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Fatty Acid Biohydrogenation, Fermentation, and Digestibility of Ration Containing Napier and King Grass with Different Harvest Ages and Altitudes: *In Vitro* Study

D. Anzhany[a], T. Toharmat[b,*], Despal[b], & A. Łozicki[c]
[a]Study Program Nutrition and Feed Science, Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University
[b]Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University
Jalan Agatis, Kampus IPB Dramaga Bogor 16680, West Java, Indonesia
[c]Division of Animal Nutrition, Institute of Animal Sciences, Warsaw University of Life Sciences, Ciszewskiego 8, 02-786, Warsaw, Poland
*Corresponding author: ttoharmat61@gmail.com
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ABSTRACT

Forage is the primary and cheapest source of fatty acids (FA), which includes conjugated linoleic acid (CLA), influencing milk FA. This study aimed to analyze the fermentation, digestibility, biohydrogenation, nutrient composition, and FA content of napier grass (NG) and king grass (KG). Grasses were collected from the Pangalengan (highland) and Dramaga (lowland) districts at three harvest ages (1, 1.5, and 2 months). The feed was then analyzed for nutrients and FA. An *in vitro* study was performed to analyze the concentrations of NH_3, VFA, protozoa populations, and biohydrogenation. No significant differences were observed in protozoa, pH, total VFA, or FA biohydrogenation. NH_3 ranged from 5.31 mM to 8.86 mM. Significant differences were found at different altitudes, with an interaction between grass type and harvest age and an interaction between the three factors. The highest NH_3 concentration was found in rations containing highland NG at 1.5 months. The DMD value was 58.27%–64.39%, and OMD was 61.07%–67.18%. Different digestibility values were observed at different harvest ages, with an interaction between altitude and harvest age. This aligned with the CF, NDF, and lignin contents in grasses. The highest was at 1.5 months NG. Significant differences were observed in the relative proportions of propionic acids. The highest value was in the ration containing the 1.5-month highland NG. Rations containing KG yielded significantly higher amounts of the C18:0 and C18:1 trans. In conclusion, the 1.5-month highland NG is a potential ration for supporting healthier FA production in milk.

Keywords: altitude; biohydrogenation products; fatty acid; grass; in vitro

INTRODUCTION

Immense population growth has contributed to the increasing national food demand. To fulfill the food demand, the government considered the food diversification program to be a food development focus on national food challenges. Food diversification encourages food availability and the quality of food consumption to be more diverse, nutritious, and balanced. Following the increasing public awareness of the importance of eating healthy food, functional foods are being considered. Functional foods are novel foods formulated to incorporate substances or live microorganisms that have possible health-enhancing or disease-preventing value at a safe and sufficiently high concentration to achieve the intended benefit (Temple, 2022).

One functional food compound is conjugated linoleic acid (CLA), derived from animals and included in the category of fatty acids (FAs). CLA is an anticancer compound found in animal products, with the highest content in milk (Duchemin *et al.*, 2013; Chen & Park, 2019). The average CLA content in ruminant milk is 0.06%–2.96% (Zongo *et al.*, 2021), and high levels in milk may increase its bioavailability (Duchemin *et al.*, 2013). Therefore, the recommended daily consumption is approximately 3 g of dietary CLA for a 70 kg person (Zongo *et al.*, 2021). CLA compounds in ruminant milk are unsaturated fatty acids produced through the activity of Δ9-desaturase in the mammary gland as well as isomerization and biohydrogenation of unsaturated fatty acids by bacteria in the rumen (Serafeimidou *et al.*, 2013). However, external factors, such as the feeding system, geography of the farm, seasonal variation in forage, and ambient temperature, strongly influence milk CLA (Serafeimidou *et al.*, 2013).

As a major component of ruminant rations, forage is the primary and cheapest source of FA in ruminant feed (Conte *et al.*, 2017). A high level of n3 FA was reported in grass compared to concentrate, contributing to the higher CLA levels in ruminant milk feed from low-input systems and natural pastures (Zongo *et al.*,

2021). Feeding sufficient fiber may prevent the negative effects of reduced rumen pH caused by common CLA-enhanced ration. Forages are essential in the synthesis of short- and long-chain FAs. In the rumen, forage FAs are degraded via lipolysis and biohydrogenation. Some FAs are desaturated in the mammary gland depending on their composition and affinity for the Δ9-desaturase enzyme (Lourenço *et al.,* 2005). Therefore, variations in the FA composition of forage also affect the FA produced in milk.

Variations in forage FAs can be caused by season, maturity, and species (Khan *et al.,* 2015; Hayashi *et al.,* 2021). Grass FA predominantly comprises three FAs: C16:0, C18:2 cis, and C18:3 n3 (Elgersma, 2015). C18:2 cis and C18:3 n3 in the rumen may be metabolized, deposited, or secreted as ruminant products (Owens & Basalan, 2016). The resulting by-product can be in the form of CLA or the CLA precursor C18:1 trans. Differences in the structure and position of the double bonds in different FA isomers result in different biological activities, and the trans10, cis12, CLA, and C18:1 trans10 isomers are related to milk fat depression (MFD).

Although forage plays an important role in FA synthesis in milk, biohydrogenation and FA profiles in CLA-enhanced rations have not been extensively discussed. The common grasses for dairy cows in Indonesia are napier grass (NG/*Pennisetum purpureum*) and king grass (KG/*Pennisetum purpuphoides*). Both grasses have a high yield, are adaptable to various soil types, and can be easily propagated (Vidal *et al.,* 2017; Hendarto & Setyaningrum, 2022). Both grasses have the potency to produce a high dry matter yield (DMY). For example, NG produces DMY 29.5 tons/ha (Vidal *et al.,* 2017), and KG produces DMY 12.663 tons/ha (Hendarto & Setyaningrum, 2022). A previous study found the best feeding system by traditional farmers in Indonesia that produces high CLA content in milk as well as high ration digestibility (Anzhany *et al.,* 2022). Modification of the previous ration is required to enhance the CLA ratio with the least negative effect by selecting the potential forages used in the ration. Therefore, this study aimed to analyze the differences in ruminal fermentation, digestibility, and FA biohydrogenation of rations comprising NG and KG of different harvest ages from lowland and highland regions.

