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Ration to Produce Milk High in Conjugated Linoleic Acid (CLA) at Smallholder Dairy Farm: An *In Vitro* Reconstruction

Dwitami Anzhany, Toto Toharmat and Despal

Department of Animal Nutrition and Feed Technology, Study Program Nutrition, and Feed Science, IPB University, Bogor, Indonesia

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Corresponding Author:
Toto Toharmat
Department of Animal Nutrition and Feed Technology, IPB University, Bogor, Indonesia
Email: ttoharmat61@gmail.com

Abstract: Smallholder dairy farms have shown the best practices for producing milk with high Conjugated Linoleic Acid (CLA). The rations need to be further investigated to explain the digestion process. This study aimed to reconstruct and evaluate the digestion process of five rations that produced milk with high CLA using *in vitro* method. The rations were collected from five traditional dairy farms (P1, P2, P3, P4, and P5) after screening 260 milk samples from 60 farms at different smallholder dairy cattle farms in West Java Province, Indonesia. Digestibility of the ration was tested using a two-stage *in vitro* method. The result shows that the five rations consist of different feeds and nutrients. The P4 was better than other rations of its simplicity, nutrient sufficiency, fermentation, digestibility, and microbial synthesis. The P4 ration consists of 40.27% Napier grass and 59.72% cooperative concentrates with the lowest crude fiber (10.20%) and the highest crude protein (12.63%). The high crude protein percentage was in line with the ammonia concentration. The P4 significantly produced the lowest molar proportion of butyrate (12.68%), the highest total digestible nutrient (59.9%), total VFA (132.23 mm), the *in vitro* dry matter and organic matter digestibility (77.95 and 74.49%), protozoa (6.31 log cell/mL) and with bacteria number 7.19 log CFU. It is concluded that a ration with 40% Napier grass and 60% concentrate is the best smallholder dairy cattle ration to produce high CLA milk.

Keywords: Dairy Ration, Milk CLA, Rumen Fermentation

Introduction

Smallholder dairy farmers accounted for 98% of dairy farming in Indonesia, with less than ten cows ownership. On average, it produced 14.9 L/head/d, lower than large-scale dairy cattle farms (28 L/head/d) (Despal *et al*., 2021a). Despite lower milk production, smallholder dairy cattle farms produced milk with higher milk components, especially milk fat, due to the negative correlation between milk production and components (Husvéth *et al*., 2010).

Milk fat is the most variable component in milk (Lestari and Abdullah, 2015; Nugroho *et al*., 2015; Zahera *et al*., 2015; Hasanah *et al*., 2020; Anzhany *et al*., 2021; Riestanti *et al*., 2021). The milk fat variation determines the variation in Total Solid (TS), which is one of the determining factors for the current milk price. However, increasing public awareness on eating healthy food beyond the nutrient function (functional food) increases the villain of fat as a harmful nutrient (Salles *et al*., 2019). Not all fatty acids are harmful to human health and require a detailed fatty acid profile to improve consumer confidence in consuming milk. Milk fatty acid profiles describe their impact on human health. They can be used to calculate the Milk-Fatty Acid health Index (MFAI) and used as price determinants (Despal *et al*., 2021b). Conjugated Linoleic Acid (CLA), a group of milk fatty acids, has been reported to have functional food characteristics that prevent atherosclerosis, cancer, diabetes, and control body weight (Jimenez *et al*., 2008).

Several factors influence milk fatty acids profile, including the CLA content in bovine milk. Among others are species, breeds feed, biohydrogenation process in the rumen, altitude, season, milk production, and animal comfort. Milk fatty acids from smallholder dairy farms in different altitudes, feeding, season, and milking time have been collected and analyzed using chemical or NIRS analysis (Martha *et al*., 2019; Anzhany *et al*., 2021; Despal *et al*., 2021a). Based on the nutrient composition in milk, both highlands and lowlands have milk quality that was not much different (Anzhany *et al*., 2021). However, traditional small-scale farms produced better milk components, primarily Unsaturated Fatty Acids (UFA) and CLA, than

large farms (Despal *et al*., 2021b). Milk fatty acids profiles between areas were more influenced by feed type used (Despal *et al*., 2021a) and cow's comfort. According to Moran (2005), cows produced less during heat stress or at Temperature-Humidity Index (THI) >78, which is in humid tropics like Indonesia is at temperature >21°C.

