



Research article

Integrating palm fatty acid distillate and mangrove fruit tannins in complete feed wafers: Effects on physical quality of wafers, rumen fermentation, and methane mitigation in vitro

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Abstract: In ruminant production systems, inefficient rumen fermentation may contribute to enteric methane production and suboptimal nitrogen utilization, potentially reducing the efficiency of dietary energy and protein use. The combination of palm fatty acid distillate (PFAD) and tannin-containing feed ingredients may influence rumen fermentation; however, the magnitude of the response depends on the ingredient source, inclusion level, and fermentation conditions. The present in vitro study investigated how the inclusion of PFAD in mangrove fruit-based tannin wafer rations influences rumen fermentation profiles and nutrient utilization. This study consisted of two stages. Stage 1 evaluated the physical characteristics of the complete-feed wafers prior to in vitro fermentation. Stage 2 conducted an in vitro evaluation of feed wafers, assessing rumen fermentation parameters (pH, NH₃, partial VFA profile), nutrient digestibility (digestibility of nutrient and fiber fractions), microbial activity (protozoan population, microbial biomass, and protein synthesis), and methane gas production. A randomized block design with four treatments was applied: T1 (wafer ration + 4% mangrove fruit), T2 (T1 + 1% PFAD), T3 (T1 + 2% PFAD), and T4 (T1 + 3% PFAD). The result showed that increasing

PFAD levels had significant effects ($P < 0.05$) on improving nutrient and fiber digestibility, microbial protein synthesis, and partial VFA production, while reducing NH_3 concentration, methane production, and protozoa population. Compared with T1, T4 reduced methane production by 32.64% and NH_3 concentration by 21.70%, while increasing microbial protein synthesis by 7.29%. Dry matter, organic matter, and crude protein digestibility increased by 8.91%, 11.20%, and 15.36%, respectively. Overall, 3% PFAD in wafer rations containing 4% mangrove fruit was associated with the most favorable responses among the evaluated treatments, including higher nutrient digestibility and microbial protein synthesis and lower methane production.

Keywords: digestibility; physical quality; wafer; biohydrogenation; protozoa

1. Introduction

Milk demand in Indonesia continues to increase annually; however, domestic dairy productivity remains relatively low. As a result, Indonesia relies on imported milk to meet industrial and household requirements. Beyond this production gap, one of the major biological constraints in dairy cattle nutrition is the inefficient use of feed energy during rumen fermentation. Approximately 2–12% of dietary energy can be lost as methane, reducing the energy available for milk synthesis and animal productivity [1–3]. Therefore, nutritional strategies that improve rumen fermentation efficiency while reducing methane output are important for enhancing dairy production and supporting emission mitigation efforts [4].

One nutritional approach to address this challenge is to include bioactive compounds to enhance fermentation efficiency and reduce methane production. Mangrove fruit is a potential feed resource containing high tannin levels (28.08%) [5,6]. Tannins are a group of polyphenolic compounds that function as natural rumen modifiers. In the rumen, tannins exhibit antiprotozoal activity that inhibits methanogenic archaea by competing for hydrogen, thereby reducing hydrogen transfer from protozoa to methanogens [7,8]. This mechanism helps reduce methane production and improve hydrogen utilization for microbial synthesis [9]. Tannins also form reversible complexes with dietary proteins, protecting them from excessive ruminal degradation (bypass protein) and allowing improved digestion in the small intestine. Moreover, tannins modulate ruminal biohydrogenation by limiting the extensive conversion of polyunsaturated fatty acids (PUFA) compared with diets without tannins.

However, the effects of tannins depend heavily on concentration, chemical structure, plant source, and interactions with the basal diet. While moderate levels can improve rumen efficiency, excessive tannin supplementation can negatively impact feed intake, fiber degradation, nutrient digestibility, and the activity of beneficial rumen microorganisms [10,11]. Therefore, determining the appropriate level of tannin supplementation is crucial for maximizing rumen function. Given their potential roles in methane mitigation, nitrogen utilization, and rumen fermentation modulation, tannin-containing mangrove fruits warrant further investigation as feed additives, particularly when combined with energy-rich ingredients, such as palm fatty acid distillate in complete feed wafers.

Palm fatty acid distillate (PFAD) is an economical byproduct rich in free fatty acids, particularly palmitic acid and oleic acid, as well as monounsaturated fatty acids (MUFA) and PUFA [12]. As a lipid source, PFAD can influence rumen fermentation by reducing the activity of carbohydrate-degrading microorganisms and lowering the availability of hydrogen for methanogenesis [13]. Additionally, the unsaturated fatty acids in PFAD can act as hydrogen scavengers via

biohydrogenation, thereby contributing to methane mitigation. Combining PFAD with tannin-rich feed ingredients can further enhance this effect by modifying rumen biohydrogenation and microbial activity [14]. Therefore, PFAD is a potential feeding strategy to improve rumen fermentation efficiency while reducing methane production.

There is potential synergy between mangrove fruit tannins and PFAD, as these components may influence rumen fermentation via complementary pathways. Mangrove fruit tannins may modulate ruminal protein degradation and protozoa population, whereas PFAD may increase dietary energy density and influence methane formation through lipid-related fermentation and biohydrogenation pathways. However, their combined effect in a densified, complete-feed wafer format remains unexplored. Therefore, evaluating these two components together may provide a more comprehensive strategy for improving rumen efficiency, nutrient utilization, and methane mitigation.

Complete feed wafer technology offers additional advantages, including high density, good palatability, extended shelf life, ease of standardization, and efficient transportation, particularly during periods of limited forage availability [15]. Wafer formulation also enables better nutritional control than loose feeds, resulting in more consistent fermentation responses. The inclusion of 10% tapioca flour serves as an effective binding agent, enhancing wafer structural properties and improving nutrient digestibility [16]. Although PFAD and feed additives containing tannins have been studied separately as methane mitigation strategies, studies evaluating their combined effects remain limited. Furthermore, the use of mangrove fruit tannins as a concentrated natural source of tannins has received less attention than conventional tannin sources. Furthermore, previous research has generally focused separately on rumen fermentation responses or feed processing characteristics.

Therefore, the uniqueness of this study lies in integrating PFAD and mangrove fruit tannins into a complete feed wafer and evaluating their stimulatory effects on wafer physical quality, rumen fermentation characteristics, nutrient utilization, and methane mitigation *in vitro*. This approach provides a more comprehensive assessment of how feed processing technologies and methane-reducing feed additives can be combined to improve feed preservation, energy utilization efficiency, and environmental sustainability in ruminant production systems.

2. Materials and methods

2.1. Ethical approval of research

This study was conducted using an *in vitro* approach without directly subjecting animals to experimental treatments. However, rumen fluid was collected from a donor animal maintained under standard management practices, and the procedure was conducted in accordance with institutional ethical guidelines. Ethical approval was obtained prior to sample collection.

2.2. Experimental design

A randomized block design was employed, consisting of four dietary treatments and five blocks. The blocking factor was defined as the source of rumen fluid, with each block representing rumen fluid obtained from a different donor animal. Within each block, all four dietary treatments were incubated under the same *in vitro* conditions. This design was adopted to minimize biological variation associated with differences in rumen inoculum among donor animals. The treatments were as follows: T1 = wafer

ration + 4% mangrove fruit; T2 = wafer ration + 4% mangrove fruit + 1% PFAD; T3 = wafer ration + 4% mangrove fruit + 2% PFAD; and T4 = wafer ration + 4% mangrove fruit + 3% PFAD.

2.3. Stage 1: Physical quality evaluation of feed wafers

Stage 1 evaluated the physical quality of the complete-feed wafers, including sensory and structural characteristics. The assessed parameters included color, aroma, texture, water absorption capacity, and wafer density. These measurements were conducted to confirm that the formulated wafers met acceptable physical quality before proceeding to the in vitro fermentation experiment.