MATERIALS AND METHODS

Feed Preparation

Feed comprising forage grass and cooperative concentrates was used. Grasses and concentrates were obtained from Pangalengan District (984–1571 masl), Bandung Regency and Dramaga District (142–200 masl), Bogor Regency. The two grass species were *Pennisetum purpureum* (NG) and *Pennisetum purpuphoides* (KG) (Table 1). Each grass species was harvested at three different harvest ages: 1, 1.5, and 2 months. Fresh grass was dried in the sun for 4–5 d. The grass was then dried at 60 °C for 3 h. Each grass and the dried concentrate were finely ground and then mixed into a ration with a 40:60 forage: concentrate ratio.

Analysis of Nutrition Content

The rations were analyzed for crude protein (CP), ash, crude fiber (CF), and ether extract (EE) (Table 2). All analyses were performed using a rapid FT-NIR Spectrometer Solids Cell (BUCHI; NIRFlex N-500, Switzerland), except for ether extract (EE). EE analysis was conducted according to the AOAC (2005) using a high-temperature EV-16 Gerhardt Instrument (Germany).

Analysis of FA of the Ration

The FA analysis of the rations (Table 3) consisted of three stages: extraction, methylation, and injection. Extractions were performed using the AOAC (2005) method for EE analysis. The ration sample (2 g) was weighed and wrapped in fat-free cotton and filter paper to form a cylindrical sleeve. The samples were hot extracted using a Soxhlet extractor with hexane. The extraction process required six reflux cycles. After removing excess hexane in the flask, the flask was heated at 102 ± 2 °C for 4 h.

Methylation was initiated by weighing the extracted lipids (30 mg) in Hach tubes. Methylation was carried out using AOAC Official Method 969.33 (2000). The lipids were methylated using 1 mL of 0.5 N NaOH in methanol and heated for 20 min. Upon reaching room temperature, 2 mL of 20% BF3 in methanol was added to the sample, which was heated again for 20 min. Finally, after reaching room temperature (25 °C), 2 mL of saturated NaCl and 1 mL of isooctane were added to the sample, which was homogenized by vortexing for 2 min. The top layer containing FA methyl ester (FAME) was carefully transferred into a vial tube and stored at -20 °C for further analysis.

FA was identified by injecting FAME into a gas chromatograph (GC-7820A; Agilent Technologies). FAME (1 μL) was injected into the GC using column CP-Sil 88 fused-silica capillary columns (100 m length × 0.25 mm diameter (i.d), and film thickness 0.20 μm; Agilent Technologies). The temperature of the injector and detector was 260 °C with a split ratio of 50:1. The analysis was performed in the 32 psi constant pressure mode, with hydrogen as the carrier gas (40 mL/min), helium as the makeup gas (25 mL/min), and unpurified air (400 mL/min). The initial temperature was 100 °C maintained for 5 min, then increased to 180 °C at 8 °C increase/min maintained for 9 min, and increased to 230 °C at 1 °C increase/min maintained for 15 min. The FA profile was identified by comparison of retention times using an authentic standard (Supelco 37 Component FAME Mix; CRM47885; Sigma-Aldrich).

In Vitro Study

The rations were tested for fermentability and digestibility *in vitro*, as described by Tilley & Terry (1963). The ration (0.5 g) was mixed with 40 mL of McDougall buffer and 10 mL of rumen fluid in a 100 mL fermenter tube. Rumen fluid was collected from two fistulated male Fristien-Holstein bulls. The cattle

Table 1. Nutrient composition of napier and king grass

Composition of nutrient (%DM)	King grass						Napier grass						SEM
	Lowland			Highland			Lowland			Highland			
	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	
Ash	13.86	15.01	10.53	15.79	14.31	10.58	14.22	12.90	12.61	14.48	16.17	11.41	0.55
CP	12.01	12.75	11.29	12.82	12.53	11.19	12.33	11.99	11.90	12.66	13.22	11.82	0.18
CF	31.15	28.45	27.94	28.38	30.67	31.20	30.78	28.65	28.88	28.23	29.94	31.55	0.39
NDF	68.96	65.11	62.47	61.74	64.00	67.18	65.96	65.04	64.75	63.76	63.21	70.06	0.73
ADF	40.96	38.83	34.5	37.63	38.60	38.35	39.00	37.83	36.83	37.97	39.75	40.72	0.50
Hemicellulose	25.58	22.69	26.55	21.08	23.91	27.17	26.59	24.61	24.95	22.99	20.95	26.92	0.63
Cellulose	32.09	29.83	27.75	28.78	30.70	31.51	29.89	30.20	28.07	28.31	30.18	32.98	0.47
Lignin	5.03	4.64	4.13	4.65	4.80	4.90	4.73	4.15	4.53	4.57	5.17	4.80	0.09
Silica	1.17	1.61	1.11	1.62	1.71	0.78	1.24	1.34	0.56	1.54	1.33	0.84	0.11
EE[^]	2.74	2.41	3.34	2.99	3.75	3.73	4.12	5.06	5.59	4.71	6.76	5.23	0.37
NFE*	40.24	41.38	46.90	40.02	38.74	43.30	38.55	41.4	41.02	39.92	33.91	39.99	0.88
TDN*	51.70	51.88	54.96	51.43	51.95	54.03	51.95	53.58	53.80	52.74	51.61	53.89	0.35

Note: CP= crude protein, CF= crude fiber, NDF= neutral detergent fiber, ADF= acid detergent fiber, EE= ether extract, *NFE= nitrogen-free extract, calculated using the following formula: NFE= 100 (%Ash + %CP + %EE + %CF); TDN= total digestible nutrients, according to Wardeh (1981); mo= month(s).

