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Fermentation parameters in the rumen of goats supplemented with polyphenol oxidase derived from *Gliricidia sepium* leaves under in vitro conditions

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Abstract. *Huda AN, Sabarudin A, Jayanegara A, Soetanto H. 2023. Fermentation parameters in the rumen of goats supplemented with polyphenol oxidase derived from* Gliricidia sepium *leaves under in vitro conditions. Biodiversitas 24: 3282-3290.* The growing demand for healthy foods derived from ruminant products is restricted by the high content of saturated fatty acids in meat or milk due to the process of rumen biohydrogenation. Recent reports from intensive studies using polyphenol oxidase (PPO), an enzyme in plants' secondary metabolism, as the inhibitor of rumen biohydrogenation cost doubt of ruminant products. PPO is assumed to increase the supply of UFA by reducing the rate of biohydrogenation due to rumen microbes' activity. Microbes of the rumen, including species of bacteria and protozoa are responsible for rumen biohydrogenation. The present study investigated the effect of adding PPO derived from *Gliricidia sepium* leaves on fermentation parameters in the rumen, including protozoa density and diversity, fatty acid profile, volatile fatty acid profile, and the efficiency of microbial protein synthesis under in vitro conditions. The experiment was carried out through an in vitro rumen fermentation method, consisting of five treatments, i.e., negative control, positive control, the addition of 0.1 ml, 0.3 ml, and 0.5 ml PPO emulsion. The most dominant type of protozoa among all treatments was *Entodinium* spp. PPO emulsion increases the efficiency of microbial protein synthesis and volatile fatty acid concentration. 0.5 mL PPO emulsion increases linoleic acid. In conclusion, this finding poses a question on the role of rumen protozoa in lipid biohydrogenation in the rumen. A further experiment on the benefits of adding PPO regarding rumen biohydrogenation under in vivo conditions is warranted.

Keywords: Lipid biohydrogenation, polyphenol oxidase, protozoa, rumen fermentation

INTRODUCTION

Lipid metabolism in the rumen is a complex system consisting of two main processes, namely lipolysis and biohydrogenation, in which rumen microbes play a significant role. The changes in ingested dietary unsaturated to saturated fatty acids remain unclear and are subject to much controversy in the literature (Zhao et al. 2016; Vargas et al. 2017; Li et al. 2023). Lipolysis is the process where lipids are converted into free fatty acids by microorganisms in the rumen. These microorganisms use the enzyme lipase to break down dietary lipids (Enjalbert et al. 2017; Ferlay et al. 2017). The lipolysis products in the form of polyunsaturated fatty acids (PUFA) stimulate the initiation of biohydrogenation, which results in the formation of saturated fatty acids (SFA) that are manifested in ruminant products such meat and milk (Ferlay et al. 2017; Morales and Ungerfeld 2015; Lanier et al. 2015). Under this condition, ruminant products may become less desirable or even undesirable by consumers, particularly those who are susceptible to or suffer from cholesterol-related diseases.

There is a growing demand for healthy food originating from animal products and therefore, it is unsurprising that massive and intensive studies have been conducted to manipulate rumen biohydrogenation in the past two decades (Kallas et al. 2014; de Neve et al. 2018; Gebreyowhans et al. 2019; Kholif et al. 2022). Lanier and Corl (2015) reviewed the positive effect of PUFA protection by coating the PUFA with formaldehyde-casein to increase the amount of PUFA transitioned to the small intestine for absorption. Therefore, the increased absorption of PUFA in the small intestine will likely enrich the amount of PUFA contained in the milk or meat. A similar benefit has been achieved through the use of fatty acyl amides or calcium salts (Kliem et al. 2013; Zymon et al. 2014; Freitas et al. 2018). Other studies have shown that phenolic compounds such as tannins also have the capability to modify the rumen (Daning et al. 2022a; Daning et al. 2022b).

Recently polyphenol oxidase (PPO) extracted from various plants has been demonstrated to influence the rumen fatty acid digestion process. (Gadeyne et al. 2015; Gadeyne et al. 2017; de Neve et al. 2018). PPO is the only copper metalloenzyme oxidoreductase that can oxidize phenol, causing a brown color change (Boeckx et al. 2015; Jukanti 2017). A similar report focused on PPO extracted from tropical lands, especially from Indonesia, which is considered the second-biggest country in the world in terms of biodiversity. The potential of PPO sourced from local biodiversity will innovate the PPO effect on

biohydrogenation even further.

Many studies have shown that the biohydrogenation process is initiated through lipolysis by the rumen microbes, especially rumen bacteria from the genus *Butirivibrio fibrisolvent* and *Anaerovibrio lipolytica* (Conte et al. 2017; Dewanckele et al. 2018). The role of other rumen microbes, such as protozoa and fungi are less clear due to the conflicting results dealing with the contribution of rumen protozoa and fungi in either lipolysis or subsequence biochemical reaction of fatty acid biohydrogenation. Newbold et al. (2015) reported an increased amount of SFA when protozoa were absent from the rumen which suggests that the most significant role in the biohydrogenation of fatty acids is attributable to rumen microbes. Fransisco et al. (2019) reported negative relationships between the concentration of ciliates and the proportion of PUFA in the rumen. Additionally, William et al. (2020) reported a study that confirmed that protozoa have no direct role in fat metabolism in the rumen. This study also conducted an analysis of the fatty acid profile.

However, a study using in vitro digestibility trials should be conducted to determine the amount and diversity of rumen protozoa with the addition of PPO. This is little information regarding the role of the rumen microbe type in biohydrogenation and its abundance after the addition of PPO compounds. Huda et al. (2022) used a simplicial of potato peels as a PPO source through in vitro research and stated that the treatment had an anti-protozoa effect in the rumen fluid.

The objective of this research was to investigate the effect of added dietary PPO derived from *Gliricidia sepium* leaves (GL) on the protozoa population and diversity in the rumen under in vitro conditions.

MATERIALS AND METHODS

Study area

This study lasted from April to August 2022 and was conducted almost exclusively in the Animal Feed and Nutrition Laboratory, Faculty of Animal Science, Universitas Brawijaya, Malang, Indonesia.

Materials

Source of inoculum

Rumen digesta was collected from three goats that were slaughtered at a small abattoir belonging to Malang municipality immediately after the animals were slaughtered. Approximately 2 L of rumen digesta was transferred into a pre-warmed Thermo-flask and transported to the laboratory. The flask content was filtered through four layers of muslin cloth, and the liquid was squeezed out to obtain a sufficient volume of rumen fluid (RF). The RF was mixed with McDougall buffer solution at a ratio 1:4 (v/v), flushed with CO_2, and the temperature was maintained at 39-40°C to simulate the rumen environment. The RF was kept fresh until it was utilized for subsequent experiments.

