Characteristics of In Vitro Fermentation and Nutrient Digestibility of Ration Supplemented with Different Level of Soybean Oil Calcium Soap

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Abstract. The in vitro experiment was aimed to evaluate the effect of soybean oil calcium soap (CaS-soybean) supplementation in ration on fermentability characteristics, microbial population and nutrient digestibility by using rumen fluid of Bali cattle. The experiment was arranged in a completely randomized block design with 4 different ration treatments with 3 replicates. The ration treatments were R₁: 40% native grass (NG) + 60% concentrate (C), R₂ (40% NG + 60% C containing 2.5% CaS-soybean), R₃: (40% NG + 60% C containing 5.0% CaS-soybean) and R₄ (40% NG + 60% C containing 7.5% CaS-soybean). The measured variables were pH, NH₃-N, total volatile fatty acids, total bacteria and protozoa (microbial populations), dry matter and, and organic matter digestibility. The data were analyzed by using Analysis of Variance and the differences between treatments were examined with Duncan's Multiple Range Test. The experimental results showed that the different feed treatments did not have any significant effect on pH, ammonia (NH₃-N) concentration, total bacteria, protozoa population, dry matter, and organic matter digestibility. However, the treatments significantly affect the total volatile fatty acids (VFA) production. Ration with 7.5% CaS-soybean (R₄) had the highest VFA concentration compared to R₁ (Control), R₂ (control ration + 2.5% CaS-soybean) and R₃ (control ration contains 5% CaS-soybean). Feed treatment supplemented with 5.0% CaS-soybean (R₃) had a higher total VFA concentration compared to control rations (R₀). In conclusion, supplementation of the different levels of CaS-soybean within concentrate created the normal of in vitro fermentation characteristics and the total production of VFA.

1. Introduction

Soybean oil has been widely used in cattle, poultry, and pork rations because it contains essential fatty acids (linoleic acid, C₁₈: 2n-6 and high oleic acid, C₁₈: 3n-3) [1]. Soybean oil fatty acid composition consists of linoleic acid (> 50%), oleic acid (21% to 25%) and linolenic acid (5% to 6%) [2]. Research on the use of soybean oil to increase the content of unsaturated fatty acids in ruminant animal products has been conducted but has not been able to provide optimal results.

Awawdeh et al. (2009) [3] found that the addition of 32 g kg⁻¹ soybean oil to barley-based feeds could increase the consumption and digestibility of crude fat. Bodyweight gain was 237 g head⁻¹.
and final body weight, but could not improve the quality of the carcass. The addition of 33 g kg\(^{-1}\) dry matter (DM) soybean oil based on high forage in beef cattle fattening feed increases the content of conjugated linoleic acid (CLA) deposited in intramuscular and subcutaneous fat, the content of vasenic acids and the profile of unsaturated fatty acids and decreases the composition of saturated fatty acids (CLA) [4].

The utilization of vegetable oils in ruminant animal rations is limited by the negative effects of polyunsaturated fatty acid (PUFA) in the rumen. PUFA in ruminant digestion undergoes many changes [5] and has negative effects on rumen fermentation, digestion of crude fiber and consumption of dry matter of feed containing barley straw (barley) and concentrate [6]. In addition, PUFA contained in ruminant feed undergo an esterification and biohydrogenation process when entering rumen digestion [7]. The biohydrogenation process in the rumen is a detoxification mechanism to avoid the anti-bacterial effect of unsaturated fatty acids which can damage the integrity of cell membranes which has implications for decreased growth and microbial activity [8]. CLA supplementation without protection would experience biohydrogenation of 91.5% in the sheep’s rumen whereas if given in protected form only 35% had biohydrogenation [9].

The negative effect of PUFA on rumen fermentation is the main reason that the utilization of vegetable oils containing high PUFA must be limited and protected so as not to interfere with rumen function. If this can be controlled and conducted appropriately, the use of vegetable oils containing high PUFA can have a positive effect on improving the productivity and quality of ruminant livestock products [1]. Differences in livestock response to the application of plant oil in cattle rations are influenced by the growth phase of cattle, the amount of fat or oil in the total ration and the levels of linoleic fatty acids contained in vegetable oils.