Table 2. Nutrients composition of the ration containing napier and king grass that comes from different altitudes and harvest ages

Treatments			Composition of nutrients (%DM)				
Type of grass	Altitude	Harvest ages (Month)	EE	CP	Ash	CF	NFE*
KG	Lowland	1	3.25	12.22	9.92	18.81	55.80
		1.5	3.79	14.29	10.26	16.65	55.01
		2	3.80	13.31	8.05	18.10	56.74
	Highland	1	4.42	13.69	8.88	16.67	56.34
		1.5	4.00	12.94	8.41	17.51	57.14
		2	3.34	12.54	8.11	18.80	57.21
NG	Lowland	1	3.29	12.49	10.64	18.34	55.24
		1.5	3.39	13.24	9.24	18.19	55.94
		2	3.17	12.53	11.17	18.09	55.04
	Highland	1	3.14	12.78	11.47	17.96	54.65
		1.5	3.53	13.76	11.26	16.99	54.46
		2	3.27	13.08	9.37	18.45	55.83
SEM			0.11	0.18	0.36	0.22	0.27

Note: ΣN Highland= 6 samples, ΣN Lowland= 6 samples, ΣN EG= 6 samples, ΣN KG= 6 samples. DM= dry matter, CP= crude protein, CF= crude fiber, EE= ether extract, *NFE= nitrogen-free extract, calculated using the following formula: NFE= 100 – (%Ash + %CP + %EE + %CF), NG= napier grass, KG= king grass.

were housed and cared for according to the guidelines of the Animal Ethics Committee of IPB University under contract no. 047/KEH/SKE/XI/2021. Rumen fluid was collected in the morning before feeding. The rumen fluid was conditioned to remain anaerobic using flowing CO_2 gas. The mixture of rumen fluid, buffer solution, and feed was incubated at 39 °C for 4 h for fermentability analysis and 48 h for digestibility analysis. After 4 h of incubation, samples were removed, and 1 mL of rumen fluid was collected under flowing CO_2 gas and mixed with 1 mL of TBFS solution for use in the protozoan population analysis. Incubation samples of up to 10 mL and H_2SO_4 were added for the relative proportion volatile FA (VFA) analysis. pH was measured using a Hanna pH meter. The remaining incubated sample was dripped with an $HgCl_2$ solution and centrifuged at 3500 rpm for 10 min to separate the residue from the supernatant. The supernatants were used for ammonia (NH_3) and total VFA concentration analysis. The residue from the 48-h incubation sample was mixed with a pepsin HCl solution and re-incubated for 48 h. After 48 h, the sample was screened with Whatman No 40-filter paper and heated at 105 °C for 24 h to calculate dry matter digestibility (DMD), and the sample was further heated at 600 °C for 4 h to calculate organic matter digestibility (OMD).

A 4-h incubation supernatant was used to analyze NH_3 concentration using the Conway microdilution method. Total VFA was determined using the steam distillation method. Total NH_3 and VFA analyses were performed according to the General Laboratory Procedures (1966). Partial VFA analysis was performed using gas chromatography as described by Cottyn & Boucque (1968). A 10 mL incubation sample was mixed with 1 mL of 95%–97% H_2SO_4. One milliliter of the sample mixture was added to 0.0003 g of sulfosalicylate dihydrate. The resulting mixture was then centrifuged at 12,000 rpm for 10 min at 7 °C. The samples were prepared for identification using gas chromatography. The total protozoan population was calculated based

Table 3. Fatty acids composition of the ration containing napier and king grass that comes from different altitudes and harvest ages