Procedures

PPO extraction and purification

This study used PPO compound as an in vitro fermentation simulation procedure obtained from extraction and purification processes. The extraction and purification method used the Aqueous Two-Phase System (ATPS) method as described by Panadare and Rathod (2018) with samples of GL as a source of PPO. GL weighing 100 g was frozen with liquid nitrogen at a ratio of 1:2 and then crushed using a mortar until it crumbled. After that, it was soaked in 10 mM phosphate buffer saline (PBS) overnight at 4°C and filtered using a muslin cloth. The resulting filtrate was combined with 25% $(NH_4)_2$ SO_4 and centrifuged at 10,000 rpm for 20 min. The supernatant was removed, and the pellet was diluted with 50 mM PBS and dialyzed using the same buffer and dialysis tube. Upon completion, crude PPO enzymes were obtained. The production of the ATPS was performed by mixing 1 g of crude enzyme, 5% (w/b) polyethylene glycol (PEG) 8000, 28.5% (w/b) PBS. and 10 g of distilled water were added; this was then centrifuged at 10,000 rpm for 20 min. The resulting supernatant from the centrifugation process is a pure PPO compound. The enzyme was assayed using a spectrophotometer based on the method described by Falguera et al. (2012) and Wu et al. (2013). Briefly, 2.8 mL of the pure PPO is mixed with 0.2 mL catechol as the substrate inside a cuvette. Immediately the cuvette was placed into a spectrophotometer and measured at a wavelength of 420 nm. The reaction between the catechol and PPO creates quinone, which changes the absorbance at a rate of 0.001 M/min.

PUFA protection with PPO emulsification

To elucidate the role of PPO derived from GL as a PUFA protector against biohydrogenation, the method described by Gadeyne et al. (2017) was applied with the modification of using a micro vibrator instead of a high-speed Ultra Turrax. The emulsion consists of pure PPO extract, whey protein isolate (WPI) as a stabilizer, and catechol as a substrate at the ratio of 1:1:98 (v/v/v), respectively. The PUFA source in this study was obtained from linseed oil (*Linum usitatissimum*). Linseed oil consists of 47% PUFA, 11% monounsaturated fatty acids (MUFA), and 7% SFA (Wang et al. 2022). The ratio of linseed oil to the emulsion mixture (PPO, WPI and catechol) was 3:10 (v/v), respectively.

In vitro digestibility procedure

In vitro digestion trials were conducted in accordance with the Tilley and Terry method (Tilley and Terry 1963; Alende et al. 2018). A complete diet consisting of 60% *Pennisetum purpureum* and 40% concentrate used by Huda et al. (2022) with dry matter (DM) content of 92.93%, organic matter (OM) 86.54%, crude protein (CP) 10.91%, complete feed (CF) 28.16%, and ether extract (EE) 2.29% of DM. The CF weighing 0.5 g, was placed into the fermenter tube, and then into the incubator at 39°C. Meanwhile, the buffer solution was prepared by mixing 10.2 g $Na_2HPO_4.12H_2O$, 21.6 g $NaHCO_3$, 1.3 g NaCl, and 1.25 g KCl and dissolved in distilled water to 440 mL. The

buffer solution was mixed with 550 mL of RF, 4.4 g $MGCl_2$, 4.4 g $CaCl_2$, and 1751 mL of distilled water until homogeneous while flushing with CO_2 to maintain anaerobic conditions. The solution, which was a mixture of buffer solution and RF, was put into the fermenter tube (50 mL) and PPO emulsion was added, according to the following treatments: (i) Negative control: CF; (ii) Positive control: CF + linseed oil; (iii) Treatment 1: CF + 0.1 mL emulsion; (iv) Treatment 2: CF + 0.3 mL of emulsion; (v) Treatment 3: CF + 0.5 mL emulsion. Each treatment consisted of 10 fermenter tubes with two repetitions analyzed at the same sampling time performed every 4, 8, 16, 24, and 48 h. Standard production uses the same method, but the sample used is elephant grass (*P. purpureum*) without the addition of linseed oil or emulsion, while blanks are made without the addition of samples, each of which has two replications. Every 4, 8, 16, 24, and 48 h an RF sample was taken from the fermenter tube to stop the fermentation process. The solution was centrifuged at 2,500 rpm for 15 min and preserved using formal saline at a ratio of 1:6. Formal saline can be made by mixing 10% formalin with 9 g NaCl and dissolving it in distilled water up to 1 L.

Population density and diversity of protozoa

Density and diversity calculations use Sedgewick Rafter chamber S52 with a volume of 1 mL^3. The observation of the protozoa was conducted without staining and a total of 20 fields were observed using a Nikon Upright Eclipse Ci-L Fluorescence Microscope. The criteria used to identify the protozoa was based on morphological characteristics described in Dehority (2018) which include the presence of cilia on the body surface area, number and location of cilia, shape and dimensions, number and location of vacuoles, and presence of terminal spines.

Fatty acid profile analysis

The concentration of long-chain fatty acids was analyzed using the Gas Chromatography GC-FID type Clarus 690 as described by Weatherly (2016). Gas chromatography analyzed the fatty acid methyl ester compound, which is formed from the sample extraction process using organic solvents, and undergoes hydrolysis and derivatization (Ostermann et al. 2014). While the short-chain fatty acids were analyzed using the Gas Chromatography Shimadzu GC-2010 Plus according to the method described by Ribeiro et al. (2018).

Efficiency of microbial protein synthesis

The method to calculate the efficiency of microbial protein synthesis is in accordance with that described by Blümmel et al. (1997). After the termination of the 48 h digestibility study, all fermenters were immersed in the iced water for an hour to ensure that the fermentation process had stopped. The fermenter content was then transferred to a centrifuge tube and centrifuged at 12,000 rpm for 10 min. The true degradability value is obtained by adding Neutral Detergent Solution (NDS) was added to the residue. The residue was heated in an oven at 105°C and put in the furnace at 550°C. The values that must be known to estimate the microbial biomass are apparent and true degradability. The apparent degradability values are obtained by following the same steps above but without adding NDS. Furthermore, the calculation of the efficiency of microbial protein synthesis was performed by multiplying the microbial biomass with the microbial N and 1000/DOMR (digestible organic matter rumen).

Data analysis

Observational data of protozoa diversity were analyzed through morphological comparison between screenshots from the microscope with references from Dehority (2018). Meanwhile, the calculation data obtained were tabulated using Microsoft Excel and an analysis of variance (ANOVA) was performed with a factorial completely randomized design (Sudarwati et al. 2019).

RESULTS AND DISCUSSION

Protozoal density and diversity under in vitro conditions

Table 1 describes the dynamic of the protozoal rumen density at the different incubation times and the effect of adding linseed oil or PPO emulsion. There was a consistent declining trend in the protozoal density with the increased incubation time in PPO treated RF, resulting in a statistically significant difference ($P < 0.01$). Treatment 1 has the lowest protozoa density in the initial 4 hours. Treatment 2 has the lowest protozoa density in 48 hours. The positive control shows a higher protozoal density compared to the treatments and is higher than the negative control. The higher values demonstrate the positive effect of linseed oil on protozoa density. The present study shows that the addition of PPO reduces the protozoal density.