2.3.1. Experimental procedures stage 1

2.3.1.1. Preparation of feed ingredient

The experimental diets consisted of forage (60%) and concentrate (40%). The nutritional composition of forage and concentrate is presented in Table 1, obtained from laboratory analyses of the feed ingredients used in the present study and not derived from the literature or feed composition databases. Proximate composition was analyzed according to AOAC procedures, while fiber fractions were determined using the Van Soest method. The feed ingredients were prepared under standardized conditions. *Tithonia diversifolia* was harvested at the late-vegetative to early-flowering stage, approximately 50–60 days after regrowth, using edible leaves and young stems. Sugarcane tops were collected from physiologically mature sugarcane plants, approximately 10–12 months old, and consisted of young leaves and tender top stems. Mangrove fruits were collected at the mature fruit stage, and only fully developed and undamaged fruits were selected. All materials were cleaned, chopped, dried under sunlight or in a low-temperature oven until a stable moisture content was achieved, ground into meal, and stored in airtight containers before diet formulation. Concentrate ingredients, including corn, rice bran, tapioca flour, minerals, and molasses, were obtained from CV Mega Mulia Sejahtera, Padang City. PFAD was purchased from PT Sinar Jaya Inti Mulya, Bandar Lampung, Lampung.

2.3.1.2. Fermentation of sugarcane tops

Fresh sugarcane tops were chopped and air-dried [17]. The materials were then spread on a plastic sheet, inoculated with 10% *Pleurotus ostreatus*, and thoroughly mixed. The inoculum was prepared as an actively growing fungal culture and mixed thoroughly with the chopped sugarcane tops to ensure uniform distribution. The prepared mixture was transferred into plastic containers, compressed to establish semi-anaerobic conditions, sealed securely, and maintained for a 21-day incubation period. At the end of fermentation, the quality of the fermented sugarcane tops was evaluated based on visible fungal mycelial growth, the absence of undesirable mold contamination, an acceptable fermented aroma, and a stable physical appearance of the substrate. Upon completion of fermentation, the sugarcane tops were dried in a laboratory oven (Memmert, Germany) at 60 °C for 48 h, then ground to obtain a uniform material.

Table 1. Nutrient composition of feed ingredients used in the present study on an as-fed basis.

Nutrient contents	Fermented sugarcane tops	Tithonia	Tofu pulp	Bran	Tapioca flour	Mangrove fruit
Crude protein	8.36	21.88	19.26	11.09	4.40	8.70
Total digestible nutrients	49.05	63.15	70.22	74.87	82.00	57.53
Dry matter	92.32	90.89	85.91	87.5	86.13	55.82
Ash	10.57	13.28	5.6	10.56	0.06	5.18
Crude fat	2.90	3.06	2.8	6.28	1.90	1.44
Crude fiber	37.98	18.32	16.8	9.23	4.60	28.76
Nitrogen-free extract	41.75	43.46	55.54	62.83	89.04	55.92
Neutral detergent fiber	80.87	60.72	44.6	48.11	2.1	62.20
Acid detergent fiber	52.63	41.78	27.37	23.08	9.64	54.01
Hemicellulose	28.24	18.94	17.28	25.03	7.54	8.19
Cellulose	33.8	35.07	25.67	11.05	1.61	22.61
Lignin	8.79	4.8	5.95	3.78	1.11	23.55
Tannin	-	-	-	-	-	28.34

Note: Nutrient composition values are expressed on a dry matter basis and represent mean values obtained from two laboratory analytical replicates of the feed ingredients used in the present study. Proximate composition was analyzed according to AOAC procedures, while fiber fractions were determined using the Van Soest method. Tannin content was analyzed in mangrove fruit as the tannin source.

2.3.1.3. Preparation of a complete feed wafer

Complete feed wafers were prepared from locally sourced forage and concentrate materials [16]. The dietary formulation was designed to provide a forage-to-concentrate ratio of 60:40 on a dry-matter basis. The formulation was adjusted to provide adequate crude protein and energy levels for supporting rumen fermentation evaluation in dairy cattle. The forage component was composed of dried *Tithonia diversifolia* and fermented sugarcane tops at a ratio of 50:50. *Tithonia diversifolia* plants were harvested at the vegetative stage and air-dried until the moisture content was reduced to below 12%. The concentrate component (40%) consisted of rice bran, soybean curd residue, ground corn, molasses, mineral mix, and salt. The basal formulation was maintained identically across all treatments, and the experimental variation was limited to the inclusion level of PFAD. All treatments contained 4% mangrove fruit as a tannin source, with PFAD added at 0, 1, 2, or 3% depending on the designated treatment.

Table 2. Ration composition and calculated nutrient content of complete feed wafer on a dry matter basis.

Feed ingredient	Ration composition (%)
Tapioca starch	10
Fermented sugarcane tops	30
<i>Tithonia diversifolia</i>	30
Rice bran	8.55
Tofu dregs	9.95
Mangrove fruit	4
Molasses	6
Salt	0.5
lime	0.5
Sulfur	0.5
Nutrient content	
Crude protein	12.68
Total digestible nutrients	61.26
Ash	8.96
Dry matter	85.09
Crude fat	2.94
Crude fiber	20.91
Nitrogen-free extract	48.08
Neutral detergent fiber	54.06
Acid detergent fiber	36.22
Hemicellulose	19.35
Cellulose	25.09
Lignin	6.04
Tannin	1.13

Note: Ration composition and nutrient content values are expressed on a dry matter basis. Nutrient content values are calculated estimates based on the nutrient composition of each feed ingredient used in the formulation, rather than direct laboratory analysis of the final mixed ration. The basal complete feed wafer was formulated for dairy cattle using a 60:40 forage-to-concentrate ratio and was kept identical across treatments, except for the PFAD inclusion level.

PFAD and mangrove fruit extract were incorporated into the ration according to the designated treatment levels. PFAD was premixed with molasses to improve mixing homogeneity, whereas the mangrove fruit extract was dissolved in warm water to ensure uniform distribution throughout the ration. All feed ingredients were weighed on a dry-matter basis and thoroughly mixed in a horizontal mixer until a homogeneous mixture was obtained. The mixture was then conditioned by adding hot water at approximately 10–15% of the total mixture weight to promote starch gelatinization and enhance binding during wafer formation. Subsequently, the conditioned mixture was compressed using a feed wafer (Indonesia) at 120°C and high pressure for 20 min to produce compact wafers with uniform size and structure. The wafers were cooled at room temperature for 24 h to stabilize their physical characteristics before being subjected to physical quality evaluation and *in vitro* nutrient digestibility analysis, as described in the following sections.

2.3.2. Physical properties analysis of the wafer

2.3.2.1. Texture, aroma, and color

Texture, aroma, and color of the wafers were evaluated according to the method described by Sholihin and Sutrisna [18]. The assessment was adapted by employing a panel-based sensory evaluation with predefined scoring criteria for wafer color, aroma, and texture. The evaluation was conducted by 20 panelists consisting of students and laboratory staff who were familiar with feed physical quality assessment and had no sensory impairments that could affect the evaluation. Before the assessment, the panelists received a brief orientation on the evaluation criteria and scoring scale for color, aroma, and texture to ensure a consistent understanding and application of the predefined criteria presented in Table 3.

Table 3. Wafer characteristics assessment criteria.

Criteria	Characteristic	Score	Description
Color	Dark brown	3-3,9	Very good
	Light brown	2-2,9	Good
	Brownish yellow	1-1,9	Fair
Aroma	Smells	3-3,9	Very good
	Odourless	2-2,9	Good
	Rancid	1-1,9	Fair
Texture	Has a firm, dense texture (not easily broken)	3-3,9	Very good
	Has a firm texture, easy to break	2-2,9	Good
	Has a wet, easily broken, and slimy	1-1,9	Fair

2.3.2.2. Wafer absorption capacity

Water absorption capacity was evaluated by recording the weight of wafers before and after immersion in water for 5 min, and was determined using the method of Trisyulianti et al. [19]. The percentage of wafer absorption was subsequently determined according to the equation below:

$$WA (\%) = \frac{B_2 - B_1}{B_1} \times 100 \quad (1)$$

Where WA = water absorption (%), B1 represents the initial sample weight (g), and B2 denotes the final weight after immersion (g).

2.3.2.3. Wafer density

The wafer density was determined using the Trisyulianti et al. [19] method, using the following formula:

$$K = W / (P \times T \times L) \quad (2)$$

Where K denotes wafer density (g/cm³), W represents the sample weight (g), P corresponds to sample length (cm), L indicates sample width (cm), and T refers to sample thickness (cm).