Several technologies have been developed to protect dietary fat in rumen digestion including in the forms of fat aldehydes, calcium soap and fatty acid amides [10]. Fatty acid calcium soap was developed as a fatty acid protection technology that is resistant to microbial enzymes and products that can prevent disruption of the rumen fermentation process by fatty acids [11]. In addition, Besides calcium fatty acid soap has higher digestibility than other sources of fatty acids (beef fat, hydrogenated glycerides, cottonseed oil, prilled fatty acids, and calcium palm fatty acid soap), it can further provide digestible energy values to increase livestock production.

Some calcium soap products have been produced commercially to increase the content of unsaturated fatty acids in the body tissues of beef cattle [10]. The application of fat in the form of calcium soap can protect some of these fatty acids from the rumen microbial biohydrogenation process [12]. The diversity of results obtained is related to the use of vegetable oil calcium soap due to differences in oil’s type, the amount of PUFA content, the quantity, and the quality of calcium soap products used, physiological and digestive factors of livestock. The variety of factors that can influence the effectiveness of utilization in ruminant rations is the main reason for the need to continue the study of the use of calcium soap. Some of these reasons are also confirmed by the data obtained in this series of studies, where the use of vegetable oil (soybean oil, palm oil, and sunflower oil) protected by calcium soap has not been able to provide a consistent fermentation performance both on the characteristics of fermentation, microbial growth and digestibility nutrients suppress the negative effects of PUFA on bacterial growth and fermentation variables. The results of previous in vitro studies showed that the use of different types of vegetable oils in the unprotected and protected forms of calcium soap did not produce different fermentation performance but the use of soybean oil in the unprotected and protected forms of calcium soap independently produced the highest total of VFA production. Although the combined effect of oil type treatment and form of supplementation results in pH levels, N-NH3 concentrations, microbial population and nutrient digestibility which are relatively similar, the combination treatment can create fermentation variables that are conducive to the continuation of the fermentation process and microbial growth.

Based on the explanation, an in vitro study was conducted to evaluate the use of calcium soap protected by soybean oil at different levels on fermentability, microbial growth, and feed nutrient digestibility.
2. Material and Method

2.1. Material

The experimental rations consisted of 40% native grass (NG) and 60% concentrate (C). The concentrate’s material consisted of tapioca waste, pollard, coconut meal, cashew fruit flour, soybean oil calcium soap (CaS-soybean), molasses, CaCO$_3$, and urea. The CaS-soybean was produced following the method developed by Kumar et al. (2006)[13] using soybean oil (Mazola brand, produced by © ACH Food Companies, Inc), distilled water, NaOH (Supplier: WuhaiXinye Chemical Industry Co., Ltd) and CaCl$_2$ (Keg King, 2/33-35 Smith Road Springvale, Vic 3171). The production of CaS-soybean was initiated by using a specified soap saponification amount in determining the amount of required NaOH concentration. The NaOH solution and soybean oil were mixed and heated on a hot plate up to a temperature of 180°C and stirred at 800 rpm rotation until achieving the NaOH solution and soybean oil dissolved completely. CaCl$_2$ solution (made from 2.35g of CaCl$_2$, and mixed into 4.7 ml of distilled water or in the scale of CaCl$_2$: water = 1:2) was added slowly to the mixture and stirred up until the CaS-soybean was formed. Then, CaS-soybean was dried up for 24 hours at 60°C and ready to be mixed with other concentrate materials.

The research was arranged in a completely randomized block design (rumen fluid collection period) to test four types of treatment rations (concentrate was containing the different levels of CaS-soybean) with three replicates (based on Bali cattle rumen fluid collection period). The ration treatment consisted of: R1 (40% NG + 60% C); R2 (40% NG + 60% C containing 2.5% CaS-soybean), R3 (40% NG + 60% C containing 5.0% CaS-soybean) and R4 (40% NG + 60% C containing 7.5% CaS-soybean). The proportion of the raw materials of each treatment is presented in Table 1. Nutrient composition of the treatment ration was compiled with 68% TDN, 13% crude protein (CP), and 3.91–6.54% ether extract (EE) by the standard nutrient requirement of Bali cattle that weighed 250 kg [14]. The nutrient composition of the treatment rations (% dry matter) is presented in Table 2.