Smallholder dairy farms in Indonesia, by default, use many forages, especially in areas with a high land carrying capacity. The forage commonly used were Napier grass, natural grass, corn stover, rice straw, and corn husk (Despal *et al*., 2021b). The altitude and the local climate were predicted to play a role in determining the quality of forage used. Less sun exposure was thought to inhibit aging in plants that impact the lignification process and a relative percentage of other nutrients. It was reported that highlands and mountains tend to have forages with higher levels of Unsaturated Fatty Acids (UFA) and CLA (Collomb *et al*., 2002). Thus it encouraged the production of more CLA and UFA in milk. The use of tofu waste to overcome low-quality forage and concentrate was thought to have also contributed to the high CLA in milk. Tofu waste is a by-product of the tofu manufacturing industry with the primary raw material of soybeans. About 68% of fatty acid in whole soybeans was linoleic fatty acids (C18:2) (Ivanov *et al*., 2010).

High-producing cows require a high energy intake. Lack of energy in feed or Negative Energy Balance (NEB) encourages body fat storage mobilization in the form of Long-Chain Fatty Acids (LCFA). Mobilization of body fat reserves affected milk's fatty acid profile, especially MUFA such as C18:1, and cis-9 (Hanuš *et al*., 2018).

Although the milk fatty acids profile and CLA of several milk samples from different dairy cattle areas have been analyzed (Despal *et al*., 2021a), the feeding management to produce the highest milk CLA and its digestion process have not been studied. Therefore, this study aimed to study feeding management and reconstruct and evaluate the digestion process of five rations that produced milk with high CLA using *in vitro* method.

Materials and Methods

Respondents' Selection and Feeding Management Study

Five cows that produce the highest milk CLA have been chosen from 260 milk samples collected from different smallholder dairy cattle farming areas in the West Java Province of Indonesia (Anzhany *et al*., 2021). The 260 milk samples were collected three times with two weeks intervals to overcome seasonal variation. The samples were also collected from different altitudes to represent the dairy farming area in the West Java Province. The five cows belong to five farmers in the Pangalengan District of Bandung Regency. The five cows' feeding management and milk quality were studied for seven days to measure feed offered, feed refusal, milk production and components, and milk fatty acids.

Feeds Collection and Analysis

Forages offered were samples of as much as 1 kg/day/farm for seven days of the observation. The concentrate from each farm was collected as much as 3 kg on the last day of observation. The forages sample was dried under the open sun for at least seven days and continued with a K11755 Swallow (Made in the UK) oven at 60°C. The concentrates were dried directly in the oven at 60°C. The forage and concentrate were finely ground with a laboratory Ossel E-250G-V2 (made in China) milling machine equipped with a 1 mm sieve filter. Feeds refusal was collected and weighted. 10% of them were sampled, dried under the open sun, and continued in the oven at 60°C before being milled using the same procedure as the feed samples.

The feed sample and feed refusal were chemically analyzed following the AOAC (2005) to measure Dry Matter (DM), ash, Crude Proteins (CP), Ether Extract (EE), and Crude Fibers (CF). The CP analysis was conducted according to AOAC (2005) but with the following modifications. The amount of 0.25-0.3 g samples was weighed and put into the Kjehdahl flask along with 20 mL concentrated H_2SO_4 (95-97%) and 0.7 g selenium mixture. The sample destruction was conducted by heating the flask using a hot plate until the color changed into a clear solution. Then, the sample was moved, cooled down, and diluted in aqua dest with an 18 dilution factor. After cooling down, 5 mL of the diluted samples were added with 10 mL of 50% NaOH solution and distilled. Five ml of 2% boric acid solution and 1 mL indicator (Brom Cresol Green and Methyl Red (BCG-MR)) were put into 100 mL Erlenmeyer to capture the ammonia released from the distillation. The distillation was stopped if the Erlenmeyer reached up to 50 mL volume. The Erlenmeyer was then titrated using 0.01 N HCl solution until the color changed from bright green into light pink.