2.4. Stage 2: *In vitro* evaluation of feed wafers

An *in vitro* approach was employed to evaluate the fermentation characteristics and digestibility of feed wafers as a preliminary step prior to *in vivo* application. The parameters examined encompassed nutrient digestibility, rumen fermentation characteristics, methane production, protozoal population, microbial protein synthesis, and individual volatile fatty acid (VFA) fractions, including acetate, propionate, butyrate, iso-butyrate, iso-valerate, *n*-butyrate, and *n*-valerate.

2.4.1. Experimental procedures stage 2

2.4.1.1. *In vitro* fermentation procedure

Feed wafers were initially ground, and 2.5 grams of each sample was transferred into 300 mL Erlenmeyer flasks, which were then used to determine the Tilley and Terry [20] method. McDougall's buffer was prepared in advance according to the formulation in Table 4 and flushed with CO₂ prior to use to create anaerobic conditions. Rumen fluid was collected from five clinically healthy dairy cattle at a local slaughterhouse, and each donor animal was considered as one experimental block. The donor cattle were physiologically mature and selected for the absence of visible digestive disorders or abnormal rumen contents at slaughter. Rumen fluid was collected immediately after slaughter, transferred into pre-warmed insulated bottles, and transported to the laboratory under anaerobic conditions. Upon arrival, the rumen fluid was filtered through four layers of cheesecloth, maintained at approximately 39 °C, and continuously flushed with CO₂, then used as the inoculum for the *in vitro* incubation. Each incubation flask received 200 mL of McDougall's buffer and 50 mL of rumen fluid. Throughout the inoculation process, CO₂ was continuously supplied to maintain anaerobic conditions and sustain microbial activity. The *in vitro* incubation was arranged in a randomized block design, with five blocks corresponding to rumen fluid from five different donor animals. Within each block, all four dietary treatments were incubated simultaneously under the same conditions, with one incubation flask assigned to each treatment. Therefore, a total of 20 incubation flasks were used, consisting of four treatments and five independent replicates per treatment. Each flask served as one experimental unit. The flasks were incubated in a shaking incubator (NBS incubator shaker, USA) at 39 °C for 48 h with an agitation speed of 90 rpm.

Table 4. Composition of McDougall's buffer solution.

Chemicals	Amount in solution (g/L)
NaHCO ₃	9.8
KCL	0.57
MgSO ₄	0.12
NaCl	0.47
CaCl ₂	0.05
Na ₂ HPO ₄	4.62

Note: Values are expressed as grams of each chemical per liter of McDougall's buffer solution (g/L).

Methane production was quantified according to the procedure described by Fievez et al. [21]. After 48 h of incubation, fermentation was terminated by placing the flasks in an ice bath. The pH of

the fermentation medium was immediately measured using a digital pH meter. The fermentation contents were then centrifuged using a refrigerated high-speed centrifuge (Hitachi, Japan) at 3000 rpm for 15 min to separate the supernatant from the residue. The supernatant was used to analyze the protozoan population, VFA, ammonia (NH₃), microbial biomass, and microbial protein synthesis. The residue was filtered using Whatman No. 41 filter paper and washed with buffer as required. Proximate analysis of the residues was conducted according to AOAC procedures [22], and fiber fraction analysis was performed according to Van Soest et al. [23].

2.4.1.2. Measurement of VFA production

Total VFA concentration was quantified by a steam distillation procedure [24]. Each sample was analyzed in duplicate measurements. Briefly, 5 mL of rumen fluid supernatant was placed into a distillation tube, acidified with 1 mL of 15% H₂SO₄, and sealed prior to distillation. The apparatus was assembled by connecting the tube to a condenser and a boiling flask containing heated water. The resulting distillate was collected in an Erlenmeyer flask containing 5 mL of 0.5 N NaOH, and the distillation was continued until a final volume of 250 mL was reached. Thereafter, phenolphthalein indicator was added, and the solution was titrated with 0.5 N HCl until the endpoint was indicated by a color change from purple to colorless. Total VFA concentration was calculated using the following formula:

$$\text{VFA (mM)} = (a - b) \times N_{\text{HCl}} \times \left(\frac{1000}{\text{sample volume (mL)}} \right) \quad (3)$$

where *a* represents the volume of HCl (mL) required for sample titration, and *b* denotes the volume of HCl (mL) used for blank titration, N HCl is the normality of HCl used for titration, and sample volume is the volume of rumen fluid supernatant used for distillation.

2.4.1.3. Measurement of ammonia (NH₃) concentration

Rumen ammonia (NH₃) concentration was quantified using a microdiffusion technique adopted from Conway and O'Malley [25]. Each sample was analyzed in duplicate measurements. Prior to analysis, the rim of the Conway dish was coated with petroleum jelly to maintain an airtight seal. One milliliter of rumen fluid supernatant was dispensed into one outer compartment to prevent initial mixing. The central compartment received 1 mL of boric acid solution containing an appropriate indicator, after which the dish was immediately sealed. The Conway dish was then gently tilted to allow contact between the supernatant and Na₂CO₃, followed by incubation at room temperature for 24 h. Upon completion of incubation, the boric acid solution was titrated with 0.005 N H₂SO₄ until a color change from blue to red was observed. Ammonia concentration was calculated using the following formula:

$$\text{NH}_3 \text{ (mg/100 mL)} = \text{Volume of titrant (mL)} \times N_{\text{H}_2\text{SO}_4} \times 17 \times 100 \quad (4)$$

2.4.1.4. Total gas and methane production

At the end of the 48-h incubation, total gas production was measured with a gas-tight syringe, following the procedure described by Fievez et al. [21]. Methane production was estimated by

absorbing CO₂ from the collected gas using 10 M NaOH. Briefly, the gas-tight syringe containing the fermentation gas was connected to another syringe containing 4 mL of 10 M NaOH, and the NaOH solution was introduced to absorb CO₂. After CO₂ absorption, the residual gas in the syringe was assumed to be methane (CH₄). Methane production was calculated from the residual gas volume and expressed as mL/g organic matter digested (mL/g DOM). No additional correction factor was applied, except for expressing methane output relative to organic matter digestibility.

2.4.1.5. Enumeration of rumen protozoa

Fresh rumen fluid was diluted with MFS solution at a 1:4 ratio, with further adjustment made when necessary to accommodate protozoal density. A small aliquot of the diluted sample was introduced into a counting chamber characterized by a depth of 0.2 mm, a grid area of 0.0625 mm², and a total of 16 × 16 counting grids [26]. Protozoa were counted under a light microscope at 100× magnification. The number of protozoa per milliliter of rumen fluid was calculated using the following formula:

$$P = \left[\frac{1}{0.2 \times 0.0625 \times 16 \times 16} \right] \times 1000 \times C \times FP \quad (5)$$

where P = protozoa population (cells/mL rumen fluid); C = total number of protozoa counted in the chamber (16 × 16 grids); FP = dilution factor (e.g., 1:4 = dilution factor of 4); 1000 = conversion factor from mm³ to cm³ (mL)

2.4.1.6. Measurement of microbial protein synthesis

Microbial biomass was estimated from the fermentation residue fraction obtained after incubation and centrifugation. The fermentation contents were centrifuged to separate the supernatant and residue, and the residue fraction was used as an indicator of microbial-associated biomass in the fermentation medium. The results were expressed as mg/100 mL of fermentation medium. Microbial protein synthesis was determined using a Lowry-based colorimetric method adapted from Lowry et al. [27]. Sample aliquots were reacted with Lowry reagents, and absorbance was measured at 750 nm using a spectrophotometer after color development. Protein concentration was calculated using a regression equation obtained from a bovine serum albumin standard curve. The microbial protein synthesis values were expressed as mg/100 mL of fermentation medium.

2.5. Statistical analysis

All experimental data were analyzed using analysis of variance (ANOVA) according to a randomized block design. Prior to ANOVA, the assumptions of normality and homogeneity of variance were evaluated. Normality of the data was assessed using the Shapiro-Wilk test, while homogeneity of variance was examined using Levene's test. When significant treatment effects were detected, treatment means were compared using Duncan's multiple range test. Statistical significance was declared at $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics software, version 21.0.

