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[Veterinary World, EISSN: 2231-0916](#) RESEARCH ARTICLE [Available at www.veterinaryworld.org/Vol. 13/April-2020/8.pdf](#) Open Access [In vitro rumen biohydrogenation of unsaturated fatty acids](#) in tropical grass-legume rations Malik Makmur , Mardiaty Zain , Fauzia Agustin, Riesi Sriagtula and Ezi Masdia Putri [Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Kampus Limau Manis, Padang, West Sumatera, Indonesia](#). Corresponding author: Mardiaty Zain, [e-mail: mardiaty@ansci.unand.ac.id](mailto:mardiaty@ansci.unand.ac.id) Co-authors: MM: malikmakmur27@gmail.com, FA: fauziaagustin [@ansci.unand.ac.id](mailto:ansci.unand.ac.id), RS: riesisriagtula [@ansci.unand.ac.id](mailto:ansci.unand.ac.id), EMP: ezimasdia@gmail.com Received: 21-11-2019, Accepted: 02-03-2020, Published online: 12-04-2020 doi:

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Makmur M, Zain M, Agustin F, Sriagtula R, Putri EM (2020) [In vitro rumen biohydrogenation of unsaturated fatty acids](#) in tropical grass-legume rations, *Veterinary World*, 13(4): 661-668. Abstract Aim: [The aim of this study was to evaluate the effects of](#) various combinations [of](#) tropical grass-legume species in rations [on the biohydrogenation](#) (BH) activity [of unsaturated fatty acids](#) (FAs), C18:0 composition, and fermentation profile in an in vitro rumen system. Materials and Methods: Samples of the following five fodder plants were used: One species of grass (*Pennisetum purpureum*) and four species of [tree legumes](#) (*Leucaena leucocephala*, *Gliricidia sepium*, *Calliandra calothyrsus*, and *Indigofera zollingeriana*). The following eight experimental diets were evaluated: 50% *P. purpureum* + 50% *L. leucocephala* (LL I); 50% *P. purpureum* + 50% *G. sepium* (GS I); 50% *P. purpureum* + 50% *C. calothyrsus* (CC I); 50% *P. purpureum* + 50% *I. zollingeriana* (IZ I); 75% *P. purpureum* + 25% *L. leucocephala* (LL II); 75% *P. purpureum* + 25% *G. sepium* (GS II); 75% *P. purpureum* + 25% *C. calothyrsus* (CC II); and 75% *P. purpureum* + 25% *I. zollingeriana* (IZ II). Each ration was replicated 3 times. In vitro rumen incubation was performed for 48 h, according to the Tilley and Terry method. Determination of the FA profiles of the forage materials and rumen fluid samples was performed using gas chromatography. Results: The percentage of polyunsaturated FA (PUFA) in the forage materials ranged from 34.18% (*P. purpureum*) to 74.51% (*C. calothyrsus*). The percentage of monounsaturated FA (MUFA) ranged from 5.06% (*P. purpureum*) to 8.71% (*L. leucocephala*). The percentage of saturated FA (SFA) was the lowest at 19.12% (*C. calothyrsus*) and highest at 60.76% (*P. purpureum*). In vitro BH of [C18:3 n-3](#), [C18:2 n-6](#), [C18:1 n-9](#), and [C18](#) PUFA in the experimental diets ranged from 72% to 100%. The BH of C18:1 n-9 in GS I (80%) and IZ I (72%) [was significantly different \(p<0.05\)](#). The percentage [of](#) C18:0 was 10-50% and [significantly different \(p<0.05\)](#) among treatments, with [the](#) highest [\(of 50%\) in](#) GS II. [No significant differences \(p>0.05\) were observed in the](#) fermentation parameters (pH, total volatile FAs, [in vitro dry matter digestibility, and in](#)