Table 1. The proportion of the raw materials of each treatment ration (% dry matter)

| Ration Ingredients (%) | Treatments |
|------------------------|------------|
|                        | R1  | R2  | R3  | R4  |
| Native Grass           | 40.00 | 40.00 | 40.00 | 40.00 |
| Concentrates:          | 60.00 | 60.00 | 60.00 | 60.00 |
| Cassava Waste          | 30.00 | 27.50 | 25.00 | 22.50 |
| Pollard                | 31.50 | 31.50 | 31.50 | 31.50 |
| Coconut Meal           | 20.50 | 20.50 | 20.50 | 20.50 |
| Molasses               | 15.00 | 15.00 | 15.00 | 15.00 |
| CaCO$_3$               | 1.50  | 1.50  | 1.50  | 1.50  |
| Urea                   | 1.50  | 1.50  | 1.50  | 1.50  |
| CaS-Soybean            | 0.00  | 2.50  | 5.00  | 7.50  |

Notes: R1 (40% NG + 60% C); R2 (40% NG + 60% C containing 2.5% CaS-soybean), R3 (40% NG + 60% C containing 5.0% CaS-soybean) and R4 (40% NG + 60% C containing 7.5% CaS-soybean).

Table 2. Nutrient composition of the treatment rations (% dry matter).

| Nutrient composition (%) | Treatments |
|--------------------------|------------|
| Dry Matter               | 76.00 | 76.05 | 76.09 | 76.13 |
| Ash                      | 6.37  | 6.64  | 6.91  | 7.17  |
| Crude Protein            | 13.21 | 13.19 | 13.17 | 13.15 |
| Crude Fiber              | 16.71 | 16.55 | 16.38 | 16.22 |
| Ether Extract            | 3.91  | 5.19  | 6.48  | 7.77  |
NFE | 59.80 | 58.43 | 57.06 | 55.68 |
TDN | 71.19 | 72.47 | 73.76 | 75.05 |
Ca | 0.52 | 0.52 | 0.51 | 0.51 |
P | 0.25 | 0.25 | 0.25 | 0.25 |

Notes:  R1 (40% NG + 60% C); R2 (40% NG + 60% C containing 2.5% CaS-soybean), R3 (40% NG + 60% C containing 5.0% CaS-soybean) and R4 (40% NG + 60% C containing 7.5% CaS-soybean).

2.2. Research Procedure
The study was conducted for 2 months, in several laboratories in the Department of Nutrition and Feed Technology, Faculty of Animal Science, Bogor Agricultural University, namely: (a) In vitro testing, measurements of total gas production were held in the Laboratory of Dairy Animal Nutrition (b) Measurement of the microbial population was conducted in the Laboratory of Microbiology, Physiology, and Nutritional Biochemistry (c) Proximate analysis of rations was performed in the chemical analytic laboratory of The Livestock Research Center, Ciawi, Bogor.

Research variables observed consisted of (i) fermentation characteristics (pH, NH$_3$-N concentration, total VFA production), (ii) population of microbes (total bacteria and protozoa), and (iii) nutrient digestibility consisted of dry matter and organic matter. The number of samples measured for each variable was Duplo in each replication of the experiment.

In vitro fermentation was conducted following Tilley dan Terry (1963) [15]. Each treatment ration sample (500 mg) and 40 ml McDougall solution was poured into a 100 ml fermenter tube. The tube was then added with 10 ml of the Bali’s cattle rumen fluid, slowly stirred and flushed with CO$_2$ gas and then incubated in the shaker water bath (produced by Memmert GmbH + Co.KG, Äußere Rittersbacher Startβe 38) set at 39°C for 24 hours. Rumen fluid of the Bali cattle, used in the treatment, was previously collected four hours after morning feeding using a stomach tube connected to a vacuum pump. The pH, VFA, NH$_3$-N, total bacteria and protozoa population measurement of the fermented fluid samples in the fermenter tube were conducted four hours after the incubation. The measurements of dry matter and organic matter digestibility were carried out by incubating a fermenter tube filled with the ration sample in the shaker bath set at 39°C for 48 hours.