FA profile (% / 100% ration fat)	King grass						Napier grass						SEM
	Lowland			Highland			Lowland			Highland			
	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	1 mo	1.5 mo	2 mo	
C4:0	0.16	0.06	0.16	Nd	0.10	0.6	0.19	0.22	nd	nd	0.52	nd	0.058
C6:0	0.08	nd	nd	0.10	nd	nd	0.19	nd	nd	nd	Nd	0.51	0.044
C8:0	0.24	0.08	0.42	0.33	0.18	0.7	0.41	0.09	0.06	nd	0.07	nd	0.062
C10:0	0.56	0.23	1.25	3.68	0.65	0.35	0.80	0.35	0.27	0.26	0.26	0.16	0.282
C11:0	0.56	0.43	1.32	0.61	0.80	0.37	0.90	0.21	0.45	0.18	0.11	0.31	0.101
C12:0	27.66	2.63	0.20	0.19	0.18	34.9	0.18	0.16	0.21	0.09	1.52	0.45	3.485
C13:0	0.03	0.11	0.06	0.12	0.38	0.24	0.25	0.36	0.32	0.39	0.27	1.48	0.110
C14:0	5.49	0.77	0.13	0.15	0.22	5.47	0.43	0.35	0.14	0.23	0.11	0.11	0.588
C14:1	0.24	5.53	8.67	4.75	0.05	0.30	7.71	8.72	7.00	0.83	6.40	5.56	0.978
C15:0	0.18	0.58	0.83	1.01	1.43	0.34	0.98	0.79	1.33	0.20	0.58	0.63	0.116
C15:1	0.27	8.55	0.43	0.28	0.29	0.31	0.30	13.38	10.69	10.78	9.50	8.78	1.542
C16:0	25.55	7.65	7.28	21.91	nd	20.44	3.84	3.18	2.25	2.28	3.29	2.11	2.586
C16:1	2.32	11.15	15.44	7.76	14.40	3.32	13.91	15.95	13.11	12.82	11.49	10.75	1.283
C17:0	0.19	0.06	0.14	0.15	0.06	0.12	1.78	1.92	1.63	nd	1.33	0.42	0.222
C17:1	2.41	13.30	18.54	8.51	17.07	3.82	15.85	2.42	15.20	14.47	12.94	12.15	1.653
C18:0	5.73	2.54	3.72	4.82	2.82	4.11	1.66	1.53	1.10	1.00	1.24	0.84	0.477
C18:1 trans	0.14	0.33	0.73	2.49	1.39	1.25	0.60	3.31	0.41	1.48	1.51	0.58	0.272
C18:1 cis	7.54	10.92	0.80	5.00	nd	3.25	3.14	3.71	2.34	2.62	nd	1.98	0.913
C18:2 trans	0.55	0.98	1.42	2.64	nd	1.14	2.00	2.22	1.75	0.37	1.46	2.28	0.238
C18:2 cis	6.70	0.77	1.06	4.54	14.34	0.98	1.99	1.07	2.40	8.45	1.52	7.37	1.214
C20:0	1.05	10.34	13.05	6.71	12.98	0.80	11.53	13.40	9.92	9.95	9.12	8.94	1.222
C18:3 n6	1.64	1.03	1.30	0.68	4.98	0.27	1.00	2.86	2.82	1.32	3.40	0.51	0.407
C20:1	0.31	0.49	1.56	0.59	1.68	0.60	0.65	0.40	0.45	1.15	1.29	0.73	0.136
C18:3 n3 (ALA)	0.13	3.85	4.13	1.43	5.48	1.20	3.57	5.13	3.71	3.61	4.26	2.29	0.471
C21:0	0.16	7.23	9.05	4.68	9.07	2.34	8.13	9.86	6.84	7.06	6.78	6.50	0.821
C20:2	4.88	0.42	1.35	4.07	0.67	4.33	0.67	1.30	1.08	1.00	0.56	1.15	0.469
C22:0	0.67	0.74	0.39	3.86	0.27	1.94	0.62	0.34	0.12	5.67	0.12	5.87	0.625
C20:3 n6	1.47	0.44	1.02	1.05	0.61	0.38	1.94	0.62	0.74	0.59	15.83	2.14	1.247
C22:1 n9	0.10	0.15	0.40	0.50	0.19	0.52	0.66	0.91	0.46	0.08	0.22	0.49	0.073
C20:3 n3	0.12	0.36	0.93	0.45	1.10	0.11	0.53	0.79	0.92	0.96	0.64	1.27	0.108
C20:4 n6	0.33	1.41	1.25	1.30	1.23	0.58	1.46	1.86	1.61	1.53	1.69	1.85	0.135
C23:0	0.16	4.52	0.11	0.15	4.49	1.33	4.73	0.51	3.88	3.44	0.42	3.64	0.570
C22:2-n6	nd	0.51	0.70	0.42	0.92	0.67	0.66	0.78	0.53	0.54	0.50	1.52	0.103
C24:0	0.63	0.32	0.47	0.67	0.50	0.43	0.37	0.47	0.17	0.16	0.36	0.36	0.045
C20:5 n3 (EPA)	0.80	0.77	0.64	3.78	0.67	2.06	5.77	0.39	5.57	5.59	0.33	5.27	0.679
C24:1	0.57	0.28	0.37	0.36	0.50	0.45	0.36	0.17	0.26	0.73	0.12	0.98	0.070
C22:6 (DHA)	0.37	0.46	0.68	0.27	0.31	nd	0.21	0.28	0.26	0.18	0.27	nd	0.053
SFA	69.11	38.3	38.59	49.13	34.11	74.48	37.01	33.73	28.69	30.91	26.08	32.35	4.487
UFA	30.89	61.7	61.41	50.87	65.89	25.52	62.99	66.27	71.31	69.09	73.92	67.65	4.487
PUFA	16.99	11.00	14.47	20.64	30.32	11.72	19.80	17.29	21.39	24.14	30.46	25.65	1.872
MUFA	13.90	50.70	46.93	30.23	35.56	13.80	43.19	48.98	49.92	44.95	43.46	42.00	3.746
PUFA/MUFA	0.55	0.18	0.24	0.41	0.46	0.46	0.31	0.26	0.30	0.35	0.41	0.38	0.031

Note: nd= not detected in this analysis. SFA= Saturated fatty acid, UFA= Unsaturated fatty acid, PUFA= Polyunsaturated fatty acid, MUFA= Monounsaturated fatty acid, PUFA/MUFA= the ratio of Polyunsaturated fatty acid and Monounsaturated fatty acid, mo= month(s).

on the coloring and dilution method of Ogimoto & Imai (1981), using a counting chamber at 40× magnification.

Biohydrogenation of Ruminal FAs

Biohydrogenation of feed was carried out *in vitro* by incubating feed in a mixture of rumen fluid and McDougall buffer, according to the method described by Tilley & Terry (Tilley & Terry, 1963). The FA profile was measured in rations incubated for 0 h and 48 h. The samples were immediately removed, dried with $HgCl_2$, and soaked in an ice bath to prepare them for FA analysis. Unanalyzed incubation samples were stored in the freezer at -20 °C.

The preparation, extraction, and methylation of the FA incubation ratios were performed according to the method described by Vargas & Angel (2021). The incubation samples were prepared in powder form or lyophilized using a freezer dryer. Lyophilized content samples (50 mg) were transferred into a new screw cap tube. Samples were extracted and methylated by mining 2148 µL methanol, 990 µL toluene, 66 µL sulfuric acid, 1000 µL dimethyl sulfoxide (DMSO), and 2000 µL hexane. All materials used were pure chemicals in tubes. The tightly

closed tube was heated in a water bath of 80 °C for 2 h. After allowing it to stand at room temperature (25 °C), the hexane layer was transferred to a 2 mL Eppendorf tube. The hexane solution was evaporated using nitrogen flux. The remaining residue was dissolved using 500 µL of dichloromethane and homogenized using a vortex for 1 min. The residue-dichloromethane solution (250 µL) was transferred into chromatographic vials and stored in freezers at -20 °C until analysis.

FA analysis was performed by injecting one µL of sample into GC (GC-7820A, Agilent Technologies). The conditions of the GC settings were as follows: GC used a CP-Sil 88 fused-silica capillary column (100 m length × 0.25 mm diameter (i.d), and film thickness of 0.20 µm; Agilent Technologies). The injector and detector temperatures were 260 °C with a split ratio of 50:1. Analysis was performed in 32 psi constant pressure mode, with hydrogen as the carrier gas (40 mL/min), helium as the makeup gas (25 mL/min), and unpurified air (400 mL/min). The initial temperature was 100 °C maintained for 5 min, then increased to 180 °C at an 8 °C increase/min maintained for 9 min, and increased to 230 °C at a 1 °C increase/min maintained for 15 min. The FA profile was identified by comparison of retention times using an authentic standard (Supelco 37 Component FAME Mix; CRM47885; Sigma-Aldrich).