[vitro organic matter digestibility](#)) among [the](#) treatments, except in NH<sub>3</sub> concentration ( $p < 0.05$ ). Conclusion: The various combinations of tropical legumes do not have significant inhibitory effects on the BH of [C18:2 n-6](#), [C18:3 n-3](#), and C18 PUFA [after in vitro incubation](#) for 48 h. Furthermore, an increase in the tropical legume ratio in the ration tends to suppress C18:0 formation after the fermentation simulation process. IZ I has the potential to reduce C18:1 n-9 (MUFA) disappearance and yield an ideal rumen fermentation profile. Keywords: biohydrogenation, in vitro rumen, tropical grass-legume rations, unsaturated fatty acid. Introduction Currently, the main challenge in ruminant nutrition research is to derive food products from ruminants with fatty acid (FA) profiles that have acceptable health standards. Unsaturated FA (UFA) groups, such as polyunsaturated FAs (PUFAs) and monounsaturated FAs (MUFAs), for example, linoleic acid ([C18:3 n-3](#)), linoleic acid ([C18:2 n-6](#)), oleic acid ([C18:1 n-9](#)), and conjugated linoleic acids (cis-9 Copyright: Makmur, [et al. Open Access. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License \(http://creativecommons.org/licenses/by/4.0/\)](#), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver ([http://creativecommons.org/publicdomain/zero/1.0/](#)) applies to the data made available in this article, unless otherwise stated. trans-11 C18:2,)) have medical effects and are main targets for the effective deposition of FAs in meat and dairy products [1,2]. In ruminants, FA metabolism in the rumen is initiated by lipolysis (which liberates the lipid fraction in the feed into free FA), followed by biohydrogenation (BH), performed by two dominant microbial groups, namely, *Butyrivibrio fibrisolvens* and *B. proteoclasticus*, to transform most UFA into saturated FAs (SFAs), especially stearic acid (C18:0), which promotes degenerative diseases in humans [3]. The manipulation of rumen BH using a forage-based ration approach elucidates two important points: (1) UFA concentrations in tropical forage species and (2) presence of BH reduction agents in the form of plant secondary metabolites. Forage is the main source of FA intake for ruminants because it has a high UFA composition (>50% FA) in chloroplast lipids, thus, has a beneficial FA profile at the level of body tissue and lipid distribution in ruminants, in addition to the reduction of volatile components and minimization of off-flavor in meat [4-6]. In addition, the presence of plant secondary metabolites, such as phenol, tannin, flavonoid, and saponin components, suppresses the activity of rumen BH at various stages of isomerization and saturation through the modulation of the rumen microbiota [7-9]. This increases the preservation of UFA and accumulation of BH intermediate metabolites in the rumen digesta. The [previous in vitro and in vivo studies](#) have indicated [that the](#) phenol and tannin contents in forage are natural indicators of the potential of a feed crop as a BH reduction agent and the anti-methanogenic activity of tropical legumes [10-12]. Thus, tropical legume compositions in the ration are important for sustainable, inexpensive, and adaptable strategies for the protection of UFA from rumen degradation. However, [there is limited information on the effects of](#) tropical grass-legume levels on the ration and on the BH of UFA. [The aim of this study was to evaluate the effects of](#) various combinations [of](#) tropical grass-legume species in rations on UFA BH activity, C18:0 composition, and fermentation profile in an in vitro system. Materials and Methods Ethical approval In this experiment, we did