2.3. Collection and measurement of samples
Rumen fermentation characteristic variables were pH (measured with pH meter, manufactured by Hanna Instruments, 3820 Packard Road, Suite 120 Ann Arbor, Michigan 48108), the concentration of NH$_3$-N analyzed with Conway micro diffusion method of Conway (1962) [16] and total VFA concentration was analyzed using steam distillation method of General Laboratory Procedure (1966). Protozoa population was counted with the aid of a microscope of 40x magnification using Fuchs Rosenthal Counting Chamber, size 4 x 4 x 0.2 mm (manufactured by Hausser Scientific, Montgomery County, Pennsylvania, USA) on which the in vitro liquid (0.5 ml) was incubated for four hours and mixed with 0.2 ml methyl green formaldehyde saline solution [17]. Bacteria in the Hungate tube were visually counted to determine the bacterial population. Before counting, the bacterial culture was diluted using the BHI medium of Ogimoto dan Imai (1981) [17] and homogenized in a roller tube.

The 48 hours incubated fermentation liquids were measured for their dry matter and organic matter digestibility. The process started with in vitro liquid, where the fermenter tube was taken out from the shaker bath after 24 hours of incubation. At the end of the first incubation period, the 2-3 drops of the HgCl$_2$ solution were added to stop the microorganism fermentation process, then centrifuged at 3500 rpm to separate the supernatant and residue. After discarding the supernatant, 50 ml of freshly-made pepsin solution were added to the residue in each tube. The tubes were then incubated at 39°C for 48 h with occasional shaking. Anaerobic conditions were not necessary during
this stage. At the end of the incubation, the supernatants were discarded and the insoluble residues were washed with water in the centrifuge. The substrate was then filtered using Whatman paperNo.41 (it is distributed by Voigt Global, Po Box 1130, Lawrence, Kansas 66044 USA), placed in a porcelain cup and then dried in an oven set at 105 °C for 8 hours at a constant weight. The dry weight of the residue was calculated. Then, it was followed by incineration set at 550°C for 4 hours to measure the ash weight.

2.4. Data Analysis
The data were analyzed using one-way ANOVA followed by Duncan’s Multiple Range Test using the IBM SPSS Statistics for Windows, Version 21.0. Armonk, New York: IBM Corp. Differences were considered significant at P<0.05 and P<0.01.

3. Result and Discussion
3.1. Fermentation Characteristics
The effect of treatment on fermentation characteristics (pH value, N-NH3 concentration, and total production of VFA) in each treatment was showed in Table 3.

Table 3 Effect of supplementation of different level of soybean oil calcium soap in the concentrate on fermentation characteristics

| Fermentation characteristics | Treatments | P-Value |
|------------------------------|------------|---------|
|                              | R1         | R2      | R3      | R4      |
| pH                           | 6.64 ± 0.17| 6.59 ± 0.17| 6.56 ± 0.20| 6.53 ± 0.26| 0.47 |
| N-NH3 (mM)                   | 9.43 ± 0.88| 10.55 ± 1.35| 10.42 ± 0.87| 10.13 ± 2.65| 0.73 |
| Total of VFA (mM)            | 125.29 ± 35.75A| 133.75 ± 33.16AB| 148.19 ±28.55B| 167.89 ± 31.3C| 0.002 |

Notes: R1: 40% natural grass (NG) + 60% concentrate (C), R2 (40% NG + 60% C, containing 2.5% SCa-soybean), R3 (40% NG + 60% C, containing 5% SCa-soybean), R4 (40% NG + 60% C, containing 7.5% SCa-soybean). Numbers in the same column followed by different letter means significantly different at the level test 1% (Duncan’s multiple range test).

The data in Table 3 showed that the application of soybean calcium soap at different levels did not affect pH levels and N-NH3 concentrations. The addition of SCa-soybean at different levels greatly affected (P<0.002) total VFA production. The highest total VFA production was in R4 treatment and the lowest total VFA production occurred in the R1 (control) treatment. The total production between rations R1 and R2 and between R2 and R3 were not significantly different but the total production of VFA of R3 was higher than the control ration.