The relative abundance of protozoal genera in the RF was affected by the different treatments and are presented in Table 2-6. There were nine identified genera across 40 samples of RF, namely *Entodinium, Buetschlia, Dasytricha, Isotricha, Charonina, Polypastron, Oligoisotricha, Diplodinium,* and *Ostracodinium.* There was a tendency of the predominant genus of *Entodinium* found across the sampled rumen fluid regardless of PPO treatment and incubation time, except in the RF samples with the addition of 0.1, 0.3, and 0.5 mL PPO emulsion at 24 h and 48 h incubation and was replaced by *Buetschlia* and *Dasytricha* (see Table 4.C, 5.C, 5.D, 6.D).

Table 3 shows that the predominant genus at all observation times was *Entodinium.* The genera that were always present at all sampling times were *Entodinium, Buetschlia,* and *Charonina,* which indicates the genus that is most frequently identified in the rumen fluid.

Based on Table 4, it is known that there is a change in the percentage of the present genus between *Entodinium* and *Buetschlia,* between 4 h and 16 h incubation, the rate of *Entodinium* genus was higher than that of *Buetschlia.* In contrast, at the 24 h incubation, *Entodinium* was lower than *Buetschlia.* At the 48 h incubation, *Buestchlia* experienced

a decrease in percentage compared to *Entodinium*, which experienced an increase.

Table 5 shows that the predominant genus percentage at 4 and 16 h was *Entodinium*, while at 24 h and 48 h, *Buetschlia* was highest. The genus *Oligoisotricha* was found at the 4, 24, and 48 h of observation.

Table 6 shows that the genus *Entodinium* had the dominant percentage at 4, 16, and 24 h, while at 48 h, the genus with the dominant percentage was *Dasytricha*. The genera *Oligoisotricha* and *Buetschlia* were found at all observation times. Among all the treatments, the genus with the most dominant percentage was the protozoa type which was easiest to observe under a microscope. The percentage difference was also due to the protozoa's locomotion apparatus, which allowed it to move. *Entodinium* was the dominant genus at all observation times in the negative and positive control treatments, whereas in the PPO treatments, the dominant genus varied.

The results of the morphological observations and identification of protozoa under a microscope are presented in Figure 1. The total protozoa portraits shown in Figure 1 are nine genera of the Genus *Entodinium*, *Dasytricha*, *Buetschila*, *Charonina*, *Oligoisotricha*, *Diplodinium*, *Ostracodinium*, *Eudiplodinium*, and *Epidinium.* The protozoa with the most significant size were *Eudiplodinium maggi* and *Epidinium cattanei*, which have the same size range, length 115-112 µm and width 73-143 µm, while the protozoa with the smallest size was *Oligoisotricha bubali* which has a length of 12-22 µm and width 8-20 µm.

Table 1. Protozoal density in the rumen fluid at different incubation times (× 10^5 cells/mL rumen fluid)

Incubation time (hours)	Polyphenol oxidase treatments					P-value
	Negative control	Positive control	Treatment 1	Treatment 2	Treatment 3	
4	2.088^{d}±0.066	1.779^{c}±0.032	1.009^{a}±0.018	1.073^{b}±0.005	1.443^{b}±0.016	0.01
8	1.190^{c}±0.033	1.593^{d}±0.074	0.869^{a}±0.010	0.937^{a}±0.025	1.044^{b}±0.097	0.01
16	1.090^{c}±0.008	1.371^{d}±0.057	0.729^{b}±0.007	0.583^{a}±0.004	0.694^{b}±0.008	0.01
24	0.933^{d}±0.016	1.090^{e}±0.041	0.625^{c}±0.008	0.496^{a}±0.009	0.566^{b}±0.041	0.01
48	0.677^{c}±0.033	0.933^{d}±0.016	0.485^{ab}±0.009	0.414^{a}±0.058	0.501^{b}±0.033	0.01

Note: Negative control: Complete feed, Positive control: Complete feed + 0.02 mL linseed oil, Treatment 1: Complete feed + 0.1 mL emulsion, Treatment 2: Complete feed + 0.3 mL of emulsion, Treatment 3: Complete feed + 0.5 mL emulsion

Table 2. Relative abundance of rumen protozoal genera in the negative control (no emulsion or linseed oil)

Genus	Incubation time (hours)			
	4	16	24	48
	Percentage of abundance (%)			
Buetschlia	1.64	16.67	3.85	6.67
Diplodinium	-	13.33	-	6.67
Charonina	8.20	-	3.85	-
Dasytricha	3.28	-	3.85	-
Entodinium	85.25	66.67	57.69	60.00
Isotricha	1.64	-	-	-
Oligoisotricha	-	3.33	30.77	20.00
Polypplastron	-	-	-	6.67

Table 3. Percentage of genera from the positive control (linseed oil only)

Genus	Incubation time (hours)			
	4	16	24	48
	Percentage of abundance (%)			
Buetschila	14.00	8.51	9.38	22.22
Charonina	6.00	8.51	3.13	3.70
Dasytricha	2.00	4.26	15.63	-
Diplodinum	12.00	2.13	-	-
Entonidium	60.00	61.70	62.50	70.37
Epidinium	-	2.13	-	-
Metadinium	-	-	-	3.70
Oligoisotricha	2.00	10.64	6.25	-
Ostracodinium	2.00	-	3.13	-
Polyplastron	2.00	2.13	-	-

Table 4. Percentage of protozoa genera from treatment 1 (0.1 mL of emulsion). A. 4 h, B. 16 h, C. 24 h, D. 48 h incubation

Genus	Incubation time (hours)			
	4	16	24	48
	Percentage of abundance (%)			
Buetschlia	6.90	24.00	38.89	28.57
Charonina	3.45	4.00	-	-
Dasytricha	6.90	8.00	-	7.14
Elytroplastron	-	-	5.56	-
Entodinium	65.52	60.00	27.78	42.86
Epidinium	6.90	4.00	27.78	-
Oligoisotricha	10.34	-	-	21.43

Table 5. Percentage of protozoa genera from treatment 2 (0.3 mL of emulsion)

Genus	Incubation time (hours)			
	4	16	24	48
	Percentage of abundance (%)			
Buetschlia	7.41	-	50.00	50.00
Charonina	3.70	-	-	-
Dasytricha	-	11.76	-	7.14
Diplodinium	14.81	-	-	-
Enoploplastron	3.70	-	-	-
Entodinium	48.15	64.71	31.25	28.57
Eudiplodinium	3.70	17.65	-	-
Metadinium	7.41	-	-	-
Oligoisotricha	3.70	-	12.50	14.29
Ostracodinium	11.11	-	-	-
Polyplastron	-	5.88	6.25	-

Table 6. Percentage of protozoa genera from treatment 3 (0.5 mL of emulsion)

Genus	Incubation time (hours)			
	4	16	24	48
	Percentage of abundance (%)			
Buetschlia	14.63	6.67	10.71	20.00
Charonina	4.88		7.14	
Dasytricha	2.44		10.71	30.00
Entodinium	53.66	53.33	46.43	25.00
Epidinium			3.57	
Eudiplodinium	4.88	26.67		
Metadinium		6.67		
Oligoisotricha	17.07	6.67		25.00
Ostracodinium			3.57	
Polyplastron	2.44	17.86		

Ruminal fatty acid profile

The fatty acid profile in the present study was based on linoleic acid (C18:2) and stearic acid (C18:0) in the rumen. Linoleic acid is a PUFA that is considered to be harmful to rumen bacteria and is the starting fatty acid form in biohydrogenation process and stearic acid is a SFA which is the final form of the biohydrogenation process (McSweeney and Fox 2020). Goat milk and meat mainly consist of trans fatty acids (C18:1) which is the intermediate process of PUFA converting into SFA. The fatty acid profile of RF in RF treated with PPO derived from GL after 48 h of incubation is presented in Table 7. The data shows an increase in the unsaturated fatty acid content, namely linoleic acid, compared to the SFAs. Linoleic acid had the highest concentration in the rumen in the 0.5 mL PPO emulsion treatment compared to the other treatments. In contrast, the most elevated stearic acid was present in the positive control compared to the other treatments.