not use live animals; thus, ethical approval is not required. Study area, forage materials, and nutrition analysis Plant species samples were collected from the pasture region ( $\pm 25$  ha) of UPT Teaching Farm, [Faculty of Animal Science, Andalas University, Padang, Indonesia](#). The geographical position of the area is  $0^{\circ}54'30''S$  and  $100^{\circ}27'48''E$  at an elevation of 250 m above sea level. The average rainfall is  $>4000$  mm/year and the climate type is wet. The soil profile in the area around the pasture is Ultisol type. Samples of five fodder plants were collected: One species of grass (*Pennisetum purpureum*) and four species of tree legumes (*Leucaena leucocephala*, *Gliricidia sepium*, *Calliandra calothyrsus*, and *Indigofera zollingeriana*) that had entered the generative phase. These plant species are used extensively in forage rations for ruminants by farmers in Indonesia. For tree legumes, leaves, flowers, and soft stems (edible parts) were sampled and as much as 3 kg of fresh material was used. [After collection, the samples were maintained indoors at 28 °C for 24 h, oven-dried at 60°C for 3 h, and milled to pass through a 1 mm sieve. Then, the samples were placed in an airtight plastic bag and stored in a refrigerator at 4°C.](#) The determination of the nutritive value of forage samples (Table-1) [\(crude protein, ether extract, and crude fiber contents\)](#) was carried out using proximate analysis [13]. The analysis of [neutral detergent fiber, acid detergent fiber, and lignin contents](#) followed the protocols [of Goering and Van Soest](#) [14]. The measurement of the gross energy of plant samples was performed using a bomb calorimeter [15]. The quantification of plant secondary metabolites (total phenols, tannins, and saponins) was performed according to the procedure of Makkar [16] and Hiai et al. [17] using a UV-visible U-1800-5930482 spectrophotometer (High-Technologies Corporation). Animals and in vitro method Rumen liquor from Kacang crossbred goats (body weight,  $\pm 20$  kg) was used as the fermentation medium. The goats were obtained from a slaughterhouse in Padang City. Before slaughter, the goats were fed roughage ad libitum. The incubation procedure was performed in vitro for 48 h according to the Tilley and Terry method [18]. The following combinations of the grass-legume species were used for in vitro experimental ration I: 50% *P. purpureum* + 50% *L. leucocephala* (LL I); 50% *P. purpureum* + 50% *G. sepium* (GS I); 50% *P. purpureum* + 50% *C. calothyrsus* (CC I); and 50% *P. purpureum* + 50% *I. zollingeriana* (IZ I). The following combinations were used for in vitro experimental ration II: 75% *P. purpureum* + 25% *L. leucocephala* (LL II); 75% *P. purpureum* + 25% *G. sepium* (GS II); 75% *P. purpureum* + 25% *C. calothyrsus* (CC II); and 75% *P. purpureum* + 25% *I. zollingeriana* (IZ II). Each treatment was replicated 3 times. The main reason for selecting *P. purpureum* is that the species has a higher content of BH reduction agents (total phenols and tannins) than other tropical grass species [6]. Next, 2.5 g of the experimental diet was weighed and placed in a 250 ml fermenter tube. Then, [50 ml of rumen fluid and 200 ml of McDougall buffer solution \(9.8 g of NaHCO<sub>3</sub>, 9.3 g of Na<sub>2</sub>HPO<sub>4</sub>, 12 H<sub>2</sub>O,](#)

Material	Crude protein	Ether extract	Energy	Crude fiber	Ash	NDF	ADF	Lignin	Cellulose	Total phenolic	Tannin	Saponin																																																											
7.02	25.15	2.51	4.80	37.63	42.58	34.12	15.24	8.18	8.56	64.01	31.63	34.19	23.39	3.48	9.14	28.36	14.85	1.98	2.65	0.94	1.15	0.00	2.2	25.20	28.46	3.96	4.11	40.90	42.81	13.09	11.50	9.07	7.04	35.73	50.72	22.47	31.45	6.68	12.96	15.24	16.98	1.11	0.66	0.19	0.16	0.00	1.78	31.90	3.64	40.76	9.95	9.43	21.91	8.37	1.62	6.09	2.46	1.13	2.5	4.31	0.37	0.93	4.42	0.41	7.43	4.51	2.02	3.56	0.38	0.22	0.54