3. Results

3.1. Digestibility of dry matter, organic matter, and crude protein

The digestibility of dry matter (DM), organic matter (OM), and crude protein (CP) was significantly influenced by dietary treatments ($P = 0.001$) (Table 5). DM digestibility increased from 63.85% in T1 to 69.54% in T4, with intermediate values in T2 (67.67%) and T3 (67.87%). A similar trend was observed from OM digestibility, which rose from 63.15% (T1) to 70.22% (T4). Likewise, CP digestibility increased progressively, from 63.13% in T1 to 72.83% in T4, with PFAD-supplemented treatments showing significantly higher values than the control.

Table 5. Nutrient digestibility of complete feed wafer containing mangrove tannin and PFAD.

Parameter	Treatment				SEM	P-Value
	T1	T2	T3	T4		
Dry matter digestibility	63.85 ± 0.53 ^c	67.67 ± 0.62 ^b	67.87 ± 0.93 ^b	69.54 ± 0.92 ^a	0.38	0.001
Organic matter digestibility	63.15 ± 0.82 ^c	66.97 ± 0.87 ^b	69.91 ± 1.19 ^a	70.22 ± 1.62 ^a	0.47	0.001
Crude protein digestibility	63.13 ± 0.48 ^c	69.82 ± 0.96 ^b	71.86 ± 1.13 ^a	72.83 ± 0.31 ^a	0.36	0.001

Notes: T1 = Wafer ration + 4% mangrove fruit, T2 = Wafer ration + 4% mangrove fruit + 1% PFAD, T3 = Wafer ration + 4% mangrove fruit + 2% PFAD, T4 = Wafer ration + 4% mangrove fruit + 3% PFAD. Values are presented as mean ± SD (n = 5 replicates/treatment).

3.2. Rumen pH and NH₃ concentration

Rumen pH was not affected by dietary treatments ($P = 0.743$), with values ranging from 6.74 (T1) to 6.82 (T2 and T4), remaining within the normal physiological range. In contrast, ruminal ammonia (NH₃) concentration differed significantly among treatments ($P = 0.001$) (Table 6). The highest NH₃ level was observed in T1 (20.78 mg/100 mL), while the lowest value was observed in T4 (16.27 mg/100 mL). PFAD-supplemented treatments (T2–T4) consistently showed lower NH₃ concentrations, indicating more efficient ruminal nitrogen utilization.

Table 6. Rumen pH and ammonia concentration in response to mangrove tannin and PFAD supplementation.

Parameter	Treatment				SEM	P-Value
	T1	T2	T3	T4		
pH	6.74 ± 0.10	6.82 ± 0.13	6.78 ± 0.13	6.82 ± 0.03	0.05	0.743
NH ₃ (mg/100mL)	20.78 ± 0.54 ^a	18.84 ± 0.59 ^b	18.33 ± 0.34 ^b	16.27 ± 1.51 ^c	0.374	0.001

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n=5 replicates/treatment).

3.3. Microbial biomass and microbial protein synthesis

Microbial biomass concentration was not significantly affected by dietary treatments ($P = 0.79$) (Table 7). Although statistically similar, microbial biomass T1 (1.365 mg/100 mL) and T4 (1.393 mg/100 mL). In contrast, microbial protein synthesis was significantly influenced by the treatment ($P = 0.012$). The lowest value was observed in T1 (136.61 mg/100 mL), whereas the highest microbial protein synthesis was observed in T4 (146.57 mg/100 mL). Treatments supplemented with PFAD (T2–T4) showed progressively higher microbial protein synthesis, with T4 being significantly higher than T1.

Table 7. Microbial biomass and microbial protein synthesis in rumen fermentation in vitro.

Parameter	Treatment				SEM	P-Value
	T1	T2	T3	T4		
Microbial biomass (mg/mL)	1.365 ± 0.01	1.375 ± 0.03	1.384 ± 0.03	1.393 ± 0.06	0.02	0.79
Microbial protein synthesis (mg/100mL)	136.61 ^c ± 4.24	138.56 ^{bc} ± 5.55	143.97 ^{ab} ± 2.97	146.57 ^a ± 4.01	2.10	0.012

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n=5 replicates/treatment).

3.4. Methane gas production and protozoa population

Methane gas production was significantly affected by dietary treatments. The highest methane production was observed in T1 (25.03 mL/g DOM), with a progressive reduction as PFAD supplementation increased (Table 8). Treatment T2 showed a lower methane output (21.42 mL/g DOM) compared to T1, followed by T3 (19.89 mL/g DOM). The lowest methane production was observed in T4 (16.86 mL/g DOM), indicating that higher inclusion of PFAD in wafer rations containing mangrove fruit effectively suppressed methanogenesis.

Table 8. Methane production and protozoa population affected by mangrove tannin and PFAD.

Parameter	Treatment			
	T1	T2	T3	T4
Methane gas production (mL/g DOM)	25.03 ± 1.59 ^a	21.42 ± 1.04 ^b	19.89 ± 1.86 ^b	16.86 ± 0.63 ^c
Protozoa population (log ₁₀ mL/cell)	5.16 ± 0.03 ^a	5.14 ± 0.01 ^{ab}	5.11 ± 0.01 ^{bc}	5.10 ± 0.01 ^c

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n=5 replicates/treatment).

A similar trend was observed for the protozoa population, which also showed significant differences among treatments, as indicated by the superscript notation. The highest protozoa population was recorded in T1 (5.16 log₁₀ cells/mL), whereas treatments supplemented with PFAD exhibited lower protozoal counts (Table 8). Protozoa population decreased slightly in T2 (5.14 log₁₀ cells/mL) and T3 (5.11 log₁₀ cells/mL), with the lowest value observed in T4 (5.10 log₁₀ cells/mL).

However, the numerical differences appear small. The consistent downward trend indicates that increasing PFAD levels contributed to reduced protozoa populations, which is closely associated with lower methane production in the rumen.

3.5. Fiber digestibility (ADF, NDF, cellulose, and hemicellulose)

Dietary treatments significantly affected fiber fraction digestibility ($P = 0.001$) (Table 9). ADF digestibility increased from 61.70% in T1 to 66.50% in T4, with intermediate values in T2 (64.71%) and T3 (64.89%). A similar pattern was observed for NDF, which rose from 62.56% (T1) to 68.26% (T4). Likewise, cellulose and hemicellulose digestibility showed progressive increases, ranging from 64.16% to 70.23% and 65.65% to 72.38%, respectively, indicating improved utilization of fibrous components with higher PFAD inclusion.

Table 9. Fiber fraction digestibility (ADF, NDF, cellulose, and hemicellulose) of the complete feed wafer in vitro.

Parameter (%)	Treatment				SEM	P-Value
	T1	T2	T3	T4		
ADF	61.70 ± 1.16 ^c	64.71 ± 1.10 ^b	64.89 ± 0.55 ^b	66.50 ± 0.88 ^a	0.46	0.001
NDF	62.56 ± 0.61 ^c	65.35 ± 0.63 ^b	66.04 ± 0.38 ^b	68.26 ± 0.73 ^a	0.29	0.001
Cellulose	64.16 ± 0.75 ^c	66.76 ± 1.68 ^b	68.12 ± 1.05 ^b	70.23 ± 0.85 ^a	0.54	0.001
Hemicellulose	66.55 ± 1.06 ^c	69.18 ± 1.15 ^b	71.14 ± 1.12 ^a	72.38 ± 0.82 ^a	0.41	0.001

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n=5 replicates/treatment).

3.6. Physical properties of wafer rations

The physical properties of the wafer rations, including aroma, texture, and color, were not significantly affected by dietary treatments ($P > 0.05$) (Table 10). Aroma scores were relatively uniform across treatments, ranging from 3.28 to 3.32, with T1 showing the highest mean value (3.32) and T2 and T4 presenting slightly lower but comparable scores. The texture values ranged from 3.12 to 3.16, with T2 showing the highest (3.16) and T1 the lowest (3.12). Color scores ranged from 3.24 to 3.27, with T1 having the highest score (3.27) and T4 showing the lowest (3.24). These results indicate that PFAD supplementation did not alter the physical properties of wafer rations.

Table 10. Physical properties of complete feed wafer supplemented with mangrove tannin and PFAD.