The addition of PPO significantly increased the PUFA concentration in the RF (linoleic acid, C 18:2), even though SFA (stearic acid, C:18:0) was also increased compared to the negative control. The addition of linseed oil increased the PUFA significantly but doubled the stearic acid percentage compared to the other treatments. The PPO shows optimal results by increasing the PUFA and SFA which result from interfering with the biohydrogenation process.

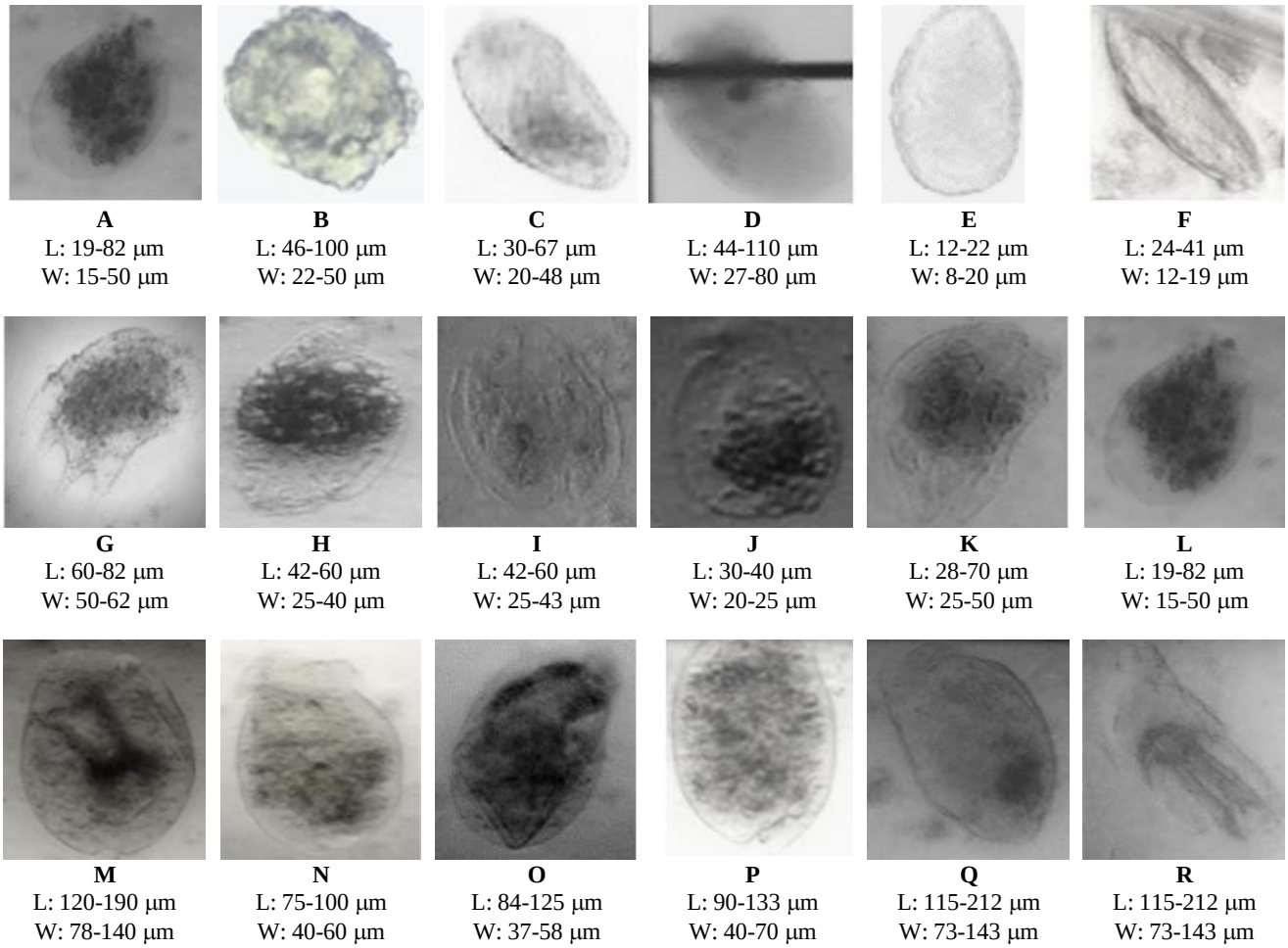

Figure 1. Protozoa genera observed under the microscope. A. *Entodinium dubardi*, B. *Dasytricha ruminantium*, C. *Buetschlia parva*, D. *Entodinium longinucleatum*, E. *Oligoisotricha bubali*, F. *Charonina ventriculi*, G. *Diplodinium dentatum*, H. *Diplodinium lobatum*, I. *Diplodinium monolobosum*, J. *Diplodinium polygonale*, K. *Entodinium caudatum*, L. *Entodinium dubardi*, M. *Polyplastron multivesiculatum*, N. *Ostracodinium trivesiculatum*, O. *Ostracodinium rugoloricatum*, P. *Ostracodinium gracile*, Q. *Eudiplodinium maggi*, R. *Epidinium cattanei*, L: length, W: width

Ruminal volatile fatty acid profile

The volatile fatty acid profile of the RF after 48 h of incubation is presented in Table 8. Table 8 shows the variation in the data of the volatile fatty acid profile. The acetic acid content increased from negative and positive controls with the treatments using PPO. The highest acetic acid content value was observed in the 0.5 mL PPO emulsion treatment at 17.52 ± 0.301 mMol. While the highest propionate and butyric acid content were present in the 0.3 mL PPO emulsion treatment, with concentrations of 4.03 ± 0.13 and 3.88 ± 0.01 mMol, respectively.