Ag/100 g dry matter. Bkcal/kg. SEM=Standard error of the mean

[0.57 g of KCl](#), [0.47 g of NaCl](#), [0.06 g of MgSO<sub>4</sub>·7H<sub>2</sub>O](#), and [0.04 g of CaCl<sub>2</sub>·2H<sub>2</sub>O](#) per liter of distilled water) were poured into the fermenter tube (1:4 ratio), CO<sub>2</sub> gas was injected into the tube for 30 s, and the tube was sealed with a rubber cap. The blank was prepared by adding only rumen fluid to three fermenter tubes. To obtain the FA profile at 0 h, we used three 50 ml plastic tubes filled with rumen stored at 0°C. Each fermenter tube was [placed in a shaker bath at 39°C](#) and constantly rotated [at 100 rpm](#) for [48 h](#). Then, the fermenter tubes were immersed in ice water to stop the fermentation process. The rubber cap was removed to measure the [pH of the rumen fluid](#) with [a pH meter](#) and the fermented liquid [was centrifuged at 1000 g for 5 min](#). [The supernatant was collected for FA determination](#), [total volatile FAs \(VFAs\) was measured using the distillation method](#) [19], and NH<sub>3</sub> production was measured using the microdiffusion method [20]. The sediment was filtered using Whatman paper no. 41 [and dried in an oven at 60°C for 24 h](#) to calculate the [digestibility of dry matter and organic](#) feed in vitro [according to the procedures of the Association of Official Analytical Chemists \(AOAC\)](#) [21]. FA determination FA methyl ester (FAME) standard solution was used (Sigma-Aldrich, Inc., USA) to determine the composition of plant FA in rumen fluid. Then, the fat content in the samples was determined as follows: (1) Direct extraction with the Soxhlet apparatus for solid samples and (2) extraction from liquid samples using the hydrolysis method. The fat extract was prepared for the methylation process (cold extraction). The procedures for making standard solutions, sample preparation (determination of fat content), and extraction of sample fat for methylation were in accordance with the procedures for FAME by the American Oil Chemists' Society [22] and AOAC [21]. FAME was injected into the gas chromatography (GC) instrument at an injector temperature of 225°C. The quantification of the FA component in the sample was performed using GC Agilent 7890B (Agilent Technologies, Inc., USA) [equipped with a capillary column](#) of 100 [m×0.25 mm×0.2 μm](#) (Supelco SPTM 2560). FAs were detected in the column using [a flame ionization detector](#) at 240°C. [The carrier gas used was N<sub>2</sub>, with a flow rate of 18.0 cm/s at a 1:100 split ratio](#). [The fraction and form of FA \(cis, trans, SFA, MUFA, and PUFA\) were determined by adjusting the retention time of methyl FA to the FAME standard, according to the protocol of Ratnayake et al.](#) [23]. The FA concentration is expressed as the percentage of total FA. Rumen BH calculations The in vitro rumen BH activity was calculated by measuring the differences between FA components at the end of incubation (48 h) and before incubation (0 h), according to the methods of Jayanegara et al. [11] and Jafari et al. [24]: BH [C18:3 n-3 \(%\) = \[\(C18:3 n-30h - C18:3 n-348h\) / C18:3 n-30h\] × 100](#) BH [C18:2 n-6 \(%\) = \[\(C18:2 n-60h - C18:2 n-648h\) / C18:2 n-60h\] × 100](#) BH [C18:1 n-9 \(%\) = \[\(C18:1 n-90h - C18:1 n-948h\) / C18:1 n-90h\] × 100](#) BH PUFA (%) = [(PUFA<sub>0h</sub> - PUFA<sub>48h</sub>) / PUFA<sub>0h</sub>] × 100 Specifically, the composition of C18:0 (48 h), the main end-product of the overall metabolism of UFA (lipolysis and BH) in the rumen, was determined using the following formula: C18:0 (%) = [C18:0<sub>48h</sub> / total C18 FA<sub>48h</sub>] × 100 In this study, we also aimed to detect intermediate BH FAs, namely, trans-C18:2 n-6; however, this intermediate metabolite was not detected after in vitro incubation for 48 h (limit of detection, 0.00164%). Statistical analysis The study design was completely randomized. The BH activity data and fermentation parameters were evaluated in JASP version 0.9.2 (University of Amsterdam, Amsterdam, Netherlands) [25]. If the treatments were significantly different ([p<0.05](#)), [Fisher's least significant difference test](#) was performed. Results FA profiles in