The use of SCa-soybean at different levels resulted in the average pH value being relatively the same in all treatments, the pH range of 6.5 to 6.64. The pH level was a normal pH range to support the continuation of the fermentation process. The average value of ruminal pH (6 to 7) is a normal and ideal pH to support microbial growth and the fermentation process in the rumen [18]. Furthermore, maintaining rumen pH levels is very important for the survival and stabilization of rumen microbial growth [19]. The level of rumen pH is influenced by the type and frequency of ration given to each ruminant livestock species. The stability of rumen pH achieved in this study was thought to be related to the effectiveness of calcium soap in protecting long-chain unsaturated fatty acids from forming complex bonds with cations that can affect rumen pH conditions and microbial requirements for cations [20]. Ruminal pH levels in the range of pH 6.53 to 6.64 are ideal pH to guarantee the stabilization of the resistance of unsaturated fatty acids and calcium ions in calcium soap products in the rumen [11]. The stability of PUFA bonds and calcium ions will prevent negative effects and the process of PUFA biohydrogenation in the rumen.

Treatment of SCa-soybean supplementation at different levels (2.5%, 5.0%, and 7.5%) in the concentrate also resulted in N-NH3 levels and total VFA production which was ideal to support microbial growth in the fermentation process in the rumen. Fermentation in the rumen could take place normally if the conditions of the rumen ecosystem had a temperature range 38 to 42°C, pH, 6.0
to 7.0, N-NH₃ concentration 6 to 21 mM and total VFA levels 70 to 150 mM [18]. The pH levels, N-NH₃ concentration, and total VFA production had correlations that were interrelated with each other in creating ideal rumen ecosystem conditions to support the continuation of the fermentation process. In addition, a decrease in rumen pH from 6.5 to pH 5.7 could result in a marked decrease in N-NH₃ production [21]. Another study reported that total VFA production in cows that consume high concentrates is positively correlated with pH, that is total VFA production decreases if the pH decreases to 5.3 [22]. Normal pH levels in this study contributed positively to N-NH₃ concentration and total VFA production within the normal range N-NH₃ levels (9.43 to 10.55 mM) and total VFA production (125.29 to 167.89 mM).

Data on pH, N-NH₃ concentration and total production of VFA which were relatively similar in all treatments could be used as an indicator that the addition of CaS-soybean at different levels in the concentrate did not have a negative impact in the ecosystem and digestion of the feed substrate. This phenomenon also means that supplementation of soybean oil protected by calcium soap was quite effective in protecting PUFA in the rumen ecosystem so that the presence of PUFA did not interfere with microbial growth and activity in degrading the feed fiber fraction. A good level of degradation of feed fiber would have implications for the formation of VFA components during the incubation process. This phenomenon was also demonstrated by the highest total VFA production achievement in the use of 7.5% CaS-soybean which reached 167.89 mM. The results of this study were in line with a previous study that supplementation of protected flaxseed oil in the form of calcium soap significantly increased total VFA production [23].

The high total production of VFA in the 7.5% CaS-soybean treatment was thought related to the high levels of PUFA linoleic acid in the CaS-soybean level, where the higher PUFA content of vegetable oils used as ingredients for calcium soap will require more calcium ions in the preparation process. A large number of available calcium ions had good potential to provide the calcium ions needed to stimulate the growth and activity of fiber-digesting bacteria in the rumen [23]. Moreover, CaCl₂ increased the capacity of calcium soap formation, its toxicity to low rumen bacteria and increased digestibility of plant cell walls compared to dicalcium phosphate [24]. Calcium minerals play a role in the synthesis and stability of microbial cell wall structures and can activate various microbial enzymes such as α-amylase and are needed by microbes to digest cellulose [25]. The increased digestibility of the feed fiber fraction further had implications for the increase in total VFA production produced in the rumen.