The efficiency of microbial protein synthesis

The efficiency value of the microbial protein synthesis is presented in Table 9. Based on these data, the efficiency value of the microbial protein synthesis increased linearly with the addition of PPO compounds, especially when compared to the negative and positive controls. The highest value was observed in the treatment with the addition of 0.3 mL PPO emulsion, with 42.679 ± 0.0005 g N/kg DOMR. The value is in agreement with Table 1, which shows that treatment 2 had the lowest amount of protozoa present which can increase the microbial protein supply to the host (Nguyen et al. 2020). In addition, the efficiency value of microbial protein synthesis is influenced by the type of feed consumed by ruminant animals. Anggraeny et al. (2015) reported that high-fiber feed would produce a relatively low-efficiency value for microbial protein synthesis, around 16 g N/kg DOMR. However, the value is higher the ruminant animals are fed a diet with a balanced energy and protein content. The lower number of protozoa results in a decrease in rumen ammonia and an increase in microbial protein (Soetanto 2019).

Discussion

Tables 2-6 describe the diurnal variation of the relative abundance of protozoa affected by adding linseed oil and PPO emulsion. There was considerable variation in the individual genus of rumen protozoa appearing throughout the different incubation times. The most frequent genera found throughout the study were *Entodinium* and *Buestclia*. Formato et al. (2022) conducted a literature review study on the relationship between polyphenol, PPO, and rumen biohydrogenation. They concluded that it was unlikely to inhibit biohydrogenation completely in the rumen; however, the substances may slow down the bacterial lipases. As a result, the addition of these substances may increase the concentration of UFA but concomitantly increases the SFA content. This concept is in agreement with our findings (see Table 2). Regarding the relative abundance of the rumen protozoa in the current study, the addition of PPO significantly decreased the abundance regardless of the PPO level. Accordingly, PPO emulsion may pose a detrimental effect on the rumen protozoa, as previously reported (Moumen et al. 2016; Enjalbert et al. 2017; Hassan et al. 2020; Ibrahim et al. 2021). A lack of protozoa can reduce nitrogen content and increase the microbial protein supply in the hosts (Nguyen et al. 2020). However, a reduction in the rumen protozoa abundance significantly increases the microbial synthesis which may increase the supply of N to the host animal.

Table 9. Efficiency of the microbial protein synthesis

Treatments	(g N/kg DOMR)
Negative control	32.301^{a}±0.0010
Positive control	33.772^{b}±0.0007
0.1 mL PPO emulsion	36.606^{e}±0.0008
0.3 mL PPO emulsion	42.679^{e}±0.0005
0.5 mL PPO emulsion	41.945^{d}±0.0006
P-value	0.01

Note: PPO: Polyphenol oxidase, DOMR: digestible organic matter rumen

Table 7. Fatty acid profile

	Linoleic acid (%)	% Increase	Stearic acid (%)	% Increase
Negative control	0.0150^{a} ± 0.0071	0	0.0150^{a} ± 0.0050	0
Positive control	0.0650^{b} ± 0.0494	333.33	0.0275^{b} ± 0.0176	83.33
0.1 mL PPO emulsion	0.0167^{a} ± 0.0057	11.33	0.0166^{a} ± 0.0057	10.67
0.3 mL PPO emulsion	0.0433^{a} ± 0.0208	188.67	0.0175^{a} ± 0.0050	16.67
0.5 mL PPO emulsion	0.0803^{c} ± 0.0005	435.33	0.0225^{ab} ± 0.0095	50
P-value	0.05		0.05	

Note: PPO: Polyphenol oxidase

Table 8. Volatile fatty acid profile

	Acetic acid (mMol)	Propionic acid (mMol)	Butyric acid (mMol)
Negative control	11.88^{a} ± 0.055	2.39^{a}±0.135	1.13^{a}±0.09
Positive control	13.37^{b} ± 0.254	2.59^{b}±0.124	1.62^{a}±0.10
0.1 mL PPO emulsion	16.58^{c} ± 0.315	4.03^{c}±0.357	3.88^{d}±0.01
0.3 mL PPO emulsion	17.38^{d} ± 0.162	2.84^{d}±0.227	2.81^{b}±0.23
0.5 mL PPO emulsion	17.52^{e} ± 0.301	3.85^{e}±0.095	3.03^{c}±0.09
P-value	0.01	0.01	0.01

Note: PPO: Polyphenol oxidase

The increase in the amount of linoleic acid as presented in Table 7 shows the interference of the biohydrogenation process through the mechanism of UFA protection by PPO emulsion (Gadeyne et al. 2015; Gadeyne et al. 2017; de Neve et al. 2018). Protozoa are thought to contribute to the process of rumen biohydrogenation. As shown in Table 7, there was an increase in the UFA content, namely linoleic acid, in the treatment with added PPO emulsion-protected linseed oil compared to added linseed oil alone. The amount of stearic acid as SFA also decreased in the PPO emulsion treatment compared to the positive control.

Feeding frequency affects the diurnal variation of the rumen protozoa density (Lengowski et al. 2016). The diversity of the rumen protozoa varies because it is influenced by feed and feed intake (Leng 2014). The study of in vitro protozoa density demonstrated different conditions from in vivo due to the extensive rumen fermentation conditions and has several sections. However, the presence of protozoa in the rumen ecology is essential to maintain stable rumen conditions and avoiding the risk of acidosis, although it increases proteolysis and ammonia concentration (Yuste et al. 2019).

Theories regarding the role of protozoa in rumen biohydrogenation are still speculative (Leng 2014; Fransisco et al. 2019). Research conducted by Ebrahimi et al. (2015) reported that increasing palm oil fronds in goat diets was thought to increase the protozoa abundance but decrease the rate of biohydrogenation. However, in a study conducted by Baldin et al. (2022), there was an increase in the biohydrogenation rate of C18:3 with an increase in protozoa abundance. In the review by Vasta et al. (2019) it was stated that protozoa play an active role in the degradation of organic matter in the rumen, including lipids. The results of the study by Jafari et al. (2016) reported that polyphenol treatment from papaya leaves caused a decrease in the number of rumen protozoa, followed by a decrease in the rate of biohydrogenation marked by an increase in C18:1n-9 (oleic acid), C18:2n-6 (linoleic acid), and C18:3n-3 (α-linolenic acid). Francisco et al. (2019) reported that protozoa had a positive correlation in hydrogenated fatty acids until the formation of t11-18:1 and c9, t11-18:2, where the further process to form stearic acid was performed by other rumen microorganisms such as bacteria.

In the absence of protozoa, the number of bacteria increases substantially (Soetanto 2019). The activity of rumen bacteria in this study is presented in Table 8 through the concentration of volatile fatty acids. Acetic acid showed an increase in the PPO treatment compared to the negative and positive controls. The 0.5 mL PPO emulsion treatment showed the highest acetic acid concentration, while the highest propionate and butyric acids were present in 0.1 mL PPO emulsion treatment. In line with the research conducted by Newbold et al. (2015), a decrease in the number of protozoa was followed by an increase in acetic acid and a reduction of butyric acid concentration, but the propionic acid concentration was not affected in the rumen. Hidayah et al. (2014) reported that linseed oil encapsulation treatment would optimize rumen fermentation in terms of an increase in propionic acid in the treatment. The results from this study showed a linear increase in propionic acid production in the PPO treatment compared to the control. Concerning the activity of the rumen bacteria, the efficiency value of the rumen microbial protein synthesis (Table 9) indicates that the addition of PPO increased the bacterial cell biomass, especially when 0.3 mL of PPO emulsion was added. The higher bacterial cell biomass value suggests that the fermentation process in the rumen was functioning optimally and could meet the livestock nutrient need. Although the PPO compound reduced the number of rumen protozoa, it did not negatively affect bacterial activity.