EE analysis was done using hot extraction according to AOAC (2005) with slight modification. About 1-1.5 g samples were weighed and put into a filter paper bag. A 2 cm diameter of cotton ball was added before the bag was closed to prevent the sample from leaking. The bag was then inserted into the soxhlet. A 250 mL flask was filled with 150 mL hexane and connected to the soxhlet. The flask was heated for extraction. Extraction was running for six cycles. At the seventh cycle, the sample was removed from the soxhlet. The solvent was evaporated until the minimum amount was left in the flask. The hexane residue in the flask containing lipid was removed before it was dried in an oven at 102±2°C for 4 h. The fat extracted was then weighted and compared to the sample weight. The sample extraction was conducted with a high-temperature EV-16 Gerhardt Instrument (Made in Germany). CF analysis was done according to Procedure Ba 6a-05 (AOCS, 2005). Nitrogen Free Extract (NFE) was calculated using the following equation:

$$NFE = 100 - (Ash + CP + EE + CF);$$

Total Digestible Nutrient (TDN) was calculated using the following equation Wardeh (1981):

$$TDN = -14.8356 + 1.3310\,(\%CP) + 0.7923\,(\%NFE) + 0.9787\,(\%EE) + 0.5133\,(\%CF).$$

Ration Reconstruction

Total feed intake and quality observed from the five cows that produced the highest milk CLA were calculated. The ration used grass, botanical mix forages, rice straw, agricultural by-product, silage, cooperative concentrate, tofu waste, soy sauce, and biscuit waste. Each farm has different ration management. The rations were reconstructed for further study in the laboratory. The composition of feedstuffs and nutrient content of the reconstructed ration can be seen in Table 1.

In Vitro Study

As an inoculant for *in vitro* fermentation, rumen fluid was collected from two Friesian-Holstein fistulated bulls (live weight 500-510 kg) kept in Animal Science Faculty, IPB University field laboratory. Cattle were fed a ration of 60% elephant grass and 40% cooperative concentrates. The ration DM offered was 2% of its body weight, distributed into two equal feeding frequencies. The nutrient composition of rations was 10.25% CP, 20.70% CF, and 60% TDN. Water was given *ad libitum*.

Fermentative and enzymatic digestibility studies were performed using the two-stage Tilley and Terry (1963) *in vitro*. The treatment corresponds to the five reconstructed rations (P1 - P5). A total of 0.5 g of ration were weighed and placed in a 100 mL fermentor tube. 40 mL McDougall solution (9.8 g of $NaHCO_3$; 4.65 g of $Na_2HPO_4.2H_2O$; 0.57 g of KCl; 0.47 g of NaCl; 0.12 g of $MgSO_4.7H_2O$; 0.04 g of $CaCl_2$ for each L of aquadest) and ten rumen fluid was added to the tube. The CO_2 gas was aerated into the tube for 15 sec and then the tube was closed with a rubber cap. The tube without a sample contains a McDougall solution and rumen liquid was prepared as a blank. Next, the tube was incubated in a 39°C water-shaker bath for 4 and 48 h.

The parameters of pH, NH_3 concentration, and total and partial VFA concentrations were taken from supernatants incubated for 4 h. After 4 h of incubation, the tube was opened and the rubber cap was removed. 1 mL rumen fluid was collected for protozoa measurement and 0.5 mL for total bacteria. Analysis of protozoa and total bacteria was done by coloring and dilution methods (Ogimoto and Imai, 1981). The sample pH was analyzed by dipping the pH meter probe into the tube. A total of 10 mL of rumen liquid was separated and stored in the freezer for further use in partial VFA analysis with the method referred to by Yulistiani *et al*. (2015). The individual fatty acid was identified using Gas Chromatography (GC). Then, the sample was added with two drops of $HgCl_2$ to stop the fermentation process and continued with separation using a centrifuge at 3000 rpm for 15 min. The supernatant was stored in the freezer until a total of NH_3 and VFA analysis was performed. NH_3 concentration analysis was conducted according to Conway the microdilution method. Analysis of total VFA concentration was performed using steam distillation methods.

Table 1: Feedstuffs composition and nutrient content of the reconstructed ration.