The calculation of biohydrogenation products was calculated using the following equation (Makmur *et al.*, 2020):

C18:3 n3 (%)= $[(C18:3\ n3_{0h} - C18:3\ n3_{48h})/C18:3\ n3_{0h}] \times 100\%$

C18:2 cis (%)= $[(C18:2\ cis_{0h} - C18:2\ cis_{48h})/C18:2\ cis_{0h}] \times 100\%$

C18:2 trans (%)= $[(C18:2\ trans_{0h} - C18:2\ trans_{48h})/C18:2\ trans_{0h}] \times 100\%$

C18:1 cis (%)= $[(C18:1\ cis_{0h} - C18:1\ cis_{48h})/C18:1\ cis_{0h}] \times 100\%$

PUFA (%)= $[(PUFA_{0h} - PUFA_{48h})/PUFA_{0h}] \times 100\%$

C18:0 (%)= $[C18:0_{48h}/\text{total C18 FA}_{48h}] \times 100\%$

C18:1 trans (%)= $[C18:1\ trans_{48h}/\text{total C18 FA}_{48h}] \times 100$

Statistical Analysis

The nutrient compositions of the grass and ration and the ration fatty acid composition used in this study had no sample replicates. Therefore, they could not be analyzed using analysis of variance (ANOVA). The fermentability and digestibility of the rations were analyzed by ANOVA with a factorial group randomized design, 12 treatments, duplo, and four blocks. These factors included grass type (NG and KG), location (lowland and highland), and harvest age (1, 1.5, and 2 months). Differences between treatments were further analyzed using Tukey's test. Data were analyzed using SPSS ver. 20.

RESULTS

Fermentability of Ration

The total protozoan population, incubation pH, NH_3 concentration, and VFA described the ration fermentability. No significant differences were observed in the total protozoan population, pH, or total VFA concentration. The results of the ration fermentation are shown in Table 4.

NH_3 concentrations for all treatments were in the 5.31–8.86 mM range and were classified as normal ranges. Significant differences ($p<0.05$) were found in the rations containing forage from different altitudes. The concentration of lowland NH_3 was lower than that of highland. There was also an interaction between the type of grass and harvest day and a significant interaction between the three factors. In NG, the youngest plants produced higher NH_3, whereas in KG, the youngest plants produced lower NH_3.

Digestibility of Ration

The digestibility percentages of the rations used in this study are listed in Table 5. The DMD was in the range of 58.27%–64.39%, and OMD was in the range of 61.07%–67.18%. Statistical analysis showed no significant differences between grass types. Differences in digestibility were observed between rations containing grass at different harvest ages, and there was an interaction between altitude and harvest age. Based on the interaction between harvest age and altitude, the lowest DMD and OMD were found in rations containing lowland grasses aged 1 month and highland grasses aged 2 months, respectively. The highest OMD was found in rations containing highland grasses aged 1 and 1.5 months, and lowland grasses aged 1.5 months. The highest DMD was found in highland grasses aged 1 and 1.5 months, and in lowlands at 1.5 and 2 months. Meanwhile, based on harvest age, the highest DMD was found at the age of 1.5 months and OMD at 1 and 1.5 months.

Relative Proportion of VFAs

The relative proportions of individual VFAs in this study were only conducted on one type of grass from one altitude, namely highland NG, at three different harvest ages. This was based on the highest digestibility of DMD and OMD, NH_3 concentration, EE content of the grasses, and FA profile of the ration. The relative proportions of VFAs in this study were 69%–72% acetic acid, 19%–21% propionic acid, 6%–9% butyric acid, and 2% valeric acid (Table 6). The propionic acid (C3) content was significantly different at different harvest ages. The highest C3 value was observed in the grass-containing rations harvested at 1.5 months of age.

FA Biohydrogenation of C18

Figure 1 shows a marked difference in the C18:0 biohydrogenation product and C18:1 trans in the rumens of rations containing different grass types. The production of C18:0 and C18:1 trans was the highest in the diet containing KG.

Different FA biohydrogenation patterns were observed for each ratio. No significant differences were observed in FA C18 or PUFA biohydrogenation. As

Table 4. *In vitro* fermentation indicators of ration containing napier and king grass that comes from different altitude and harvest ages

Treatment of grasses in the ration			Fermentability variables			
Type of grass	Altitude	Harvest ages (Month)	Protozoa (log cell/mL)	pH	NH_3 (mM)	Total VFA (mM)
King grass	Lowland	1	6.44	6.95	5.39^{a}	151.33
		1.5	6.47	6.95	6.16^{ab}	145.91
		2	6.40	6.95	7.24^{bcd}	163.60
	Highland	1	6.60	6.95	8.08^{cde}	169.69
		1.5	6.67	6.99	8.52^{def}	146.37
		2	6.44	6.95	8.38^{def}	156.41
Napier grass	Lowland	1	6.41	6.95	6.31^{ab}	161.17
		1.5	6.61	6.96	6.84^{bc}	154.40
		2	6.49	6.93	5.32^{a}	142.94
	Highland	1	6.54	6.94	9.58^{f}	149.92
		1.5	6.46	6.94	7.16^{bcd}	158.87
		2	6.52	6.94	8.86^{ef}	186.18
	Statistical analysis					
	p-value					
	Type		0.960	0.202	0.875	0.653
	Altitude		0.168	0.855	0.000	0.286
	Harvest ages		0.328	0.393	0.775	0.487
	Type*Altitude*Harvest ages		0.311	0.393	0.026	0.101
	Altitude*Harvest ages		0.543	0.874	0.119	0.635
	Type*Harvest ages		0.469	0.874	0.044	0.693
	Type*Altitude		0.211	0.361	0.630	0.580
	SEM		0.041	0.011	0.372	3.920

Note: Means in the same column with different superscripts differ significantly ($p<0.05$).