the forage material The total concentration of FA (% ether extract) in the tropical forage ranged from 2.37% (P. purpureum) to 4.45% (G. sepium), with an average of 3.90% (Table-2). The percentage of PUFA in the forage material ranged from 34.18% (P. purpureum) to 74.51% (C. calothyrsus). A high percentage of PUFA was also observed in I. zollingeriana, G. sepium, and L. leucocephala. The percentage of MUFA ranged from 5.06% (P. purpureum) to 8.71% (L. leucocephala). In addition, the percentage of SFA was the lowest at 19.12% (C. calothyrsus) and highest at 60.76% (P. purpureum). The highest [proportion of C18:3 n-3](#) (>70% total FA) was observed [in](#) C. calothyrsus, followed by I. zollingeriana. The lowest [proportion of C18:3 n-3](#) was observed [in](#) L. leucocephala [and](#) P. purpureum. [The highest](#) proportion [of C18: 2 n- 6 was detected in](#) C. calothyrsus and L. leucocephala and the lowest in G. sepium and P. purpureum. Furthermore, the highest [proportion of C18: 0 was observed in](#) G. sepium and L. leucocephala and the lowest in C. calothyrsus. BH of FAs For C18:3 n-3, all experimental diets showed a BH rate of up to 100% (Figure-1). For [C18:2 n-6](#), [C18:1 n-9](#), and C18 PUFA, [the](#) BH rates [were](#) 97-99%, 72-95%, and 94-99%, respectively. For the appearance [Table-2: Fatty acid composition of](#) forage materials for in vitro fermentation. Fatty acids Forage materials SEM

	P. purpureum	L. leucocephala	G. sepium	C. calothyrsus	I. zollingeriana
C14:0A	1.69	2.35	22.78	19.53	4.64
C16:0A	8.00	5.06	8.47	12.66	13.65
C18:0A	20.25	36.24	6.75	2.35	60.76
C18:1n-9A	41.41	5.06	8.71	34.18	49.88
C18:2n-6A	2.37	4.25	1.12	0.25	21.12
C18:3n-3A	6.37	8.76	2.70	4.94	4.66
C20:0A	11.69	20.83	43.15	53.68	2.47
SFAA	1.96	39.33	19.12	5.17	6.37
MUFAA	55.73	74.51	4.45	4.08	1.37
PUFAA	17.16	4.12	4.35	15.33	47.83
Total FAB	1.83	32.27	4.35	63.16	4.37
FAB	0.01	0.12	0.06	0.04	0.09
A% total FA	0.31	0.01	0.18	0.04	0.39
B% ether extract	0.39	0.39			

[SEM=Standard error of the mean, P. purpureum=](#) Pennisetum purpureum, [L. leucocephala=](#) Leucaena leucocephala, [G. sepium=](#) Gliricidia sepium, [C. calothyrsus=](#) Calliandra calothyrsus, [I. zollingeriana=](#) Indigofera zollingeriana

Figure-1: Biohydrogenation activity (%) among experimental diets. LL I=50% Pennisetum purpureum + 50% Leucaena leucocephala; LL II=75% P. purpureum + 25% L. leucocephala; GS I=50% P. purpureum + 50% Gliricidia sepium; GS II=75% P. purpureum + 25% G. sepium; IZ I=50% P. purpureum + 50% I. zollingeriana; IZ II=75% P. purpureum + 25% I. zollingeriana; CC I=50% P. purpureum + 50% Calliandra calothyrsus; CC II=75% P. purpureum + 25% C. calothyrsus. Different superscript (a,b) indicate [significant differences \(p<0.05\)](#). of C18:0, [no significant difference was](#) observed (p>0.05); a BH rate of 10-50% was observed. For the appearance of C18:0 (Figure-2), the GS II treatment ration was recorded to have the highest BH (50%), followed by CC II (48%). The lowest occurrence of C18:0 (10%) was in the LL I ration, followed by the GS I (17%). [No significant difference was observed](#) (p>0.05) [for the disappearance of](#) C18:2 n -6, with [the](#) lowest loss observed in IZ I and GS I; each showed a BH rate of 97%. This trend was consistent with the BH rate of C18:1 n-9 in GS I (80%) and IZ I (72%), which showed [a significant difference \(p<0.05\)](#) among all groups. [The](#) appearance [of](#) trans-C18:2 n-6 (FA intermediate) in 48 h was 0%. BH was lowest when the IZ I ration containing 50% I. zollingeriana was used, except when the GS I ration, containing 50% G. sepium, was used. The production of C18:0 was the highest when the rations containing 75% P. purpureum, namely, GS II and CC II, were used. Rumen fermentation profile After 48 h of incubation, the tropical grass-legume rations showed favorable rumen fermentation characteristics (Table-3). No significant differences, except for NH<sub>3</sub> production, were observed in all treat-