	Ration				
Composition	P1	P2	P3	P4	P5
Feedstuffs (% DM)					
Grasses	7.76	21.49	4.52	40.27	47.88
Botanical mix forages	11.53	9.06	1.60		8.34
Rice straw	29.56	33.49	17.43		
Agricultural by-product			0.79		6.34
Maize silage			19.67		
Cooperative concentrate	46.02	25.24	39.33	59.73	37.44
Tofu waste	5.13	10.72	9.97		
Soy sauce waste			2.26		
Biscuit waste			4.43		
Nutrient					
DM (%)	89.05	90.04	89.50	88.57	89.03
OM (% DM)	88.29	86.14	86.98	88.28	89.42
Ash (% DM)	11.71	13.86	13.02	11.72	10.58
CP (% DM)	5.71	8.90	9.59	12.63	12.04
EE (% DM)	3.48	6.86	6.73	4.45	2.04
CF (% DM)	12.74	15.85	11.85	10.20	16.16
NFE (% DM)	66.36	54.53	58.81	61.00	59.18
TDN (% DM)	55.29	55.06	57.19	59.90	58.37

For digestibility measurement, a similar fermentation was conducted for 48 h and the fermentation was canceled by adding two drops of saturated $HgCl_2$ solution. Supernatant and sediment were separated using a centrifuge at 3000 rpm for 15 min. The sediment was transferred back into the fermentor tube and 50 mL of pepsin-HCl solution was added. The tube was incubated aerobically for 48 h in a 39°C water-shaker bath. After 48 h the tube was filtered using a vacuum machine and accommodated in Whatman filter paper no. 41. The remaining sediments in the filter paper were dried in a 105°C oven for 24 h to calculate the digest coefficient of dry materials (IVDMD). The sample was then burned in a 600°C furnace for 4 h to calculate the coefficient of organic matter (IVOMD). IVDMD and IVOMD were carried out with procedures according to AOAC (2005).

Statistical Analysis

The research used a randomized block design with five treatments and four blocks. The ration fermentation profiles and digestible were analyzed using ANOVA in SPSS version 20. The significant difference ($P<0.05$) in the parameters was further interpreted as per Duncan's Multiple Range Test.

Results

Ration Ferment Ability Profiles

The rumen pH was significantly different among the five treatments, 6.79-6.89. The lowest pH value was P1 (Table 2), while P4 was the highest. However, all the pH was normal for rumen microbial growth. Based on Table 2, the NH_3 concentration differed significantly between the rations, 5.06-9.17 mm. The ammonia concentration from P1, P2, and P3 rations was lower than P4 and P5. All ammonia concentrations in the rations were normal for microbial growth (4.99-17.61 mm). Ammonia concentrations in P1, P2, and P3 are close to the lowest range (4.99 mm), while P4 and P5 are slightly higher (8 mm) than the optimum ammonia level. Total VFA concentrations differed significantly between the rations, 82.23-132.23 mm, where P1 and P4 were higher than P2 and P5. The VFAs concentration in P2 and P5 were close to the minimum concentration for normal microbial growth, while P1 and P4 were slightly higher than the optimum level.

Partial VFA describes short-chain fatty acids of C2-C5. A significant difference in the molar percentage of partial VFAs was found in butyrate (C4) and iso-valerate (iC5) (Table 3). However, all the molar percentages were within the normal ratio to support rumen environmental physiology. The lowest butyrate percentage was found in the P4 (8%), while the highest molar percentage of C4 was found in P2 (10.99%). The lowest iso-valerate was in P1 and P5 (1.5 and 1.46%, respectively), and the lowest value was found in P2 and P3 (2.03 and 2.16%, respectively). In general, the molar proportion of the fifth treatment were 62A:22P:14B (P1), 61A:23P:14B (P2), 62A:22P:12B (P3), 60A:21P:13B (P4) and 66A:19P:13B (P5).

The protozoa and bacteria populations are shown in Table 4. The total protozoa population was in the log of the normal range (6 mL/cells). The significantly different protozoa population among the treatments was found in the P1 (6.08±0.12) and it shows the lowest value among all the rations. In contrast, the total bacterial population was not significantly different. The value (the log range 7 CFU/mL) was under the normal range.