Based on the research results, *Entodinium* was the most common genus observed, especially in the negative and positive control treatments. In the treatment with added PPO emulsion (T1-T3), other genera were also dominant, namely *Buetschlia* in T1 and T2 and *Dasytricha* in T3. Other genera that were identified included *Charonina, Isotricha, Polypastron*, and *Oligoisotricha* in the negative control, while *Diplodinium*, *Ostracodinium*, and *Metadinium* were also found in the positive control. Another protozoa genus found in T1 was *Eudiplodinium*, and *Enoplopastron* in T2. *Entodinium* was the dominant protozoa and played a significant role in degrading the crude fiber feed (Henderson et al. 2015). *Eudiplodinium*, *Dasytricha*, *Ostracodinium*, *Enoplopastron*, and *Polypastron* also have the ability to digest crude fiber with different activity variations (Castillo-González et al. 2014; Aprilia et al. 2021). Protozoa in the holotrich category have a strong preference for dietary starch (Patel and Ambalam 2018). *Dasyctricha* is another holotricha that utilizes soluble carbohydrates. These abilities of feed degradation are considered better than that of bacteria. The high digestive ability of protozoa is a positive attribute; however, not all protozoa can nourish the host animal as not all protozoa can pass from the rumen to the lower digestive tract (Soetanto 2019). The presence of *Dasytricha* and *Isotricha* does not have a detrimental effect like that of *Entodinium* on bacterial protein intake. Therefore, fermentative becomes less efficient due to the activity of *Entodinium*. *Entodinium* is common in adult ruminants, while *Buetschlia* is more common in calves (Duarte et al. 2018). Duarte et al. (2018) reported that *Buetschlia* was dominant in calves aged 6-8 months allowed to feed in low-nutrient pastures of *Brachiaria*, compared to other genera. *Buetschlia* is classified as a holotricha protozoa and is commonly found in the rumen of domestic and wild ruminants (Patel and Ambalam 2018; Firkins et al. 2020). *Buetschilia* was the most identified genus of ruminal ciliates in Nellore calves that were raised in tropical pastures during the dry season without feed supplementation (Duarte et al. 2018; dos Santos et al. 2021).

Nevertheless, there was no evidence found supporting this in this study as *Isotricha* was only observed in very low density in the negative control treatment and they were absent in the other treatments. Therefore, it is unlikely that protozoa play a significant role in fatty acid biohydrogenation in the rumen.

In conclusion, the addition of PPO emulsion to RF in this study revealed that the protozoal abundance decreased regardless of the PPO level, suggesting that PPO had a detrimental effect on the rumen protozoa. Concomitantly, the concentration of both UFA (linoleic acid) and SFA (stearic acid) increased significantly, but the increased increment of UFA was greater than that of SFA. This finding poses the question of the role of the rumen protozoa in lipid biohydrogenation in the rumen. Additionally, there was a negative correlation between the relative abundance of rumen protozoa and the increase of microbial protein synthesis, indicating that the growth of rumen microbe increases when the number of rumen protozoa is decreased.

The protozoa density decreased with the increase in vitro rumen fermentation incubation time. A decrease in protozoa density also occurred with the addition of PPO. The results of this study indicate that there is a relationship between the role of protozoa in rumen biohydrogenation and the use of PPO compounds as rumen manipulation biomarkers. This was determined through the observed increase in the linoleic acid content (C 18:2) and decrease in stearic acid content (C 18:0) in the RF when using PPO emulsions. Further experiments on the benefits of adding PPO regarding rumen biohydrogenation under in vivo conditions are warranted.

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REFERENCES

Alende M, Lascano GJ, Jenkins TC, Koch LE, Volpi-Lagreca G, Andrae JG. 2018. Technical note: Comparison of 4 methods for determining in vitro ruminal digestibility of annual ryegrass. Prof Anim Sci 34 (3): 306-309. DOI: 10.15232/pas.2017-01688.

Anggraeny, YN, Soetanto H, Kusmartono, Hartutik. 2015. Sinkronisasi suplai protein dan energi dalam rumen untuk meningkatkan efisiensi pakan berkualitas rendah. Wartozoa 25 (3): 107-116. DOI: 10.14334/wartazoa.v25i3.1155. [Indonesian]

Aprilia RM, Huda AN, Sumitro AM, Kusmartono S, H. 2021. Rumen protozoa diversity of Indonesian indigenous cattle. Jurnal Ilmu-Ilmu Peternakan 31 (3): 267-273. DOI: 10.21776/ub.jiip.2021.031.03.10. [Indonesian]

Baldin M, Garcia D, Zanton GI, Hao F, Patterson AD, Harvatine KJ. 2022. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) on milk fat, rumen environment and biohydrogenation, and rumen protozoa in lactating cows fed diets with increased risk for milk fat depression. J Dairy Sci 105 (9): 7446-7461. DOI: 10.3168/jds.2022-21910.

Blümmel MH, Steingass H, Becker K. 1997. The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. Br J Nutr 77 (6): 911-921. DOI: 10.1079/BJN19970089.

Boeckx T, Winters AL, Webb KJ, Kingston-Smith AH. 2015. Polyphenol oxidase in leaves: is there any significance to the chloroplastic localization? J Exp Bot 66 (12): 3571-3579. DOI: 10.1093/jxb/erv141.

Castillo-González A, Burrola-Barraza M, Domínguez-Viveros J, Chávez-Martínez A. 2014. Rumen microorganisms and fermentation. Arch Med Vet 46 (3): 349-361. DOI: 10.4067/S0301-732X2014000300003.

Conte G, Serra A, Mele M. 2017. Dairy cow breeding and feeding on the milk fatty acid pattern. In: Watson RR, Collier RJ, Preedy VR. Nutrients in Dairy and Their Implications for Health and Disease. Elsevier Inc, London. DOI: 10.1016/B978-0-12-809762-5.00002-4.

Daning DRA, Hanim C, Widyobroto BP, Yusiati LM. 2022a. Characteristics of ruminal fatty acids using in vitro culture system by addition of galangal (*Alpinia galangal*) essential oil. Adv Biol Sci Res 18: 65-69. DOI: 10.2991/absr.k.220207.014.

Daning DRA, Yusiati LM, Hanim C, Widyobroto BP. 2022b. Dietary supplementation of galangal (*Alpinia galangal*) essential oil affects rumen fermentation pattern. Adv Anim Vet Sci 10 (2): 323. DOI: 10.17582/journal.aavs/2022/10.2.323.334.

de Neve N, Vlaeminck B, Gadeyne F, Claeys E, Van der Meeren P, Fievez V. 2018. Promising perspectives for ruminal protection of polyunsaturated fatty acids through polyphenol-oxidase-mediated crosslinking of interfacial protein in emulsions. Anim 12 (12): 2539-2550. DOI: 10.1017/S1751731118000423.