ments. The pH ranged from 6.7 to 7 and the ideal pH and total VFA values were achieved in the IZ II (7) and IZ I (6.9). NH<sub>3</sub> production (mmol) was significantly different ( $p < 0.05$ ) among the experimental diets, and the best production was achieved using IZ I (19.7) and GS I (18.6), followed by IZ II (15.4). The in vitro digestibility parameters showed no significant differences ( $p > 0.05$ ) among treatments. The in vitro dry matter digestibility (IVDMD) was in the range of 57.2-67.5% and was achieved using GS II, IZ II, and LL II. The in vitro organic matter digestibility (IVOMD) ranged from 56.2% to 67.2%, and the best rations were GS II, IZ I, and IZ II. IVDMD and IVOMD were the lowest in CC I. Table-3: Rumen fermentation profile of experimental diets after 48 h incubation. Parameters Experimental diets SEM

	LL I	LL II	GS I	GS II	IZ I	IZ II	CC I	CC II	pH	Total VFA (mmol)	NH <sub>3</sub> (mmol)	IVDMD (%)	IVOMD (%)
	6.8	6.9	118.3	111.7	12.5a	13.5b	61.0	65.7	60.7	65.4	6.9	6.7	112.5
	99.2	18.6b,c	14.6b	59.1	67.5	60.3	67.3	6.9	7.0	120.0	125.8	19.7c	15.4b,c
	64.9	66.6	67.2	66.9	6.8	6.8	114.2	117.8	11.2a	11.5a	57.2	63.1	56.2
	62.1	0.03	2.76	1.11	1.31	1.44	LL I=50% P. purpureum + 50% L. leucocephala; LL II=75% P. purpureum + 25% L. leucocephala; GS I=50% P. purpureum + 50% G. sepium; GS II=75% P. purpureum + 25% G. sepium; IZ I=50% P. purpureum + 50% I. zollingeriana; IZ II=75% P. purpureum + 25% I. zollingeriana; CC I=50% P. purpureum + 50% C. calothyrsus; CC II=75% P. purpureum + 25% C. calothyrsus. <u>IVDMD=In vitro dry matter digestibility. IVOMD=In vitro organic matter digestibility.</u>						

Different superscript (a, b, c) indicate significant differences ( $p < 0.05$ ). SEM= Standard error of the mean. P. purpureum= Pennisetum purpureum, L. leucocephala= Leucaena leucocephala, G. sepium=Gliricidia sepium, C. calothyrsus=Calliandra calothyrsus, I. zollingeriana=Indigofera zollingeriana

Figure-2: C18:0 composition of rumen fluid after 48 h incubation (% total C18 FA). LL I=50% Pennisetum purpureum + 50% Leucaena leucocephala; LL II=75% P. purpureum + 25% L. leucocephala; GS I=50% P. purpureum + 50% Gliricidia sepium; GS II=75% P. purpureum + 25% G. sepium; IZ I=50% P. purpureum + 50% Indigofera zollingeriana; IZ II=75% P. purpureum + 25% I. zollingeriana; CC I=50% P. purpureum + 50% Calliandra calothyrsus; CC II=75% P. purpureum + 25% C. calothyrsus. Different superscript (a, b) indicate significant differences ( $p < 0.05$ ).

Discussion Currently, it is still a challenge to deliver beneficial FAs efficiently from ruminants to livestock products. Various approaches have been used to reduce FA metabolism and SFA intake and form an FA profile that conforms to health standards. The use of forage as the main source of PUFA for livestock and its ability to reduce BH activity through the antimicrobial activity of its phenol components makes this approach cheaper, easier to implement, and more sustainable than alternative methods. Tropical legume species are promising feed ingredients because of the ability of plants, through the de novo synthesis of long-chain FAs, to produce a dominant proportion of C18: 3 n- 3 and C18:2 n-6, followed by a small portion of C18: 1 n-9 [26]. In addition, tropical legume species contain variable secondary metabolites, such as condensed tannins, hydrolyzable tannins, total phenols, polyphenol oxidase (PPO) enzymes, essential oils, and saponins, capable of modifying BH at various stages [2,9]. In this study, the differences in tropical forage species affected the FA component levels (% total FA) in their edible parts. In general, the level of C18:3 n-3 was the highest, followed by C16:0 and C18:2 n-6. C. calothyrsus legume species had a better FA profile than the other species. The superiority of tropical legume species over grass with respect to the FA profile was also observed. The only type of tropical grass used was P. purpureum and, in this