Table 2: The result of pH, NH3 concentration, and total VFA concentration of ration fermentation *in vitro*

Treatment	pH	NH_3 (mm)	Total VFA (mm)
P1	6.79±0.20^{a}	5.49±1.29^{a}	131.97±19.86^{b}
P2	6.83±0.15^{ab}	5.06±1.00^{a}	82.23±16.89^{a}
P3	6.82±0.17^{ab}	5.75±1.30^{a}	112.20±38.29^{ab}
P4	6.89±0.13^{b}	9.41±1.88^{b}	132.23±30.59^{b}
P5	6.83±0.17^{ab}	9.17±1.65^{b}	83.42±28.30^{a}

$^{a, ab, b}$Superscript on the different column and same row show significantly different ($P<0.05$)

Table 3: The result of partial VFA concentration of ration fermentation *in vitro*

	Treatment				
Partial VFA (%)	P1	P2	P3	P4	P5
C2	61.61±3.71	60.77±5.65	62.14±1.94	63.16±4.68	66.32±7.35
C3	22.09±1.95	22.53±2.94	21.78±0.59	20.66±0.89	19.03±2.35
iC4	4.05±2.08	2.46±2.42	2.63±2.53	4.15±4.05	3.61±4.47
C4	10.33±1.05^{ab}	10.99±1.11^{b}	9.89±1.35^{ab}	8.71±1.04^{a}	9.19±0.94^{ab}
iC5	1.50±0.23^{a}	2.03±0.39^{b}	2.16±0.36^{b}	1.91±0.10^{ab}	1.46±0.15^{a}
C5	1.15±0.13	1.23±0.13	1.40±0.16	1.41±0.21	1.18±0.18
C2/C3	2.69±0.53	2.76±0.68	2.86±0.13	3.07±0.32	3.44±0.77

C2 = acetate; C3 = propionate; iC4 = iso-butyrate; C4 = butryrate; iC5 = iso-valerate; and C5 = valerate. C2/C3 = acetate/propionate ratio
$^{a, ab, b}$Superscript on the different row and same column show significantly different ($P<0.05$)

Table 4: Result of total protozoa and bacterial populations from the fermentation of rations *in vitro*

Treatment	Total protozoa (log cell/ml)	Total bacteria (log CFU/ml)
P1	6.08±0.12[a]	7.34±0.42
P2	6.35±0.22[b]	7.20±0.33
P3	6.36±0.17[b]	7.42±0.33
P4	6.31±0.08[b]	7.19±0.17
P5	6.40±0.17[b]	7.24±0.14

[a, ab, b]Superscript on the same column and different row show significantly different (P<0.05)

Table 5: The result of the ration digestibility of dry matter and organic matter *in vitro*

Treatment	IVDMD (%)	IVOMD (%)
P1	71.59±7.88[b]	67.91±5.69[c]
P2	67.00±6.86[a]	62.09±5.37[a]
P3	71.23±7.05[b]	67.87±5.30[bc]
P4	77.95±6.49[c]	74.49±3.81[d]
P5	68.64±4.48[b]	66.14±3.70[ab]

[a, ab, b, bc, c, d]Superscript on the same column and different rows show significantly different (P<0.05)

Ration Digestibility

The ration digestibility of dry matter and organic matter was done *in vitro*. Based on the results, there were significant differences in the ration digestibility of the five observed rations. Based on Table 5, the ratio digestibility in this study was normal, in the range of 67.00-77.95% for IVDMD and 62-74.4% for IVOMD. The highest IVDMD and IVOMD were in the P4, followed by the P1 and P3. The lowest IVDMD and IVOMD were P2, followed by P5.

Discussion

Ration Fermentability Profiles

The pH rumen value variation is 5.5-7.0 (Mottram *et al*., 2008). According to, the acceptable pH range in the rumen to support the physiology and maintain the population balance is 6-7. The P1 showed the lowest pH values and the high production of propionate. The propionate, valerate, and caproate value increases as the pH decreases from 7 to 5 (Erfle *et al*., 1982). In contrast, the considerable production of acetate/propionate ratio (C2/C3) led to an increase in the pH value of rumen fluids (Jiao *et al*., 2017). However, in this study, the difference in pH value was not related to the acetate/propionate ratio in all the treatments.