Table 5. *In vitro* digestibility indicators of ration containing napier and king grass that comes from different altitude and harvest ages

Treatment of grasses in the ration			Digestibility variables					
Harvest ages (Month)	Altitude	Type of grass	DMD (%)			OMD (%)		
			Mean	H	H*A	Mean	H	H*A
1	Lowland	KG	59.90	61.38^{ab}	59.69^{a}	63.17	64.52^{b}	63.14^{ab}
		NG	59.49			63.11		
	Highland	KG	63.02		63.06^{b}	65.85		65.91^{c}
		NG	63.09			65.98		
1.5	Lowland	KG	63.93	62.81^{b}	62.53^{b}	66.64	65.60^{b}	65.18^{c}
		NG	61.14			63.72		
	Highland	KG	61.79		63.09^{b}	64.84		66.01^{c}
		NG	64.39			67.18		
2	Lowland	KG	60.86	60.39^{a}	61.73^{b}	63.34	63.07^{a}	64.21^{bc}
		NG	62.60			65.09		
	Highland	KG	58.27		59.05^{a}	61.07		61.92^{a}
		NG	59.83			62.77		
	Statistical analysis							
	p-value							
	Type			0.473			0.381	
	Altitude			0.522			0.433	
	Harvest ages			0.014			0.003	
	Type*Altitude*Harvest ages			0.165			0.100	
	Altitude*Harvest ages			0.002			0.003	
	Type*Harvest ages			0.430			0.290	
	Type*Altitude			0.147			0.112	
	SEM			0.543			0.535	

Note: Means in the same column with different superscripts differ significantly ($p<0.05$). Mean= average data of 3 factors combination (harvest age, altitude, and type), H= average data of one factor (harvest age), H*A= average data of 2 factors combination (harvest age and altitude). DMD= dry matter digestibility, OMD= organic matter digestibility, NG= napier grass, KG= king grass.

shown in Figure 2, there were several FAs with values less than zero or negative (-).

DISCUSSION

Fermentability of Ration

The protozoan population was 10^6, which is classified as the normal range for protozoan populations (McDonald *et al.*, 2020). Protozoans also play an important role in methanogenesis. In rations with low forage quality, high fiber content encourages high methane production (Ku-Vera *et al.*, 2020). Several studies have been conducted to identify ruminal protozoa (Newbold *et al.*, 2015; Li *et al.*, 2018).

The final pH was in the range of 6.93–6.95. This value was classified as suitable for maintaining the stability of rumen conditions. Rumen stability can be calculated based on the rumen stability value (RSV) related to ration fiber content, cow age, milk yield, milk quality, and the feeding system used (NRC, 2001). Microorganisms preferentially utilize more digestible carbohydrates and delay fiber digestion until a more digestible substrate is fermented, thereby lowering the

Table 6. *In vitro* relative proportion of VFA of ration containing napier and king grass that comes from different altitude and harvest ages

Treatment of grasses in the ration			Relative proportion of VFA						
Altitude	Type of grass	Harvest ages (Month)	C2 (%)	C3 (%)	iC4 (%)	nC4 (%)	iC5 (%)	nC5 (%)	C2/C3 (%)
Highland	NG	1	71.54	18.51	0.60	7.46	0.92	0.98	2.69
		1.5	70.79	20.70	0.61	5.74	1.03	1.14	2.76
		2	68.82	19.90	0.66	8.52	0.96	1.16	2.86
	Statistical analysis								
	p-value: Harvest ages		0.443	0.096	0.789	0.555	0.264	0.126	0.929
	SEM		1.810	1.542	0.046	0.834	0.157	0.124	0.133

Note: C2= acetic acid, C3= propionic acid, iC4= iso-butyric acid, nC4= butyric acid, iC5= isovaleric acid, nC5= valeric acid, C2/C3= acetic acid/propionic acid ratio, NG= napier grass.

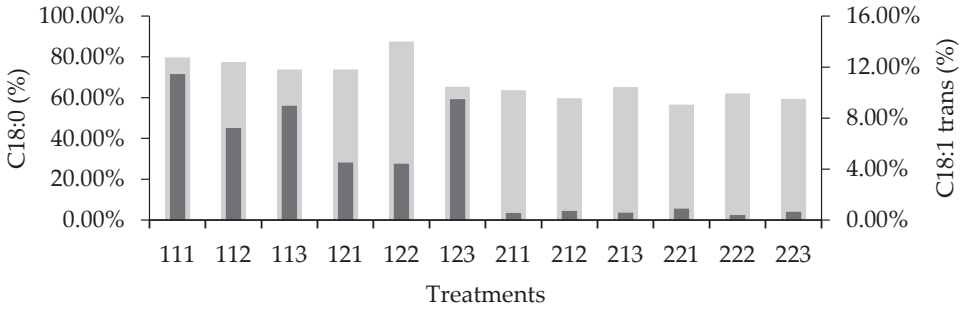

Figure 1. Biohydrogenation fatty acids products of C18:0 ( ) and C18:1 trans ( ) in the rumen of rations containing different grass types. 111= KG lowland 1 month; 112= KG lowland 1.5 months; 113= KG lowland 2 months; 121= KG highland 1 month; 122= KG highland 1.5 months; 123= KG highland 2 months; 211= NG lowland 1 month; 212= NG lowland 1.5 months; 213= NG lowland 2 months; 221= NG highland 1 month; 222= NG highland 1.5 months; 223= NG highland 2 months. KG= king grass; NG= napier grass.