grass, C16:0 (22.78%) was the main FA, followed by [C18:3 n-3](#) (20.25%) and [C18:2 n-6](#) (12.66%). In this study, the FA composition was similar to that reported by Khan et al. [27], who measured the composition and FA content in 12 tropical forage species at various growth stages (early, medium, and late). They observed that the average FA composition (% total FA) in tropical forage is C18:3 n-3 (53%), [C16:0](#) (22%), and [C18:2 n-6](#) (14%) and FA content is 8.65, 3.61, and 2.38 g/kg dry matter, respectively. Jayanegara et al. [11] also detected C18:3 n-3 content in *L. leucocephala* and *Paspalum* grass (367 and 278 g/kg FAME, respectively). However, these results contradict those of a meta-analysis study by Glasser et al. [28], who reported that the addition of more legume species than grass species reduces the content of C18:3 n-3 in sub-tropical pastures. This can occur because of the genetic factors of each group of plants, which have different formation performances for long-chain FAs; legume species tend to have more dominant proportions of PUFA, especially C18:3 n-3 FA, than other plant types. This is supported by a comparative study of legume species, forbs, and grass-clover mixtures that showed that legume plant species contain C18:3 n-3 FA and the highest concentrations of FA compared to the other tested plant and ration groups [29]. In addition to plant species, the composition of forage FA is influenced by factors such as: (i) Cultivars, (ii) growth stage at harvest, (iii) feed preservation treatment, and (iv) nitrogen fertilizer application [28,30]. The results of the 48 h incubation on the FA metabolism of the grass-legume experimental diet showed that the BH level of each UFA was different. This may have been caused by the fact that the unsaturation level of the FA is positively correlated with rumen BH activity [31]. The C18:3 n-3 disappearance rates reached 100% and, regarding C18:2 n-6, up to 99%. This result is attributable to rumen microbial preferences for different BH processes for each component of an FA in the diet. An in vitro 24 h study conducted by Lejonklev et al. [32] showed that a high rate of BH for UFA ([C18:3 n-3](#) and [C18:2 n-6](#)) is positively correlated with the incubation period. *G. sepium* (GS I) and *I. zollingeriana* (IZ I) legumes at a 50% ratio in the experimental diet showed a significant reduction in the BH of [C18:1 n-9](#). The results of a study by Van Ranst et al. [33] showed that in the 24 h in vitro incubation of perennial ryegrass, red clover silage (50:50) provides the protective abilities of PUFAs ([C18:2 n-6](#) and [C18:3 n-3](#), with a BH level of 69.9% and 75.4%, respectively). This is related to the activity of the enzyme PPO, which can encapsulate lipids in plants and inhibit the first step of lipid metabolism in ruminants (lipolysis). PPO is in the sub-tropical legume species red clover (*Trifolium repens*) [34]. In tropical legumes, the total phenol content plays an important role in the modification of the metabolism of rumen FAs. Jayanegara et al. [11] suggested that the total phenol content in *Persea americana* leaves is 73 g/kg dry matter and these leaves are able to change the BH pattern and increase PUFA bypass and the production of [cis-9 trans-11 C18:2](#), or conjugated linoleic acid, which has health effects. However, the total phenol content in the tropical forage species was lower than that able to modulate BH [11]. In this study, the appearance of C18:0 was not different from that in the previous studies on the use of tropical and sub-tropical plants as BH reduction agents [11,35]. In vitro studies have consistently shown that phenols reduce the accumulation of [C18:0](#) and increase [trans-11 C18:2](#) in the rumen digesta [9]. However, in this study, trans-C18:2 was not detected in the 48 h period. This might have been because no intermediate FAs were available after the BH of UFA, resulting in stearic acid as the final product. This deduction is supported by Jafari et al.