The pH differentiation was still within the normal range. The pH condition must stay balanced to maintain the cow's physiological stability and the absorption process of fermented products. Ruminant saliva is a buffer medium with a pH value and buffer capacity in cows is in the range of 8.2-8.5 and 80%, respectively (Aschenbach *et al*., 2011). McDougall solution is a substitute for saliva in ruminants *in vitro* fermentation. The solution contains high buffer capacity and aims to maintain the rumen pH stability during *in vitro* fermentation.

The optimum range of NH_3 concentrations in the rumen varies by 85-300 mg/L, or equivalent to 4.99-17.61 mm. Calsamiglia *et al*. (2010) stated that rumen fluid needs to produce NH_3 concentration as much as 5-11 mmol/L (or mm) to support optimal bacterial growth. Ammonia concentrations in this study were below the optimum range. The three treatments with the lowest concentration of NH_3 (P1, P2, and P3) contain rice straw at different levels.

The high concentrations of NH_3 in P4 and P5 were in line with the low *in vitro* Organic Matter Digestibility (IVOMD). Mentioned that it is more realistic to connect NH_3 concentrations with fermented OM, especially since the quantity of nitrogen utilized by rumen bacteria is close to constant values. High concentrations of NH_3 in P4 might cause by the high degradation of feed proteins. This hypothesis was supported by the high iso-C4 molar percentage of P4 that the branch-chain VFA concentration was a derivative of the branch-chain amino acid (Saro *et al*., 2014). Some rumen microbes require branched-chain fatty acids derived from proteins and amino acid degradation (Erfle *et al*., 1982).

In contrast to P4, a high concentration of NH_3 in P5 was followed by a low molar percentage of iso-C4. The protein content in P4 was due to the high crude protein in concentrate feed, while the P5 was caused by the high crude protein content in forage (Table 1). The different type of feedstuffs affects the ability to degrade feed protein in the rumen. It was reported that 45% forage in the ration produces better protein degradation in the rumen, protein utilization, and rumen fermentation (Lascano *et al*., 2016).

The total VFA concentration for the normal function of rumen bacteria was 70-150 mm. The total VFA concentration of the five treatments was in the normal range and was significantly different. The low VFA was related to a lower percentage of P2 and P5. The high concentration of total VFA was in P4 and P1. It was supported by Wang *et al*. (2020) that reported the VFA concentration increases as the percentage of forage decreases.

The relationship between feed concentrate to forages ratio was not seen in the percentage of partial VFA production. There were no significant differences in the acetate/propionate ratio which was in the range of 2.69-3.72. Wang *et al.* (2020) reported that cows fed high in concentrate produce more butyrate than cows fed high forage. This statement was different from the results of this study which the ratio with the highest concentrate (P4) produces less butyrate (C4).

Referring to, the molar proportion of VFA partial in cattle fed mature forage and herbage is 0.64:0.22:0.11:0.03 (acetate: Propionate: Butyrate: Other, respectively). Owens and Basalan (2016) also reported that the VFA partial percentage proportion of acetate, propionate, and butyrate in forage-based rations is 65:20:15, respectively. The concentration was similar to the molar proportion of the P5 treatment and slightly higher than the acetate ratio in the P1-P4 treatment.

The normal total protozoa population was 10^6 mL/cells. The total of protozoa in this study was slightly higher than in other studies in the log range of 5.79-4.64 mL/cells (Moate *et al*., 2020). The decrease in the total protozoa population in P1 was due to the content of the NFE ration, but it was still in the normal range. The P1 has the highest NFE content of the reconstruction rations. NFE is a part of readily soluble and highly digestible carbohydrates, including monosaccharides, disaccharides, and polysaccharides (Aling *et al*., 2020). The decrease of protozoa, fungi, and methanogens occurs in high starch content rations (Belanche *et al*., 2012). The experiment of Moate *et al*. (2020) showed that wheat utilization as a starch source declines the total protozoa population, particularly in *Entodinium* spp and *Dasytricha* spp species.

As much as 70% of the total rumen bacteria play an essential role in the digestion and fiber feed breakdown in the rumen (Forsberg and Lam, 1977). The total bacteria population in this study was well below the normal value, 10^9 - 10^{10}/mL rumen fluid. *In vitro* fermentation at various forage sources have a total bacterial population value in log 9 (Iqbal *et al*., 2018).