Physical properties (score)	Treatment				SEM	P-Value
	T1	T2	T3	T4		
Aroma	3.32±0.61	3.28±0.54	3.29±0.51	3.28±0.64	0.28	0.872
Texture	3.12±0.52	3.16±0.50	3.15±0.62	3.13±0.54	0.27	0.843
Color	3.27±0.71	3.27±0.57	3.26±0.63	3.24±0.52	0.30	0.750

Note: Treatment descriptions are provided in Section 2.2. Scoring scale physical properties score 1–1.9 = fair, 2–2.9 = good, 3–3.9 = very good. Values are presented as mean ± SD (n=5 replicates/treatment).

3.7. Water absorption and wafer density

The water absorption capacity of the wafer was not significantly affected by dietary treatments ($P = 0.523$), and wafer density did not differ significantly among treatments ($P = 0.485$) (Table 11). Wafer absorption values were relatively consistent across treatments, ranging from 82.08% to 82.88%. The lowest value was observed in T1 (82.08%), while the highest water absorption occurred in T3 (82.88%). Density values ranged from 0.62 to 0.65 g/cm³, with T2 exhibiting the highest density (0.65 g/cm³) and T4 showing the lowest value (0.62 g/cm³). The minimal variation observed suggests that PFAD supplementation did not affect the water absorption, compactness, or structural stability of the wafer.

Table 11. Water absorption and density of complete feed wafer.

Parameter	Treatment				SEM	P-Value
	T1	T2	T3	T4		
Water absorption of wafer (%)	82.08 ± 0.46	82.32 ± 0.63	82.88 ± 0.44	82.87 ± 0.53	0.23	0.523
Wafer density (g/cm ³)	0.63 ± 0.41	0.65 ± 0.45	0.64 ± 0.64	0.62 ± 0.51	0.25	0.485

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n = 5 replicates/treatment).

3.8. Partial VFA profile

Dietary treatments significantly affected the partial VFA profile ($P=0.001$). The proportion of acetate ranged from 52.78% to 61.75% across treatments, indicating a shift in acetate dominance with increasing PFAD inclusion. In contrast, propionate concentration increased clearly, with values ranging from 20.13% to 27.76%, reflecting a gradual redirection of rumen fermentation toward more energetically efficient pathways. Branched-chain and minor VFAs also responded to the treatments. Iso-butyrate concentrations ranged between 1.49% and 1.72%, while n-butyrate exhibited a wider variation, from 1.41% to 5.06%, suggesting enhanced butyrate production at higher PFAD levels. Similarly, iso-valerate increased from 0.55% to 1.98%, and n-valerate ranged from 0.14% to 1.62%. Overall, these changes indicate that increasing PFAD supplementation promoted a more favorable rumen fermentation pattern, characterized by higher propionate and branched-chain VFA production.

Table 12. Partial VFA profile of rumen fermentation in vitro.

Partial VFA	Treatment (%)				SEM	P-Value
	T1	T2	T3	T4		
Acetate	52.78 ± 1.38 ^c	57.55 ± 1.52 ^b	57.58 ± 0.96 ^b	61.75 ± 0.49 ^a	0.55	0.001
Propionate	20.13 ± 0.47 ^d	23.51 ± 0.33 ^c	24.43 ± 0.35 ^b	27.76 ± 0.68 ^a	0.23	0.001
Iso-butyrate	1.49 ± 0.57 ^b	1.53 ± 0.07 ^{ab}	1.55 ± 0.05 ^{ab}	1.72 ± 0.29 ^a	0.06	0.001
N-butyrate	1.41 ± 0.33 ^d	2.49 ± 0.15 ^c	3.23 ± 0.04 ^b	5.06 ± 0.50 ^a	0.13	0.001
Iso-valerate	0.55 ± 0.78 ^d	0.87 ± 0.23 ^c	1.50 ± 0.08 ^b	1.98 ± 0.11 ^a	0.08	0.001
N-valerate	0.14 ± 0.09 ^c	0.64 ± 0.04 ^b	0.69 ± 0.22 ^b	1.62 ± 0.17 ^a	0.07	0.001

Note: Treatment descriptions are provided in Section 2.2. Values are presented as mean ± SD (n = 5 replicates/treatment).

4. Discussion

4.1. Digestibility of dry matter, organic matter, and crude protein

Increasing PFAD inclusion from 0% to 3% in complete feed wafer rations containing mangrove fruit was associated with higher dry matter and organic matter digestibility under the present in vitro conditions. These treatment-associated responses indicate that PFAD inclusion at the tested levels did not impair substrate degradation in the fermentation system. The highest dry matter and organic matter digestibility values were observed.

Compared with rations without PFAD, the inclusion of PFAD, particularly at 3%, was associated with a more favorable rumen fermentation response, as indicated by higher nutrient digestibility. The presence of mangrove tannins contributed to a more balanced fermentation pattern by through their rumen-modulating properties. The combined inclusion of PFAD as a lipid source and mangrove tannins as plant polyphenols therefore appears to enhance nutrient utilization. These findings are consistent with previous studies involving dietary lipid supplementation and tannin-containing feed additives. [7].

Crude protein digestibility also increased with higher PFAD inclusion, and this response was accompanied by lower NH_3 concentration and higher microbial protein synthesis. In the absence of PFAD, rapid protein degradation may reduce nitrogen-use efficiency. Supplementation up to 3% PFAD may have supported microbial activity, which partly explains the higher microbial protein synthesis. The unsaturated fatty acid fraction in PFAD has been reported to enhance microbial protein synthesis efficiency without disrupting rumen fermentation, thereby improving energy efficiency and nitrogen utilization in the rumen [28]. In addition, mangrove tannins may moderate excessive ruminal protein degradation through reversible protein–tannin interactions, thereby reducing NH_3 accumulation during in vitro fermentation [29]. Thus, the combination of PFAD as an energy source and mangrove tannins as a protein-protective agent may contribute to the observed nutrient digestibility and nitrogen-related fermentation responses, consistent with the roles of lipids and tannins in enhancing ruminant nutritional efficiency [30].

4.2. Rumen pH and NH_3 concentration

The relatively stable fermentation pH across all treatments indicates that the fermentation process was not disrupted by the inclusion of 4% mangrove fruit and PFAD up to 3%. This stability suggests that the dietary treatments maintained a favorable fermentation environment, as reflected by pH values within the optimal range for rumen fermentation. Mangrove tannins may have contributed to this response by acting as natural rumen-modifying compounds, helping maintain balanced fermentation conditions [31]. Overall, these findings indicate that the combination of PFAD and mangrove tannins supported rumen pH stability under the present in vitro conditions.

In contrast, NH_3 concentration tended to decrease as PFAD level increased, which may reflect improved nitrogen utilization efficiency in the rumen fermentation system. In rations without PFAD, dietary protein may be more extensively degraded to ammonia, whereas PFAD supplementation up to 3% may have supported microbial protein synthesis. Moreover, mangrove tannins may contribute to lower NH_3 accumulation by forming protein-tannin complexes, thereby reducing excessive ruminal protein degradation [32]. The combination of PFAD as a lipid source and mangrove tannins as protein-

protective compounds may therefore help improve the balance between energy and nitrogen availability, as reflected in lower NH_3 concentrations and higher microbial protein synthesis [33].

4.3. Microbial biomass and microbial protein synthesis

The relatively similar rumen microbial biomass across treatments indicates that PFAD supplementation up to 3% in wafer rations containing 4% mangrove fruit did not markedly change microbial biomass under the present in vitro conditions. Comparable biomass values between T1 and T4 suggest that the tested PFAD level maintained microbial-associated biomass in the fermentation medium. This response indicates that PFAD inclusion up to 3% was compatible with the rumen microbial biomass response in mangrove fruit-based complete feed wafers [7].

In contrast, microbial protein synthesis increased with increasing PFAD level, with the highest value observed in T4 and the lowest in T1. The increase in microbial protein synthesis despite unchanged microbial biomass suggests a favorable microbial protein synthesis response, particularly in the incorporation of available nitrogen into microbial protein. This response was consistent with the lower NH_3 concentration observed in PFAD-supplemented treatments, indicating that less nitrogen accumulated as ammonia and more nitrogen was directed toward microbial protein formation [34].

The improved microbial efficiency in PFAD-supplemented treatments may be related to a more favorable balance between energy availability and nitrogen utilization during fermentation. PFAD supplementation provided lipid-derived energy that supported microbial protein formation, while mangrove tannins moderated excessive protein degradation through reversible protein–tannin interactions. Together, these responses were consistent with greater microbial protein synthesis in PFAD-supplemented treatment. Similar responses have been reported in studies evaluating dietary lipids and tannin-containing feed additives as strategies to support microbial synthesis in ruminant fermentation systems [35–37].

