lambs was pooled to produce a single inoculum batch, and the experiment was conducted in a single *in vitro* run. A limitation of this study was the use of a single pooled rumen inoculum batch and one experimental run.

Therefore, the eight syringes per treatment represented incubation replicates rather than independent biological replicates, and the findings should be interpreted within the conditions of this experiment. Consequently, the incubation units represented replicates within the same pooled inoculum rather than independent donor-level biological replicates, limiting inference regarding between-animal variability. Furthermore, the *in vitro* system cannot reproduce all physiological processes occurring *in vivo*, including feed intake, passage rate, absorption of fermentation products, host–microbiome interactions, and longer-term microbial adaptation. Therefore, the present findings should not be directly extrapolated to animal performance or methane emissions under practical feeding conditions.

CONCLUSION

PP inclusion modified selected *in vitro* rumen fermentation characteristics in sheep. Increasing PP inclusion enhanced gas production during the early incubation period and increased NH₃-N, total VFA, and acetate concentrations. However, PP did not significantly affect final gas production at 48 h, CH₄ production, rumen pH, IVDMD, or IVOMD. Among the evaluated treatments, PP-20 produced the highest total VFA concentration while maintaining CH₄ production, pH, and degradability comparable to the other treatments.

The findings indicate that PP, an agro-industrial by-product, has potential as an alternative feed ingredient for ruminant diets. Based on the measured *in vitro* fermentation responses, 20% inclusion can be considered a candidate level for further evaluation. However, this inclusion level should not yet be interpreted as an optimal feeding recommendation under practical production conditions.

This study simultaneously evaluated multiple fermentation responses, including gas and CH₄ production, pH, NH₃-N, VFA profiles, IVDMD, and IVOMD across graded PP inclusion levels. This approach provided an integrated assessment of how PP inclusion affects *in vitro* rumen fermentation.

The experiment was conducted *in vitro* using a single pooled rumen-fluid batch obtained from five donor lambs in a single experimental run. Therefore, the incubation units did not represent independent donor-level biological replicates, and the statistical inference is restricted to the pooled inoculum batch evaluated. In addition, the *in vitro* system does not account for animal-level responses such as feed intake, nutrient absorption, passage rate, microbial adaptation, animal performance, health, or whole-animal CH₄ emissions.

Further *in vivo* studies using independent animals are required to validate the effects of PP inclusion, particularly the candidate 20% inclusion level. Future studies should assess feed intake, nutrient digestibility, animal performance, rumen microbial responses, nitrogen utilization, and CH₄ emissions under practical feeding conditions. Further characterization of PP bioactive compounds, particularly tannins, may also help clarify their contribution to the observed fermentation responses.

PP modified selected rumen fermentation characteristics without adversely affecting pH, degradability, or CH₄ production under the conditions of this *in vitro* study. PP-20 represents a promising candidate for further investigation; however, its nutritional benefits and potential environmental implications require confirmation through appropriately designed *in vivo* studies before recommendations for practical sheep feeding can be made.

DATA AVAILABILITY

All data generated and analyzed during this study are included in this manuscript.

GENERATIVE AI DECLARATION

ChatGPT (OpenAI) was used to improve the English language and clarity of the manuscript. The authors carefully reviewed and edited the suggested changes and take full responsibility for the final manuscript.

AUTHORS' CONTRIBUTIONS

DR: Conceptualization, methodology, supervision, validation, and writing–review and editing. IH: Conceptualization and methodology. KNJ: Conceptualization, formal analysis, writing–original draft, and writing–review and editing. KRGA, LMM, and NBR: Analyzed the data, writing–original draft, validation, and writing–review and editing. All authors have read, reviewed, and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This research and the article processing charge were supported by the Equity WCU Program, funded by the Indonesian Endowment Fund for Education (LPDP) on behalf of the Indonesian Ministry of Higher Education,

Science and Technology (Contract Nos. 4303/83/DT.03.08/2025 and 3927/UN6.RKT/HK. 07.00/2025), under the Research Collaboration–International Research Network (Grant No. 3876/UN6.3.1/PT.00/2025).

The authors sincerely thank the Biotechnology Research and Testing Laboratory, Faculty of Animal Husbandry, Universitas Padjadjaran, and the Central Laboratory, Universitas Padjadjaran, for providing facilities and scientific and technical support. The authors also thank Dimas Permana for invaluable support during this study.

COMPETING INTERESTS

The authors declare that they have no competing interests.

PUBLISHER'S NOTE

Veterinary World remains neutral with regard to jurisdictional claims in the published institutional affiliations.

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