The low fiber content of the rations (10.20-16.16%) caused the low bacterial population. The decrease of the cellulolytic bacteria population is found in low fiber content rations (Iqbal *et al*., 2018). The statement was supported by other studies that the population of fiber-breaking bacteria increases in high NDF feed (Huws *et al*. 2010; Saro *et al*., 2014). Feed protein also plays a role in determining microbial population. Ruminants can survive with low-protein feed by increasing urea mobilization through saliva (Reynolds and Kristensen, 2008). However, over a long period, the use of low-protein feed can lead to a decrease in bacteria, protozoa, anaerobic fungi, and methanogen rumen concentration (Belanche *et al*., 2012). The crude protein content in reconstruction rations was relatively low, in the range of 5.71-12.63%. The sensitivity of bacteria to N rumen concentrations differs among the groups of bacteria. Cellulolytic bacteria tend to be more sensitive to low concentrations of N rumen (Belanche *et al*., 2012). Besides fiber and protein content in the feed, the methods used and the different *in vitro* environmental conditions significantly affect the quantitative distribution of rumen bacteria (Weimer *et al*., 2011).

Ration Digestibility

The Dry Matter Digestibility (DMD) in the two-stage *in vitro* method was in the range of 55.0-77.9% (Tilley and Terry, 1963). The DMD can describe the feed digestibility. A feed with higher energy synchronization and nitrogen release rates generally has a higher level of nutritional digestibility (Chanjula *et al*., 2004). It was related to the bacteria population, metabolism, and enzymatic activity of ammonia assimilation *in vitro* fermentation (Chanjula *et al*., 2004). P4 has the highest digestibility, NH_3, and VFA total concentration compared to the other treatments. P4 consisted of less feedstuff composition than others. It was 40.27% forages and 59.73% concentrate feed, as shown in Table 1.

DMD in ruminants shows the amount of feed digested in the rumen. The high DMD of a feedstuff indicates the high-quality feedstuff (Aling *et al*., 2020). The lowest DMD and OMD were found in P2, followed by P5. The P2 consists of 33.49% rice straw with IVDMD 67.00% and IVOMD 62.09%. The digestibility value of ration using rice straw in this research was higher than Widodo *et al.* (2012), which reported that the IVDMD and IVOMD values are 60.93-64.53 62.92-65.14%, respectively.

The similarity of the P2 and P5 IVOMD patterns was due to the high forage utilization on both rations and it was 64.04% in P2 and 62.56% in P5. The quality of fiber fractions plays a significant role in influencing the digestibility of both treatments. NDF content of forages strongly affects the digestibility and capacity of the rumen to consume and was directly related to pdNDF (potential degradable NDF). The pdNDF is a fraction of NDF that disappears after incubation for an extended period and then remains iNDF (undegradable NDF) that cannot be digested by microbes (Teimouri, 2017). Forage feed with the same NDF content can have distinctive iNDF content (Teimouri, 2017).

High concentrations of total VFA in P1, P3, and P4 also produce a high OMD. Ma *et al.* (2020) stated a real correlation between OMD and gas production. The high OMD leads to increase microbial fermentation activity and accelerates the rate of gas production (Al-Masri, 2003). The concentration of OMD in dry matter was often used to describe the concentration of energy in feed. The increase of VFA illustrates the increased rumen fermentation rate, leading to higher organic matter and NDF digestibility (Wanapat *et al*., 2013). This statement was in line with the results of this study.

Conclusion

The ration with 40% grass and 60% concentrate is the best smallholder dairy cattle ration to produce high CLA milk with the best fermentation profile. It is supported by the highest NH_3, VFA, DMD, and OMD; the microbial population is also in the normal range. It is recommended that farmers from the same area use the formula to produce high CLA milk to promote consumer health and better farmer income.

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Author's Contributions

Dwitami Anzhany: Participated in all experiments, coordinated the data-analysis and contributed the data-analysis and contributed to the writing of the manuscript

Toto Toharmat and Despal: Designed the research plan, and organized and supervised the study.

Ethics

The inoculum of ruminal fluid was taken from two FH fistulated males. The ruminal fluid was collected before morning feeding at Field Laboratory, Animal Science Faculty, IPB University. The bulls were maintained following the animal welfare standards of the IPB University. All protocols were approved by the Animal Science Faculty, IPB University.

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