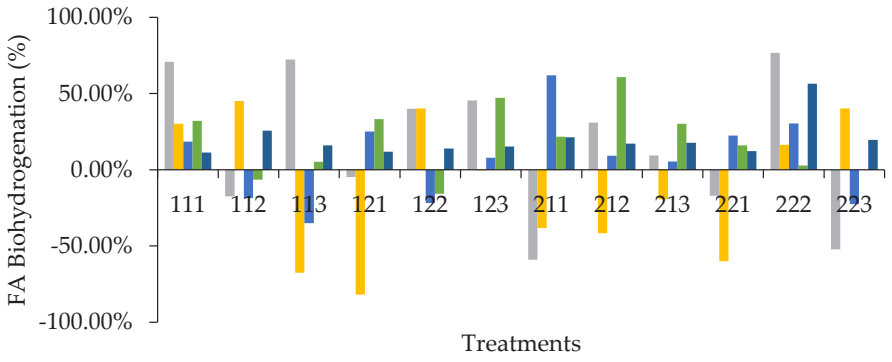

Figure 2. Fatty acids biohydrogenation of C18:1 cis ( ), C18:2 trans ( ), C18:2 cis ( ), C18:3 n3 ( ), PUFA ( ), C18:1 n9 in the rumen of rations containing different grass types. 111= KG lowland 1 month; 112= KG lowland 1.5 months; 113= KG lowland 2 months; 121= KG highland 1 month; 122= KG highland 1.5 months; 123= KG highland 2 months; 211= NG lowland 1 month; 212= NG lowland 1.5 months; 213= NG lowland 2 months; 221= NG highland 1 month; 222= NG highland 1.5 months; 223= NG highland 2 months. KG= king grass; NG= napier grass.

pH of the rumen (Mertens & Grant, 2020). Other studies have shown that rations containing high-quality forage are less affected by pH reduction than those containing low-quality forage. In this study, using a concentrate at 60% of the ratio did not affect the final pH of the incubation. In addition to pH, the proper quantity and physical form of fiber in dairy rations are important for maintaining normal milk fat percentages (Despal *et al.*, 2021).

Rumen fermentation has evolved to nourish the host animals. The main products of fermentation are VFAs, which are high-energy-potential compounds that are absorbed through the rumen wall or gastrointestinal tract and undergo further host metabolism (Weimer, 2022). The total VFA concentration in this study was in the range of 142.94–186.18 mM. This value is slightly higher than the normal range of total VFA, which is 70–150 mM (or equivalent to 5–10 g/L) (McDonald *et al.*, 2020). This may be due to the high concentration ratio used in this study. In this study, the forages: concentrate (F:C) ratio was 40:60. This may also be related to the NFE content in the diets. However, the ration with a similar F:C ratio reported a normal total VFA concentration, which was 132.23 mM (Anzhany *et al.*, 2022). The total VFA concentration directly describes the energy content of the forage (Zhang *et al.*, 2019). Volatile fatty acids (VFAs) are the primary energy source for dairy cows and other ruminants. VFAs account for approximately 70% of the total energy absorbed by ruminants (Tian *et al.*, 2022).

The usual range of rumen NH_3 concentrations is 85–300 mg/L (or equivalent to 4.99–17.61 mM) (McDonald *et al.*, 2020). Differences in planting location or altitude affect climatic and planting systems. Differences in location led to differences in rainfall, which affected CP content. The CP content of grass during the rainy season is higher than during the dry season (Hayashi *et al.*, 2021). Higher precipitation tends to prevent plants from maturing and promotes the accumulation of nutrients, resulting in higher CP content (Hayashi *et al.*, 2021). Protein degradation by ruminal microbes produces ammonia as an intermediate product (McDonald *et al.*, 2020). In this study, the differences in ration composition depended on the type of forage used. The CP content of the ration containing highland forage in this study was higher than that of the ration containing lowland forage.

The NH_3 differences between harvest ages might be correlated with nutrient composition. Nutrients in grasses might be affected by external soil fertility factors. This is because soil provides nutrients for grass absorption. The CF content of grass was affected by soil fertility. Grasses may devote more resources for rapid growth in nutrient-rich soils, producing less fibrous and more delicate plant materials. Poor soil fertility management can reduce forage yield and performance (Silveira & Kohmann, 2020). Maintaining soil fertility depends on the source, amount, and frequency of fertilizer application, which is primarily determined by target dry matter production. Lower sun intensity and lower to moderate temperatures in the highlands may prevent the maturity of grasses. In general, protein accumulation in grasses at moderate temperatures is higher than at high temperatures (Burgess & Huang, 2016).

Digestibility of Ration

Nutrient quality analysis and *in vitro* feed digestibility simulations could be a step toward evaluating rations, including forage. This value was slightly lower than the digestibility values in similar ratios, according to Anzhany *et al*. (2022), namely, DMD 77.95% and OMD 74.49%. The rations used in this study were modified from those used by Anzhany *et al*. (2022) to produce a CLA-enhancing ratio. However, the digestibility in this study was similar to the higher NG content reported by Riestanti *et al.* (2021), namely 63.82% DMD and 64.15% OMD with 58.28% NG, 33.62% concentrate, and 8.10% soybean curd. The differences in digestibility may be due to the differences in the rumen inoculum used. Forage digestibility can range from 45% to 85%, depending on the forage quality (NRC, 2001). Forage in this study was of lower quality with NDF in the 61.74%–70.06% range compared to the high-quality fodder Alfalfa, whose NDF ranged between 28.9% and 65.9% (Tucak *et al.*, 2021). Forages used in dairy feed have potentially degradable NDF ranging between 26% and 90% of the NDF (Harper & McNeill, 2015). However, the concentrate reaching 60% dry matter was considered to have increased the digestibility of the rations in this study.

The pattern of the highest digestibility was consistent with the pattern of CF content in the rations, where rations with the highest CF content had the lowest DMD and OMD values. Rations containing highland grasses showed a pattern of increasing CF content and decreasing CP content with age. Among rations containing lowland grasses, the ration containing grass that was 1 month of harvest age had the highest CF content and the lowest CP. Generally, younger ages at harvest contain higher CP and lower CF content owing to the higher leaf proportion and lack of reproductive stages. However, stress factors from environmental conditions, such as drought and nutrient deficiency in young grass, might reduce the capacity to produce and accumulate proteins, indirectly affecting fiber content. Dramaga, Bogor, as a lowland region, has higher rainfall than Pangalengan, but the highlands have a lower ambient temperature. It was presumed that the combination of poor soil fertility and high temperatures in lowland regions results in plant stress and thus may allocate more resources to defense mechanisms, reduce protein synthesis, and produce more fibrous material. The CF pattern in the lowlands and highlands aligned with the pattern of NDF and lignin content in the forage. NDF digestibility is negatively affected by lignin and linked to phenolic acids (Raffrenato *et al.*, 2017).