Dehority BA. 2018. Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa. CRC Press, Boca Raton. DOI: 10.1201/9781351073912.

Dewanckele L, Vlaeminck B, Hernandez-Sanabria E, Ruiz-González A, deBruyne S, Jeyanathan J, Fievez V. 2018. Rumen biohydrogenation and microbial community changes upon early life supplementation of 22:6n-3 enriched microalgae to goats. Front Microbiol 9: 573. DOI: 10.3389/fmicb.2018.00573.

dos Santos TAX, Fernandes LMG, Carvalho PPX, Júnior VSM, Fonseca SA, Chaves AS, Duarte ER. 2021. Performance and microbiota of the digestive tract of Nellore calves supplemented with fungi isolated from bovine rumen. Vet World 14 (10): 2686-2693. DOI: 10.14202/vetworld.2021.2686-2693.

Duarte ER, Abrão FO, Oliveira Ribeiro IC, Vieira EA, Nigri AC, Silva KL, Virgínio Júnior GF, Prates Barreto SM, Geraseev LC. 2018. Rumen protozoa of different ages of beef cattle raised in tropical pastures during the dry season. J Appl Anim Res 46 (1): 1457-1461. DOI: 10.1080/09712119.2018.1530676.

Ebrahimi M, Rajion MA, Meng GY, Shokryzadan P, Sazili AQ, Jahromi MF. 2015. Feeding oil palm (*Elaeis guineensis*, Jacq.) fronds alters rumen protozoal population and ruminal fermentation pattern in goats. Italian J Anim Sci 14 (3): 3877. DOI: 10.4081/ijas.2015.3877.

Enjalbert F, Combes S, Zened A, Meynadier A. 2017. Rumen microbiota and dietary fat: a mutual shaping. J Appl Microbiol 123 (4): 782-797. DOI: 10.1111/jam.13501.

Falguera V, Sánchez-Riaño AM, Quintero-Cerón JP, Rivera-Barrero CA, Méndez-Arteaga JJ, Ibarz A. 2012. Characterization of polyphenol oxidase activity in juices from 12 underutilized tropical fruits with high agroindustrial potential. Food Bioprocess Technol 5 (7): 2921-2927. DOI: 10.1007/s11947-011-0521-y.

Ferlay A, Bernard L, Meynadier A, Malpuech-Brugère C. 2017. Production of trans and conjugated fatty acids in dairy ruminants and their putative effects on human health: a review. Biochim 141: 107-120. DOI: 10.1016/j.biochi.2017.08.006.

Firkins JL, Yu Z, Park T, Plank JE. 2020. Extending burk Dehority's perspectives on the role of ciliate protozoa in the rumen. Front Microbiol 11: 123. DOI: 10.3389/fmicb.2020.00123.

Formato M, Cimmino G, Brahmi-Chendouh N, Piccolella S, Pacifico S. 2022. Polyphenols for livestock feed: sustainable perspectives for animal husbandry? Molecules 27 (22): 7752. DOI: 10.3390/molecules27227752.

Francisco AE, Santos-Silva JM, Portugal APV, Alves SP, Bessa RJB. 2019. Relationship between rumen ciliate protozoa and biohydrogenation fatty acid profile in rumen and meat of lambs. PLoS One 14: 1-21. DOI: 10.1371/journal.pone.0221996.

Freitas JE, Takiya CS, Del Valle TA, Barletta RV, Venturelli BC, Vendramini THA, Mingoti RD, Calomeni GD, Gardinal R, Gandra JR, Bettero VP, Ferreira de Jesus EF, Oliveira MDS, Rennó FP. 2018. Ruminal biohydrogenation and abomasal flow of fatty acids in lactating cows fed diets supplemented with soybean oil, whole