4.4. Methane gas production and protozoa

Methane production decreased progressively as the PFAD level increased from 0% to 3% in wafer rations containing mangrove fruit. This response indicates that PFAD supplementation was associated with lower methane output under the present in vitro conditions. The reduction may be associated with the lipid fraction of PFAD and tannin compounds from mangrove fruit, which have been reported to influence rumen fermentation and methane formation [38].

The decrease in methane production was accompanied by a reduction in protozoa population at higher PFAD levels. Protozoa are closely associated with rumen methanogenesis because they provide ecological niches and hydrogen substrates for methanogenic archaea [39]. The lower protozoal population may partly explain the treatment-associated reduction in methane production.

This finding suggests that the combination of PFAD and mangrove tannins may help reduce methane formation by influencing protozoal abundance and fermentation characteristics. Moreover, the lower methane output may indicate reduced gaseous energy loss during fermentation. However, this interpretation should be considered within the scope of the in vitro system used in this study.

Methane production in the present study was estimated using the NaOH absorption approach, in which CO_2 was absorbed, and the residual gas volume was assumed to be methane. This method provides a useful indirect estimate of methane production in vitro fermentation studies and has been

widely used to screen dietary methane mitigation strategies. Overall, the concurrent reduction in methane production and protozoa population suggests that PFAD supplementation in mangrove fruit-based complete feed wafers was associated with lower estimated methane production under the present *in vitro* conditions. In contrast, further validation *in vivo* would strengthen its relevance in animal models [40,41].

The decrease in methane production despite higher acetate concentration indicates that acetate formation was not directly translated into greater methanogenesis in the present *in vitro* system. Acetate production is commonly associated with hydrogen release during rumen fermentation; however, methane formation also depends on the availability of active methanogens and hydrogen transfer from protozoa-associated fermentation. In this study, the lower protozoa population in PFAD-supplemented treatments suggests reduced protozoa-associated hydrogen transfer to methanogenic archaea. In addition, the lipid fraction of PFAD may have contributed to alternative hydrogen utilization through fatty acid biohydrogenation. Thus, the concurrent increase in acetate and decrease in methane indicate that the acetate and methane responses did not change in parallel under PFAD and mangrove tannin supplementation.

4.5. Fiber digestibility (ADF, NDF, cellulose, and hemicellulose)

Fiber fraction digestibility increased with increasing PFAD level in wafer rations containing mangrove fruit. The higher digestibility of ADF, NDF, cellulose, and hemicellulose indicates that PFAD supplementation up to 3% did not impair fiber utilization under the present *in vitro* conditions. This interpretation should be made carefully because dietary lipids can inhibit fiber degradation, particularly when free fatty acids coat feed particles or interfere with the attachment and activity of cellulolytic microorganisms [41].

In the present study, the positive response of fiber digestibility may be associated with the moderate PFAD inclusion level, stable rumen pH, and uniform distribution of ingredients within the complete feed wafer matrix. The stable pH observed across treatments indicates that the fermentation environment remained favorable for structural carbohydrate degradation [42]. Moreover, processing the ration into wafer form may have helped distribute PFAD more evenly within the feed matrix, thereby limiting excessive lipid accumulation on individual fiber particles [43].

Therefore, the increased digestibility of ADF, NDF, cellulose, and hemicellulose should not be interpreted as direct stimulation of cellulolytic bacteria by PFAD. Rather, it indicates that 3% PFAD was compatible with fiber degradation in this mangrove fruit-based complete feed wafer formulation. Further studies evaluating cellulolytic bacterial populations and fibrolytic enzyme activity would help confirm the microbial mechanisms involved [44,45].

4.6. Physical properties of wafer rations

The physical properties, including aroma, texture, and color, were relatively stable across PFAD levels. These results indicate that adding up to 3% PFAD to mangrove-based wafer rations did not substantially alter the measured sensory-related physical attributes. The similar aroma scores across treatments suggest that the inclusion of PFAD did not adversely affect the panel-based aroma assessment of the wafers. Likewise, the comparable texture scores indicate that the wafer structure

remained relatively consistent after the addition of PFAD, suggesting that the mixing and pressing processes maintained wafer compactness and physical integrity [46].

PFAD inclusion did not markedly change the visual appearance of the wafers during processing. Table color is an important indicator of uniform processing and initial physical quality, particularly in the production of complete feed wafers [47,48]. Overall, these findings suggest that PFAD supplementation at up to 3% maintained the measured physical quality of mangrove fruit-based complete feed wafers, particularly in aroma, texture, and color. However, implications for animal preference, voluntary intake, and animal acceptance should be further confirmed under feeding conditions [49].

4.7. Water absorption and wafer density

Water absorption capacity remained stable across PFAD levels, indicating that PFAD inclusion up to 3% did not alter the wafer's water absorption capacity. This stability suggests that the wafer matrix structure was preserved despite the addition of lipids and that PFAD and mangrove tannins did not interfere with particle bonding during pressing, thereby maintaining porosity and water absorption characteristics. The relatively consistent values indicate that the inclusion of PFAD did not alter the measured water absorption characteristics of the wafers [50].

Wafer density values were also relatively uniform across treatments, indicating that PFAD and mangrove tannins did not substantially affect compactness. Stable density implies that the pressing process produced wafers with consistent physical integrity, which is beneficial for handling, storage, and distribution [51]. Overall, these findings indicate that PFAD inclusion up to 3% maintained the measured water absorption capacity and wafer density [52].

4.8. Partial VFA profile

Increasing PFAD inclusion from 0% to 3% in mangrove fruit-based completed-feed wafers altered the partial VFA profile, particularly by increasing acetate and propionate concentrations. The higher acetate concentration may be associated with enhanced fermentation of structural carbohydrates, which is consistent with the greater fiber digestibility observed in PFAD-supplemented treatments. Likewise, the increased propionate concentration indicates a shift toward a more gluconic fermentation pattern, as propionate serves as the primary precursor for gluconeogenesis in ruminants. These findings suggest that moderate PFAD inclusion influenced ruminal fermentation characteristics. However, this interpretation should be based on the overall fermentation response, including pH, NH_3 concentration, digestibility, and methane production, rather than on individual VFA changes alone [53,54].

The increase in minor VFAs, including iso-butyrate, n-butyrate, iso-valerate, and n-valerate, may reflect changes in substrate degradation and nitrogen-related fermentation. Branched-chain VFAs are commonly associated with the degradation of branched-chain amino acids and can contribute to the nutritional requirements of certain rumen microorganisms [55]. In the present study, higher branched-chain VFA concentrations in PFAD-supplemented treatments were accompanied by lower NH_3 concentration and higher microbial protein synthesis, indicating a consistent nitrogen-related fermentation response. Mangrove tannins may have contributed to this response by moderating ruminal protein degradation through protein-tannin complex formation, while PFAD may have provided an additional lipid-derived energy source for fermentation [56].

Overall, the VFA profile observed in this study indicates that PFAD supplementation up to 3% in mangrove fruit-based complete feed wafers was associated with treatment-related changes in the in vitro fermentation pattern, characterized by higher acetate and propionate concentrations and minor VFA concentrations. These responses should be interpreted as part of an integrated fermentation profile rather than as sole evidence of changes in energy partitioning or hydrogen utilization. Further evaluation under in vivo conditions would be useful to confirm the nutritional implications of these VFA responses for dairy cattle [57]

Acetate concentration increased with PFAD supplementation, while estimated methane production and the protozoal population decreased simultaneously. The higher acetate concentration indicates active degradation of fibrous components, whereas the lower methane output suggests that hydrogen released during fermentation was not predominantly directed toward methanogenesis. The increase in propionate concentration may also have contributed to alternative hydrogen utilization because propionate formation acts as a competing hydrogen sink in rumen fermentation.

5. Conclusions

The addition of 4% mangrove fruit and 3% PFAD (T4) to complete feed wafers improves several measured in vitro fermentation parameters, including nutrient digestibility, microbial protein synthesis, and methane reduction, while maintaining rumen pH, microbial biomass, and wafer physical quality within acceptable ranges. These findings indicate favorable treatment-associated responses under the present in vitro conditions; however, validation under in vivo conditions is necessary before recommendations for use in animals can be made.