The increase in total VFA production along with the use of calcium soap levels was thought related to the increase in the concentration of glycerol available in the rumen which was one of the precursors in the VFA synthesis process. The increase in the amount of glycerol in the rumen along with the increase in the level of calcium soap could be understood as the implication of the increase in dietary fat content (Table 7) used in this study. This phenomenon was also related to the potential for the separation of linoleic acid bonds from calcium ions from soybean calcium soap products when entering rumen digestion. Long-chain unsaturated fatty acids such as linoleic acid and linolenic acid were difficult to be protected from the biodehydrogenation of rumen bacteria compared to oleic fatty acids [10]. The increase in total VFA production in line with the increase in CaS-soybean levels which was not followed by an increase in microbial population and nutrient digestibility (dry matter and organic matter) was thought to be an implication of the influence of CaS-soybean PUFA (linoleic acid) which was separated from their calcium ions. Increased levels of PUFAs further poison and suppress the growth and activity of bacteria involved in the process of degradation of compounds contained in feed substrates, especially fiber fractions. This phenomenon was shown by microbial population data (Table 5), where the concentrate containing 7.5% CaS-soybean produced the highest total VFA production but started to disrupt the growth of the total bacterial population compared to other treatments. This research data confirmed the theory that even though the use of fatty acid calcium soap products was intended to reduce the negative effects of fat in the rumen, the amount must still be controlled because of the potential for the separation of calcium linoleic-ion bonds from calcium soap products while in the rumen ecosystem.
3.2. Microbial Population
The effect of different levels of CaS-soybean supplementation in the concentrate on microbial population was showed in Table 4.

Table 4  Effect of different level of soybean oil calcium soap in the concentrate on microbial population

| Microbial Population | Treatments | P-Value |
|----------------------|------------|---------|
|                      | R1         | R2      | R3       | R4       |
| Total of Bacterial   | 8.10 ± 0.61| 6.78 ±1.59| 7.61±1.02| 6.71 ± 1.30| 0.20 |
| (log CFU ml⁻¹)       |            |         |          |          |
| Protozoa (log sel ml⁻¹) | 4.69 ± 0.64| 5.00 ± 0.60| 4.97 ± 0.30| 5.00 ± 0.18| 0.75 |

Notes: R1 (40% natural grass (NG) + 60% concentrate (C)), R2 (40% NG + 60% C, containing 2.5% SCa-soybean), R3 (40% NG + 60% C, containing 5% SCa-soybean), R4(40% NG + 60% C, containing 7.5% SCa-soybean).

The use of SCa-soybean at different levels in the concentrate did not affect the total population of bacteria and protozoa. The total bacterial population and the protozoa population between treatments were not significantly different. Nevertheless, the use of CaS-soybean at the 7.5% level began to suppress the total bacterial population. The total bacteriapopulation in the R4 treatment (6.71 ± 1.30 log CFU ml⁻¹) when compared to the control ration (8.10 ± 0.61 log CFU ml⁻¹) and R3 (7.61 ± 0.61 log CFU ml⁻¹).

The total growth of the depressed bacteria was probably related to high levels of linoleic fatty acids which are still potentially separated from their calcium ions during the incubation process. However, in principle, the addition of various levels of CaS-soybean in this study had not yet had a significant negative impact on protozoa populations, fermentation characteristics and nutrient digestibility. The pH levels (6.53 to 6.64), N-NH₃ levels (9.43 to 10.55 mM) and optimal total VFA production (125.29 to 167.89 mM) obtained were still in the relatively normal range for the continuation of the fermentation process and microbial growth. The stabilization of the fermentation variable was very important and was the main requirement for supporting the growth and activity of microbes in the rumen. Fermentation in the rumen could be normal if the ecosystem conditions are at temperatures 38°C to 42°C, pH 6.0 to 7.0, N-NH₃ concentrations6 to 21mM and total VFA levels 70 to 150 mM [18]. Rumen N-ammonia compound is the main source of nitrogen in the process of rumen microbial protein synthesis. Microbial protein synthesis is crucial for ruminants because microbial protein synthesized in the rumen provides 50% of the total amino acids for beef cattle. Another study reported that growth and synthesis of microbial proteins depends on the adequacy of energy (ATP) produced from fermentation of organic matter in the rumen and the availability of nitrogen resulting from the degradation of non-protein nitrogen sources (NPN) and proteins and other nutrients such as sulfur, phosphorus, and other minerals [26].