Nadeau *et al.* (2019) reported the influence of forage age on organic matter digestibility. The harvest age of forage is associated with its nutrient content. As grasses mature, structural carbohydrates increase, and CP decreases, which may reduce ruminal microbial protein synthesis (Mwangi *et al.*, 2022). In this study,

the effect of harvest age on nutrients in lowland grasses was inconsistent with the literature. Differences in forage nutrient quality can be caused by external factors such as nitrogen fertilizer application in the field, land fertility, and weather (Massey *et al.*, 2020; Silveira & Kohmann, 2020; Hayashi *et al.*, 2021).

Fermentability, especially that of fermentable organic matter, is correlated with rumen NH_3 (McDonald *et al.*, 2020). Increased nutrient digestibility is associated with the increased rumen microbial activity resulting from the increased rumen protein digestibility (RDP), which improves ruminant productivity (Putri *et al.*, 2021). Forages harvested early in the maturity phase have higher fiber and protein contents and fiber digestibility (Nadeau *et al.*, 2015). In addition to fiber, other factors affect nutrient digestibility. The cross-linking of phenolic compounds between cell walls has a more significant influence on NDF digestibility than on lignin content in grass (Raffrenato *et al.*, 2017). Polysaccharides in plant cell walls are partially cross-linked via phenolic compounds, which may decrease the microbial degradation of dietary fiber (Bunzel *et al.*, 2018).

Relative Proportion of VFAs

Feed is degraded and fermented by a complex consortium of ruminal microbes. Acetic content was slightly higher, while propionic and butyric acids were slightly lower than those reported in the literature on cattle-fed mature ryegrass and herbage with relative proportions of VFAs as follows: acetic acid 0.64; propionic acid 0.22; butyric acid 0.12; and others 0.03; and cattle-fed long hay and concentrates at a ratio of 0.4:0.6, was as follows: TVFA 96, acetic acid 0.61, propionic acid 0.18, butyric acid 0.13, and others 0.08 (McDonald *et al.*, 2020). This may have been caused by the nutrient contents of the ration and forage. The propionic acid concentration in this study showed the same pattern as the EE and CP content in the rations and lignin in the three forages. The presence of lignin can increase acetic acid levels and decrease propionic acid levels under anaerobic conditions (He *et al.*, 2022).

The results showed a significantly lower propionic acid content at 1 month compared to that at 1.5 and 2 months, where the highest lignin content was found at 1.5 months, followed by high CP. Both dry matter digestibility and organic matter were the highest in rations containing highland NG aged 1.5 months, which is consistent with the proportion of propionate. Propionic acid is a glucogenic acid that can be converted into glucose via gluconeogenesis (Zhang *et al.*, 2016). VFA production is directly related to DMD, where rations with high digestibility and quality result in higher VFA levels and vice versa (Rira *et al.*, 2015).

FA Biohydrogenation of C18

As shown in Table 3, the PUFA content of the diet containing NG was higher than that of the diet containing KG. Plant factors can manipulate the FA composition in the rumen and milk (Toral *et al.*, 2018). Feed fatty acid metabolism in the rumen occurs in two stages: lipolysis and biohydrogenation. The biohydrogenation process must occur because unsaturated FA has a greater negative impact on rumen microbes than saturated FA (Enjalbert *et al.*, 2017). The recovery of PUFAs from milk may be influenced by the content of PUFAs, polyphenols, and tannins and the flow rate associated with biohydrogenation intermediates in the rumen (Dewhurst *et al.*, 2006).

As shown in Table 3, higher C18:0 content was found in the rations containing KG. Dietary C18:3 n3 and C18:2 n6 are the main precursors of C18:3 n3 and cis9, trans11 CLA in milk. However, different mechanisms of lipolysis and biohydrogenation in the rumen can alter the concentrations of C18:3 n3 and CLA in milk. The first involves factors that affect lipolysis. The lipolysis of ester bonds is the first step in limiting lipid metabolism in the rumen (Harfoot & Hazlewood, 1997). Differences in the chemical composition of the diets affected the rate of lipolysis *in vitro*. Rations with high N and starch contents have a more extensive lipolysis rate (Dewhurst *et al.*, 2006).

As shown in Table 2, the average ratio of KG resulted in higher levels of protein and starch (NFE). Therefore, diets containing KG were considered to have a higher lipolysis rate, which affected the biohydrogenation of PUFAs, resulting in C18:0 biohydrogenation products and higher C18:1 trans. The low levels of C18:0 and C18:1 trans were presumed to be caused by partial biohydrogenation. Inhibition of the biohydrogenation process may be due to the phenol content of NG forage. *P. purpureum* (NG) has higher total phenol (1.98) and tannin (0.94) contents than *P. purpurhoides* (KG) (Makmur *et al.*, 2019). Inhibition of C18:3 n3 biohydrogenation affects biohydrogenation intermediates, decreasing C18:1 trans concentrations (Dewhurst *et al.*, 2006).

C18:0 was the dominant FA absorbed by lactating dairy cattle, which was 2.5 times higher than C16:0 that entered the small intestine (Loften *et al.*, 2014). Approximately 50% or more of the C18:1 in milk results from C18:0 desaturase activity in the udder glands (Enjalbert *et al.*, 2000). The desaturase index describes the desaturase activity. High index values are often accompanied by a decrease in milk fat depression (MFD) incidence (Rico & Harvatine, 2013). A higher FA profile of biohydrogenation at 48 h than at 0 h resulted in negative values (-). This refers to the biohydrogenation formula described in the Materials and Methods section.

CONCLUSION

The NG rations were better than KG based on the EE content and PUFA C18 profile. The NG rations produced higher fermentation and digestibility than the KG rations, especially those with grass aged 1.5 months. Bio-hydrogenated products that have a lower NG ratio have the potential to be further modified to improve the quality of milk FA, which is beneficial for health.

CONFLICT OF INTEREST

Despal serves as an editor of the Tropical Animal Science Journal but has no role in the decision to publish this article. We also certify no conflicts of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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