Author contributions

Conceptualization, methodology, and writing of the original draft: Novirman Jamarun, Roni Pazla and Fauzia Agustin; supervision, experimentation, writing of the original draft: Qurrata Aini, Zaitul Ikhlas and Gusri Yanti; writing of the original draft, review, and editing: Bella Veliana Utami, Yelly Fitri and Selli Wahyuni. All authors have read and agreed to the published version of the manuscript.

Use of AI tools declaration

No Artificial Intelligence (AI) tools were used in the development of this manuscript.

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Conflict of interest

The authors state that no competing interests exist.

References

1. Putri EM, Pazla R, Jamarun N, et al. (2024) Optimizing ruminant feed efficiency: The synergistic effects of BMR sorghum and *Tithonia diversifolia* on nutrient digestibility, rumen function, and methane mitigation. *J Anim Behav Biometeorol* 12: 2024022. <https://doi.org/10.31893/jabb.2024022>
2. Elihasridas E, Pazla R, Jamarun N, et al. (2024) Effect of tannin degradation of mangrove fruit (*Sonneratia alba*) on nutrient degradation, protozoa population and methane gas production. *Czech J Anim Sci* 69: 292–301. <https://doi.org/10.17221/38/2024-CJAS>
3. Benchaar C, Hassanat F (2020) Frequency of diet delivery to dairy cows: Effect on nutrient digestion, rumen fermentation, methane production, nitrogen utilization, and milk production. *J Dairy Sci* 103: 7094–7109. <https://doi.org/10.3168/jds.2020-18199>
4. Garnsworthy PC, Difford GF, Bell MJ, et al. (2019) Comparison of methods to measure methane for genetic evaluation of dairy cattle. *Animals* 9: 837. <https://doi.org/10.3390/ani9100837>
5. Jamarun N, Pazla R, Jayanegara A, et al. (2025) Evaluation of lime treatment in reducing tannin content and enhancing nutrient utilization of mangrove fruits (*Sonneratia alba*) as an animal feed ingredient. *Emir J Food Agric* 37: 1–11. <https://doi.org/10.3897/ejfa.2025.126901>
6. Elihasridas E, Jamarun N, Yanti G, et al. (2023) Pre-treatments of *Sonneratia alba* fruit as the potential feed for ruminants using *Aspergillus niger* at different fermentation times: Tannin concentration, enzyme activity, and total colonies. *Int J Vet Sci* 12: 755–761. <https://doi.org/10.47278/journal.ijvs/2023.021>
7. Pazla R, Antonius A, Ikhlaz Z, et al. (2025) The potential of tannin from *Sonneratia alba* fruit in defaunation of rumen protozoa and reducing methane gas production: an approach to increase livestock digestive efficiency. *Czech J Anim Sci* 70: 223–233. <https://doi.org/10.17221/130/2024-CJAS>
8. Pazla R, Zain M, Ikhlaz Z, et al. (2025) Optimizing mangrove-derived tannin extract for sustainable ruminant feeding: a strategy to balance nutrient digestibility and methane mitigation. *J Anim Behav Biometeorol* 13: 2025012. <https://doi.org/10.31893/jabb.2025012>
9. Ungerfeld E (2020) Metabolic hydrogen flows in rumen fermentation: Principles and possibilities of interventions. *Front Microbiol* 11: 589. <https://doi.org/10.3389/fmicb.2020.00589>
10. Baihaqi ZA, Widiyono I, Suwignyo B, et al. (2022) Alternative strategies of plant metabolite secondary “tannin” for methane emissions reduction on ruminant livestock: A review of the last 5 years literature. *Adv Anim Vet Sci* 10: 599–606. <https://doi.org/10.17582/journal.aavs/2022/10.3.599.606>
11. He T, Yi G, Li J, et al. (2023) Dietary supplementation of tannic acid promotes performance of beef cattle via alleviating liver lipid peroxidation and improving glucose metabolism and rumen fermentation. *Antioxidants* 12: 1774. <https://doi.org/10.3390/antiox12091774>
12. Calder P (2015) Functional roles of fatty acids and their effects on human health. *JPEN J Parenter Enteral Nutr* 39: 18S–32S. <https://doi.org/10.1177/0148607115595980>
13. Cheong K, Zhang Y, Li Z, et al. (2023) Role of polysaccharides from marine seaweed as feed additives for methane mitigation in ruminants: A critical review. *Polymers* 15: 3153. <https://doi.org/10.3390/polym15153153>
14. Henke A, Westreicher-Kristen E, Molkentin J, et al. (2017) Effect of dietary quebracho tannin extract on milk fatty acid composition in cows. *J Dairy Sci* 100: 6229–6238. <https://doi.org/10.3168/jds.2016-12149>

15. Jamarun N, Ikhlas Z, Zain M, et al. (2024) Feed wafers from fermented sugarcane tops and *Tithonia diversifolia* with added tapioca starch: Effects on physical quality and in-vitro parameters for ruminant feed. *Open Vet J* 14: 3599. <http://dx.doi.org/10.5455/OVJ.2024.v14.i12.41>
16. Ikhlas Z, Jamarun N, Zain M, et al. (2024) Physical properties of sugarcane (*Saccharum officinarum*) and tithonia (*Tithonia diversifolia*) shoot-based wafers with different adhesive types. *Online J Anim Feed Res* 14: 287–294. <https://dx.doi.org/10.51227/ojafr.2024.33>
17. Pazla R, Jamarun N, Warly L, et al. (2021) Lignin content, ligninase enzyme activity and in vitro digestibility of sugarcane shoots using *Pleurotus ostreatus* and *Aspergillus oryzae* at different fermentation times. *American J Anim Vet Sci* 16: 192–201. <https://doi.org/10.3844/ajavsp.2021.192.201>
18. Sholihin M, Sutrisna R (2015) Pengaruh lama penyimpanan terhadap kadar air kualitas fisik dan sebaran jamur wafer limbah sayuran dan umbi-umbian. *J Ilm Pet Terpadu* 3: 48–54. <https://doi.org/10.23960/jipt.v3i2.p%25p>
19. Trisyulianti E, Suryahadi V, Rakhma N (2003) Pengaruh Penggunaan Molases dan Tepung Gaplek Sebagai Bahan Perekat Terhadap Sifat Fisik Wafer Ransum Komplit. *Med Pet* 26: 35–40.
20. Tilley JMA, Terry DR (1963) A two-stage technique for the in vitro digestion of forage crops. *Grass Forage Sci* 18: 104–111. <https://doi.org/10.1111/j.1365-2494.1963.tb00335.x>
21. Fievez V, Babayemi OJ, Demeyer D (2005) Estimation of direct and indirect gas production in syringes: A tool to estimate short chain fatty acid production that requires minimal laboratory facilities. *Anim Feed Sci Technol* 123–124: 197–210. <https://doi.org/10.1016/j.anifeedsci.2005.05.001>
22. AOAC (2005) *Official methods of analysis*. 18th ed. Washington, DC: Association of Official Analytical Chemists.
23. Van Soest PV, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74: 3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
24. Kromann RP, Meyer JH, Stielau WJ (1967) Steam distillation of volatile fatty acids in rumen ingesta. *J Dairy Sci* 50: 73–76. [https://doi.org/10.3168/jds.S0022-0302\(67\)87356-9](https://doi.org/10.3168/jds.S0022-0302(67)87356-9)
25. Conway EJ, O'Malley E (1942) Microdiffusion methods: Ammonia and urea using buffered absorbents. *Biochem J* 36: 655–661. <https://doi.org/10.1042/bj0360655>
26. Ogimoto K, Imai S (1981) *Atlas of Rumen Microbiology*, JSSP, Tokyo.
27. Lowry OH, Rosebough NG, Farr AL, et al. (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265–275. [https://doi.org/10.1016/S0021-9258\(19\)52451-6](https://doi.org/10.1016/S0021-9258(19)52451-6)
28. Ponnampalam E, Kearns M, Kiani A, et al. (2024) Enrichment of ruminant meats with health-enhancing fatty acids and antioxidants: feed-based effects on nutritional value and human health aspects. *Front Anim Sci* 5: 1329346. <https://doi.org/10.3389/fanim.2024.1329346>
29. Dentinho MTP, Bessa RJB (2016) Effect of tannin source and pH on stability of tannin–protein complexes. *Rev Ciênc Agrár* 39: 114–121. <https://doi.org/10.19084/rca15062>
30. Jalč D, Čertík M, Kundrikova K, et al. (2018) Effect of unsaturated C18 fatty acids (oleic, linoleic and alpha-linolenic acid) on ruminal fermentation and production of fatty acid isomers in an artificial rumen. *Vet Med* 52: 87–94. <https://doi.org/10.17221/2012-vetmed>
31. Jamarun N, Pazla R, Arief A, et al. (2020) Chemical composition and rumen fermentation profile of mangrove leaves (*Avicennia marina*) from West Sumatra, Indonesia. *Biodiversitas* 21: 5230–5236. <https://doi.org/10.13057/biodiv/d211126>