soybeans, or calcium salts of fatty acids. J Dairy Sci 101 (9): 7881-7891. DOI: 10.3168/jds.2017-13666.
Gadeyne F, De Neve N, Vlaeminck B, Fievez V. 2017. State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods. Eu J Lipid Sci Technol 119 (5): 1-22. DOI: 10.1002/ejlt.201600345.
Gadeyne F, Van Ranst G, Vlaeminck B, Vossen E, Van Der Meeren P, Fievez V. 2015. Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover. Food Chem 171: 241-250. DOI: 10.1016/j.foodchem.2014.08.109.
Gebreyowhans S, Lu J, Zhang S, Pang X, Lv J. 2019. Dietary enrichment of milk and dairy products with n-3 fatty acids: a review. Intl Dairy J 97: 158-166. DOI: 10.1016/j.idairyj.2019.05.011.
Hassan FU, Arshad MA, Ebeid HM, Rehman MS, Khan MS, Shahid S, Yang C. 2020. Phytogenic additives can modulate rumen microbiome to mediate fermentation kinetics and methanogenesis through exploiting diet-microbe interaction. Front Vet Sci 7: 575801. DOI: 10.3389/fvets.2020.575801.
Henderson G, Cox F, Ganesh S, Jonker A, Young W, Global Rumen Census Collaborators, Janssen PH. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci Rep 5: 14567. DOI: 10.1038/srep14567.
Hidayah N, Suharti S, Wiryawan KG. 2014. In vitro rumen fermentation of ration supplemented with protected vegetable oils. Media Peternakan 37 (2): 129-135. DOI: 10.5398/medpet.2014.37.2.129. [Indonesian]
Huda AN, Sumitro AM, Hanifah NA, Aprodita LRB, Lana RB, Dja'far SM, Aprilia RM, Sabarudin A, Soetanto H. 2022. Antiprotozoal properties of potato peels and linseed oil and their effect on in vitro gas production. IOP Conf Ser: Earth Environ Sci 977 (1): 1-8. DOI: 10.1088/1755-1315/977/1/012128.
Ibrahim NA, Alimon AR, Yaakub H, Samsudin AA, Candyrine SCL, Mohamed WNW, Noh AM, Fuat MA, Mookiah S. 2021. Effects of vegetable oil supplementation on rumen fermentation and microbial population in ruminant: a review. Trop Anim Health Prod 53: 1-11. DOI: 10.1007/s11250-021-02863-4.
Jafari S, Meng GY, Rajion MA, Jahromi MF, Ebrahimi M. 2016. Manipulation of rumen microbial fermentation by polyphenol rich solvent fractions from papaya leaf to reduce green-house gas methane and biohydrogenation of C18 PUFA. J Agric Food Chem 64 (22): 4522-4530. DOI: 10.1021/acs.jafc.6b00846.
Jukanti A. 2017. Polyphenol Oxidases (PPOs) in Plants. Springer Nature Singapore Pte Ltd, Singapore. DOI: 10.1007/978-981-10-5747-2.
Kallas Z, Realini CE, Gil JM. 2014. Health information impact on the relative importance of beef attributes including its enrichment with polyunsaturated fatty acids (omega-3 and conjugated linoleic acid). Meat Sci 97 (4): 497-503. DOI: 10.1016/j.meatsci.2014.03.015.
Kholif AE, Olafadehan OA. 2022. Dietary strategies to enrich milk with healthy fatty acids - a review. Ann Anim Sci 22 (2): 523-536. DOI: 10.2478/aoas-2021-0058.
Kliem KE, Reynolds CK, Humphries DJ, Kirkland RM, Barratt CES, Livingstone KM, Givens DI. 2013. Incremental effect of a calcium salt of cis-monounsaturated fatty acids supplement on milk fatty acid composition in cows fed maize silage-based diets. J Dairy Sci 96 (5): 3211-3221. DOI: 10.3168/jds.2012-6211.
Lanier JS, Corl BA. 2015. Challenges in enriching milk fat with polyunsaturated fatty acids. J Anim Sci Biotechnol 6: 1-9. DOI: 10.1186/s40104-015-0025-0.
Leng RA. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim Prod Sci 54 (5): 519-543. DOI: 10.1071/AN13381.
Lengowski MB, Zuber KHR, Witzig M, Möhring J, Boguhn J, Rodehutscord M. 2016. Changes in rumen microbial community composition during adaption to an in vitro system and the impact of different forages. PLOS ONE 11 (2): e0150115. DOI: 10.1371/journal.pone.0150115.
Li Y, Gao J, Lv J, Lambo MT, Wang Y, Wang L, Zhang Y. 2023. Replacing soybean meal with high-oil pumpkin seed cake in the diet of lactating Holstein dairy cows modulated rumen bacteria and milk fatty acid profile. J Dairy Sci 106 (3): 1803-1814. DOI: 10.3168/jds.2022-22503.
McSweeney PLH, Fox PF. 2020. Advanced Dairy Chemistry: Lipids, Fourth Edition Volume 2: Lipids. Springer Nature, New York. DOI: 10.1007/978-3-030-48686-0.
Morales R, Ungerfeld EM. 2015. Use of tannins to improve fatty acids profile of meat and milk quality in ruminants: a review. Chilean J Agric Res 75 (2): 239-248. DOI: 10.4067/S0718-58392015000200014.
Moumen A, Azizi G, Chekroun KB, Baghour M. 2016. The effects of livestock methane emission on the global warming: a review. Intl J Global Warm 9 (2): 229-253. DOI: 10.1504/IJGW.2016.074956.
Newbold CJ, de la Fuente G, Belanche A, Ramos-Morales E, McEwan NR. 2015. The role of ciliate protozoa in the rumen. Front Microbiol 6: 1313. DOI: 10.3389/fmicb.2015.01313.
Nguyen SH, Nguyen HDT, Hegarty RS. 2020. Defaunation and its impacts on ruminal fermentation, enteric methane production and animal productivity. Livest Res Rural Dev 32 (4).
Ostermann AI, Müller M, Willenberg I, Schebb NH. 2014. Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography - a comparison of different derivatization and extraction procedures. Prostaglandin Leukot Essent Fat Acid 91 (6): 235-241. DOI: 10.1016/j.plefa.2014.10.002.
Panadare D, Rathod VK. 2018. Extraction and purification of polyphenol oxidase: a review. Biocatal Agric Biotechnol 14: 431-437. DOI: 10.1016/j.bcab.2018.03.010.
Patel S, Ambalam P. 2018. Role of rumen protozoa: metabolic and fibrolytic. Adv Biotechnol Microbiol 10 (4): 79-84. DOI: 10.19080/AIBM.2018.10.555793.
Ribeiro WR, Vinolo MAR, Calixto LA, Ferreira CM. 2018. Use of gas chromatography to quantify short chain fatty acids in the serum, colonic luminal content and feces of mice. Bio-Protocol 8 (22): e3089. DOI: 10.21769/BioProtoc.3089.
Soetanto H. 2019. Ilmu Pengantar Nutrisi Ternak Ruminansia. UB Press, Malang. [Indonesian]
Sudarwati H, Nasir MH, Nurgiartiningsih VMA. 2019. Statistika dan Rancangan Percobaan Penerapan dalam Bidang Peternakan. UB Press, Malang. [Indonesian]
Tilley JMA, Terry RA. 1963. A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci 18 (2): 104-111. DOI: 10.1111/j.1365-2494.1963.tb00335.x.
Vargas JE, Andrés S, Snelling TJ, López-Ferreras L, Yáñez-Ruíz DR, García-Estrada C, López S. 2017. Effect of sunflower and marine oils on ruminal microbiota, in vitro fermentation and digesta fatty acid profile. Front Microbiol 8: 1124. DOI: 10.3389/fmicb.2017.01124.
Vasta V, Daghio M, Cappucci A, Buccioni A, Serra A, Viti C, Mele M. 2019. Invited review: plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: experimental evidence and methodological approaches. J Dairy Sci 102 (5): 3781-3804. DOI: 10.3168/jds.2018-14985.
Wang L, Dong B, Yang T, Zhang A, Hu X, Wang Z, Chang G, Chen G. 2022. Dietary linseed oil affects the polyunsaturated fatty acid and transcriptome profiles in the livers and breast muscles of ducks. Front Nutr 9: 1030712. DOI: 10.3389/fnut.2022.1030712.
Weatherly CA, Zhang Y, Smuts JP, Fan H, Xu C, Schug KA, Lang JC, Armstrong DW. 2016. Analysis of long-chain unsaturated fatty acids by ionic liquid gas chromatography. J Agric Food Chem 64 (6): 1422-1432. DOI: 10.1021/acs.jafc.5b05988.
Williams CL, Thomas BJ, McEwan NR, Rees Stevens PR, Creevey CJ, Huws SA. 2020. Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. Front Microbiol 11: 720. DOI: 10.3389/fmicb.2020.00720.
Wu J, Gao J, Chen H, Liu X, Cheng W, Ma X, Tong P. 2013. Purification and characterization of polyphenol oxidase from *Agaricus bisporus*. Intl J Food Prop 16 (7): 1483-1493. DOI: 10.1080/10942912.2011.595029.
Yuste S, Amanzougarene Z, de la Fuente G, de Vega A, Fondevila M. 2019. Rumen protozoal dynamics during the transition from milk/grass to high-concentrate based diet in beef calves as affected by the addition of tannins or medium-chain fatty acids. Anim Feed Sci Technol 257: 1-10. DOI: 10.1016/j.anifeedsci.2019.114273.
Zhao T, Ma Y, Qu Y, Luo H, Liu K, Zuo Z, Lu X. 2016. Effect of dietary oil sources on fatty acid composition of ruminal digesta and populations of specific bacteria involved in hydrogenation of 18-carbon unsaturated fatty acid in finishing lambs. Small Rum Res 144: 126-134. DOI: 10.1016/j.smallrumres.2016.06.012.
Zymon M, Strzetelski J, Skrzyński G. 2014. Aspects of appropriate feeding of cows for production of milk enriched in the fatty acids, EPA and DHA. A review. J Anim Feed Sci 23 (2): 109-116. DOI: 10.22358/jafs/65698/2014.