The relatively similar protozoapopulation between treatments might be related to the effect of the use of calcium soap which was not harmful to the protozoan growth. It is known that protozoa are more sensitive to fat than bacteria. The effect of the treatment that did not inhibit the growth of protozoa had implications for achieving optimal pH stabilization incubation levels. Protozoa have an important role in supporting (maintaining) stabilization of rumen pH due to the ability of protozoa to digest starch particles faster [19]. The normal condition of the fermentation ecosystem was also related to the role of calcium soap in suppressing the negative effects and toxicity of PUFA on microbial growth and the fermentation process during the incubation process. This was by the purpose of using fatty acid calcium soap so that PUFA did not interfere with fermentation in the rumen [24], was not toxic to rumen bacteria and prevented the digestion of crude fiber [27].

Although the pH leveland VFA concentration in this study were within the normal range, the treatment containing 7.5% CaS-soybean began to suppress the total bacterial growth when compared
to the total bacterial population in the control treatment and ration treatment containing 5% CaS-soybean.

3.3. Nutrient Digestibility
The effect of different levels of CaS-soybean on dry matter and organic matter digestibility was highlighted in Table 5. The result of the analysis of variance showed that supplementation of different levels of CaS-soybean did not affect dry matter and organic matter digestibility value.

Table 5 Effect of supplementation of different level of soybean oil calcium soap in the concentrate on nutrient digestibility

| Nutrient Digestibility (%) | Treatments | Nilai P |
|----------------------------|------------|---------|
|                            | R1         | R2      | R3      | R4      |         |
| Dry matter                 | 71.04 ± 2.03 | 70.94 ± 2.07 | 72.00 ± 1.14 | 71.19 ± 0.70 | 0.73    |
| Organic matter             | 74.63 ± 1.64 | 74.31 ± 2.85 | 75.28 ± 3.15 | 74.35 ± 0.47 | 0.79    |

Notes: R1 (40% natural grass (NG) + 60% concentrate (C)), R2 (40% NG + 60% C, containing 2.5% SCa-soybean), R3 (40% NG + 60% C, containing 5% SCa-soybean), R4 (40% NG + 60% C, containing 7.5% SCa-soybean).

Dry matter and organic matter digestibility values that did not differ in all treatments were data that confirmed that the addition of CaS-soybean at different levels could create a stable and ideal rumen ecosystem condition to support microbial growth in conducting fermentative activities or the process of degradation of the feed substrate. To avoid the negative influence of fat on ruminants, the addition of fat in the ration should be no more than 6% to 7% of the total dry matter of ration [28]. The theory is confirmed by the data of this study where the application of CaS-soybean at the 7.5% level began to suppress the total bacterial population.

The effect of the addition of CaS-soybeans which began to suppress the total bacterial population might also due to the quality and durability of the soybean calcium soap product which had not been achieved optimally in protecting PUFA in incubation processes. Calcium linoleic soap is not completely protected and escapes from the rumen bacterial biohydrogenation process [29]. Nearly 33% of calcium linoleic acid soap has a separation in the rumen. Another study reported that long unsaturated fatty acids such as linoleic and linolenic are more difficult to be protected from rumen microbial biohydrogenation than oleic fatty acids [10]. The degree of breakthrough and resistance of calcium soap from the microbial biohydrogenation process and its separation in the rumen were influenced by the manufacturing techniques and storage processes of calcium soap products. The binding of the fatty acid complex and the calcium ion which was stable and did not decompose in the rumen would prevent unsaturated fatty acids from inhibiting the growth and microbial activity in degrading the feed substrate [11]. Oil coating on feed particles and microbes could prevent microbes from digesting the compounds contained in the feed substrate [30]. This, in turn, had implications for decreasing the percentage of total digestibility of dietary nutrients.

4. Conclusion
Supplementation of different levels of soybean oil protected calcium soap in concentrate had no negative effect on the rumen ecosystem and results in the normal fermentation characteristics to support the sustainability of feed fermentation in the rumen. Application of SCa-soybean up to level 7.5% results in the highest of VFA total production, but it starts to indicate the negative effect on bacterial growth and nutrient digestibility.

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