32. Martello H, Paula N, Macedo B, et al. (2019) Effects of tannins and monensin in a feedlot diet on in vitro ruminal fermentation. *Semina Cienc Agrar* 40: 3223–3232. <https://doi.org/10.5433/1679-0359.2019v40n6Supl2p3223>
33. Reynal S, Broderick G (2005) Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. *J Dairy Sci* 88: 4045–4064. [https://doi.org/10.3168/jds.s0022-0302\(05\)73090-3](https://doi.org/10.3168/jds.s0022-0302(05)73090-3)
34. Rira M, Morgavi D, Popova M, et al. (2022) Microbial colonisation of tannin-rich tropical plants: Interplay between degradability, methane production and tannin disappearance in the rumen. *Animal* 16: 100589. <https://doi.org/10.1016/j.animal.2022.100589>
35. Calsamiglia S, Ferret A, Reynolds CK, et al. (2010) Strategies for optimizing nitrogen use by ruminants. *Animal* 4: 1184–1196. <https://doi.org/10.1017/S1751731110000911>
36. Chikunya S, Demirel G, Enser M, et al. (2004) Biohydrogenation of dietary n-3 PUFA and stability of ingested vitamin E in the rumen, and their effects on microbial activity in sheep. *Br J Nutr* 91: 539–550. <https://doi.org/10.1079/BJN20031078>
37. Fonseca NVB, Cardoso AS, de Souza Bahia A, et al. (2023) Additive tannins in ruminant nutrition: An alternative to achieve sustainability in animal production. *Sustainability* 15: 4162. <https://doi.org/10.3390/su15054162>
38. Gunun P, Wanapat M, Gunun N, et al. (2015) Effects of condensed tannins in Mao (*Antidesma thwaitesianum* Muell. Arg.) seed meal on rumen fermentation characteristics and nitrogen utilization in goats. *Asian-Australas J Anim Sci* 29: 1111–1119. <https://doi.org/10.5713/ajas.15.0552>
39. Ku-Vera J, Jiménez-Ocampo R, Valencia-Salazar S, et al. (2020) Role of secondary plant metabolites on enteric methane mitigation in ruminants. *Front Vet Sci* 7: 584. <https://doi.org/10.3389/fvets.2020.00584>
40. Min B, Castleberry L, Allen H, et al. (2019) Associative effects of wet-distiller's grains plus solubles and tannin-rich peanut skin supplementation on in vitro rumen fermentation, greenhouse gas emissions, and microbial changes. *J Anim Sci* 97: 4668–4681. <https://doi.org/10.1093/jas/skz317>
41. Belanche A, Abecia L, Holtrop G, et al. (2011) Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. *J Anim Sci* 89: 4163–4174. <https://doi.org/10.2527/jas.2010-3703>
42. Utami BV, Zai M, Elihasridas E, et al. (2025). Comparative effects of Gamal (*Gliricidia sepium*)-based diets supplemented with Gambier (*Uncaria gambir*) or direct-fed microbials on rumen fermentation, nutrient digestibility, microbial protein synthesis, and methane mitigation in beef cattle: An in vitro evaluation. *Vet World* 18: 3855–3869. <https://doi.org/10.14202/vetworld.2025.3855-3869>
43. Edwards J, Huws S, Kim E, et al. (2008) Advances in microbial ecosystem concepts and their consequences for ruminant agriculture. *Animal* 2: 653–660. <https://doi.org/10.1017/s1751731108002164>
44. Ibrahim N, Alimon AR, Yaakub H, et al. (2021) Effects of vegetable oil supplementation on rumen fermentation and microbial population in ruminant: A review. *Trop Anim Health Prod* 53: 422. <https://doi.org/10.1007/s11250-021-02863-4>
45. Ningrat R, Zain M, Suryani H (2016) Effects of doses and different sources of tannins on in vitro ruminal methane, volatile fatty acid production and on bacteria and protozoa populations. *Asian J Anim Sci* 11: 47–53. <https://doi.org/10.3923/ajas.2017.47.53>

46. Vasta V, Daghighi M, Cappucci A, et al. (2019) Plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: Experimental evidence and methodological approaches. *J Dairy Sci* 102: 3781–3804. <https://doi.org/10.3168/jds.2018-14985>
47. Niu X, Xing Y, Wang J, et al. (2024) Effects of *Caragana korshinskii* tannin on fermentation, methane emission, community of methanogens, and metabolome of rumen in sheep. *Front Microbiol* 15: 1334045. <https://doi.org/10.3389/fmicb.2024.1334045>
48. Pujaningsih R, Tampoebolon B, Widiyanto W, et al. (2019) Evaluation of the effectiveness of the use of papaya fruit latex in making herbal medicated multinutrition block as a local goat feed supplement. *Anim Prod* 20: 39–44. <https://doi.org/10.20884/1.jap.2018.20.1.687>
49. Nurfitriani R, Wulandari S, Bahariawan A, et al. (2024) The physical quality of elephant grass silage with tannin extract from coffee husk. *IOP Conf Ser Earth Environ Sci* 1302: 012054. <https://doi.org/10.1088/1755-1315/1302/1/012054>
50. Despal D, Irmadani D, Permana IG, et al. (2022) Effect of different unsaturated fatty acids sources on in vitro fermentability and digestibility of ration in dairy cattle. *Online J Anim Feed Res* 12: 154–459. <https://doi.org/10.51227/ojafr.2022.20>
51. Norrapoke T, Pongjornmit T (2025) Effect of high-quality pellet feed level on voluntary feed intake, nutrient digestibility and rumen fermentation in beef cattle. *Sci Rep* 15: 15343. <https://doi.org/10.1038/s41598-025-96455-z>
52. Pena I, De Carvalho C, Fiorentini G, et al. (2017) Supplementation with lipid sources alters the ruminal fermentation and duodenal flow of fatty acids in grazing Nellore steers. *Anim Feed Sci Technol* 227: 142–153. <https://doi.org/10.1016/j.anifeedsci.2017.02.017>
53. Zainuddin M, Rosnah S, Noriznan M, et al. (2014) Effect of moisture content on physical properties of animal feed pellets from pineapple plant waste. *Agric Agric Sci Procedia* 2: 224–230. <https://doi.org/10.1016/j.aaspro.2014.11.032>
54. Ikhlas Z, Jamarun N, Zain M, et al. (2025) The influence of tapioca flour as a binder in “complete feed wafers” composed on the crude fibre digestibility, crude fat digestibility, and nitrogen free extract (NFE) digestibility. *IOP Conf Ser.: Earth Envi Sci* 1502: 012021. https://doi.org/10.1088/17551315/1502/1/012021?urlappend=%3Futm_source%3Dresearchgate.net%26utm_medium%3Darticle
55. Haque MN (2018) Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *J Anim Sci Technol* 60: 15. <https://doi.org/10.1186/s40781-018-0175-7>
56. Mitchell K, Lee C, Socha M, et al. (2023) Supplementing branched-chain volatile fatty acids in dual-flow cultures varying in dietary forage and corn oil concentrations. II: Biohydrogenation and incorporation into bacterial lipids. *J Dairy Sci* 106: 7548–7565. <https://doi.org/10.3168/jds.2022-23193>
57. Sun X, Wang Q, Yang Z, et al. (2022) Altering methane emission, fatty acid composition, and microbial profile during in vitro ruminant fermentation by manipulating dietary fatty acid ratios. *Fermentation* 8: 310. <https://doi.org/10.3390/fermentation8070310>



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