[24], who performed 24 h in vitro experiments using goat rumen fluid and *Carica papaya* leaves as the source of polyphenols and detected the production of intermediate BH FAs (% of identified FA) such as [trans-11 C18:1](#) (10- 14%), [cis-9 trans-11 C18:2](#) (4-6%), and [trans- 10 cis- 12 C18:2](#) (<2%). Supplementation of the ration with tropical legumes results in appropriate fermentation in the rumen. In this study, the best pH, total VFA, and NH<sub>3</sub> values were achieved using the rations with *I. zollingeriana* (IZ I and IZ II). In this study, the pH range (6.9 to 7) of the rumen fluid on the basis of the *I. zollingeriana* (freshly cut) ration was at an ideal level compared to the pH ranges reported by the previous studies that used feed preservation technology in the form of *Indigofera* silage and pellet [36,37]. The NH<sub>3</sub> production differed significantly ( $p < 0.05$ ) among groups and was highest in IZ I. This indicates that the inclusion of *I. zollingeriana* at a high proportion provided sufficient organic material for rumen microbes, increased the fermentability profile, and initiated an increase in the rate of the synthesis of rumen microbial proteins. Putri et al. [38] showed the superiority of *I. zollingeriana* over some tropical forage species (*P. purpureum*, *L. leucocephala*, and *G. sepium*); NH<sub>3</sub> and total VFA contents were 36.23 mM and 162.17 mM, respectively, at pH 6.85. The IVDMD and IVOMD values showed that digestibility was observed when the legume-based diet of *G. sepium* and *I. zollingeriana* was used. Suharlina et al. [39] reported significantly different digestibility (dry and organic matter) results for *I. zollingeriana*, *G. sepium*, and *Medicago sativa*, with the highest digestibility rates (IVDMD, 68.92%; IVOMD, >65%) achieved using *I. zollingeriana*. Conclusion The various combinations of tropical legumes do not have a significant inhibitory effect on the BH of [C18:2 n-6](#), [C18:3 n-3](#), and C18 PUFA [after in vitro incubation](#) for 48 h. Furthermore, an increase in the tropical legume ratio in the ration tends to suppress C18:0 formation after the fermentation simulation process. IZ I has the potential to reduce C18:1 n-9 (MUFA) disappearance and yield an ideal rumen fermentation profile. Authors' Contributions MM, MZ, FA, RS, and EMP formulated the experimental design and did experimental work at the laboratory. MM drafted the manuscript. EMP did data analysis under the guidance of MZ, FA, and RS. All the [authors read and approved the final](#) version of the [manuscript](#). Acknowledgments This study [was supported by](#) Basic Research Scheme by DRPM, [Ministry of Technology Research and Higher Education of Indonesia](#) 2019 with contract number T/36 [/UN.16.17/PT.01.03/PD-PP/2019](#). This research would not have been possible without the technical assistance of the staff [in the Laboratory of Ruminant Nutrition, Faculty of Animal Science](#) of [Andalas University](#). [Competing Interests](#) The authors [declare that they have no competing interests](#). [Publisher's Note](#) Veterinary World [remains neutral with regard to jurisdictional claims in published institutional](#) affiliation. References 1. Anjo, E. (2015) Grazing increases the unsaturated fatty acid concentration of milk from grass-fed cows: A review of the contributing factors, challenges and future perspectives. *Eur. J. Lipid Sci. Technol.* 117(9): 1345-1369. 2. Toral, P., Monahan, F., Hervás, G., Frutos, P. and Moloney, A. (2018) Review: Modulating ruminal lipid metabolism to improve the fatty acid composition of meat and milk. Challenges and opportunities. *Animal*, 12(S2): S272-S281. 3. Parodi, P.W. (2016). Dietary guidelines for saturated fatty acids are not supported by the evidence. *Int. Dairy J.*, 52: 115-123. 4. Fruet, A., Trombetta, F., Stefanello, F., Speroni, C.S., Donadel, J., Souza, A.N.M., Júnior, R., Tonetto, C.J., Wagner, R., De Mello, A. and Nörnerberg, J. (2018) Effects of feeding legume-grass pasture and different concentrate levels on fatty acid profile, volatile compounds, and

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