

## Anacardic Acid Isolated From Cashew Nut Shell (*Anacardium occidentale*) Affects Methane and Other Products in the Rumen Fermentation

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### ABSTRACT

Biofat is a hexane extract containing several bioactive compounds with anacardic acid as the major compound. This study aimed to examine the effect of anacardic acid on rumen fermentation, especially methane and its degradation in the *in vitro* rumen fermentation. The study was arranged in a completely randomized block design. The treatments were control (substrate or complete feed), biofat (substrate + 0.75  $\mu$ L/mL biofat), and anacardic acid (substrate + 0.75  $\mu$ L/mL anacardic acid). Measured variables were total gas production, methane, pH, concentration of ammonia ( $\text{NH}_3$ ), dry matter degradability (DMD), organic matter degradability (OMD), and neutral detergent fiber degradability (NDFD) in the rumen. The chromatogram GC-MS analysis results indicated that the anacardic acid isolation process of the biofat produced nearly pure isolate (99.44%), and significantly decreased the production of methane by 51.21% and 39.62%, respectively. Anacardic acid degradation pattern in the *in vitro* rumen test showed a shifting of retention factor (Rf) value after anacardic acid being incubated with the degradation of anacardic acid occurred after 24 h of fermentation. In conclusion, anacardic acid isolated from biofat has a dominant role to reduce the *in vitro* methane production. Anacardic acid is very potential to be used as a methane reducing agent.

**Keywords:** anacardic, cashew nut shell, methane, rumen fermentation

### ABSTRAK

Biofat adalah produk bioindustri dari kulit cangkang mete yang mengandung beberapa senyawa bioaktif dengan senyawa utama adalah asam anakardat. Penelitian bertujuan untuk menguji efektivitas asam anakardat pada fermentasi rumen, khususnya gas metana dan degradasinya di dalam fermentasi rumen secara *in vitro*. Penelitian menggunakan rancangan acak kelompok. Perlakuan: kontrol (substrat atau pakan komplit), biofat (substrat + biofat dengan level 0,75  $\mu$ L/mL), asam anakardat (substrat + asam anakardat dengan level 0,75  $\mu$ L/mL). Peubah yang diukur: produksi gas total, metana, pH, konsentrasi amonia ( $\text{NH}_3$ ), degradasi bahan kering (DBK), degradasi bahan organik (DBO), dan degradasi serat (DNDF) dalam rumen. Hasil isolasi asam anakardat menghasilkan rendemen sebanyak 8 g dari 10 g biofat. Hasil analisis GC-MS menunjukkan bahwa proses isolasi asam anakardat dari biofat menghasilkan senyawa murni (99,44%) dan dapat menurunkan produksi metana secara signifikan dibanding kontrol, sebesar 51,21% oleh biofat dan 39,62% oleh anakardat. Hasil degradasi asam anakardat dalam fermentasi rumen secara *in vitro* menunjukkan bahwa perubahan nilai faktor retensi (Rf) setelah diinkubasi. Disimpulkan bahwa asam anakardat yang diisolasi dari biofat memegang peran dominan untuk menurunkan metana pada fermentasi pakan di dalam rumen secara *in vitro*. Asam anakardat lambat didegradasi oleh bakteri rumen sehingga efektivitasnya dalam menekan gas metana sangat potensial.

**Kata kunci:** anakardat, kulit cangkang mete, metan, fermentasi rumen

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## INTRODUCTION

The main product of cashew trees (*Anacardium occidentale*) are cashew nuts, while the by products are the pseudo-fruits and the cashew nut shells. The processing of cashew nut shell produces bioindustrial product called biofat or better known as CNSL (*Cashew Nut Shell Liquid*). Biofat contains bioactive substance called anacardic acid. Biofat or CNSL has been widely used for pharmaceutical, cosmetics, adhesives, insecticides, or mosquito repellent (Risfaheri, 2011) and is lately used as animal feed additives for ruminants to suppress methane production in the rumen (Watanabe *et al.*, 2010; Shinkai *et al.*, 2012; Mitsumori *et al.*, 2014). Methane is one of the gases that damage the ozone layer and had 23 times greater effect than carbon dioxide (Vlaming, 2008). By suppressing the production of methane in the rumen, biofat was predicted to be a very useful product to create a friendly environment of farm condition (Mitsumori *et al.*, 2014). Biofat contained 70% anacardic acid, 20%-25% cardol, and the rest was cardanol and methyl cardol (Saenab *et al.*, 2016). Information about the factors in biofat/CNSL that are important in suppressing methane in the rumen is not yet known. Watanabe *et al.* (2010) assumed that anacardic acid is the main factor that reduce methane production in the *in vitro* fermentation. However, they did not isolate anacardic acid from CNSL to prove the role of anacardic acid in reducing methane.

The mechanism of decreasing methane production according to Watanabe *et al.* (2010) was that the phyto-genic compounds contained in cashew nut shells (including anacardic acid) selectively inhibited some gram (+) bacteria in the rumen, so that the increased gram (-) bacteria are apparently associated with propionate production. Additionally, the anacardic acid acts as an antibacterial surfactant that can damage the membrane of the gram (+) bacteria. Therefore, this study aimed to examine the effectiveness of anacardic acid addition to substrate in the *in vitro* rumen fermentation, especially on methane production and to study the anacardic acid degradation in the *in vitro* rumen fermentation.

## MATERIALS AND METHODS

The research was conducted at the Laboratory of Indonesian Research Institute for Animal Production (IRIAP) Ciawi, Bogor, Indonesia. The GC-MS analysis was conducted in the Jakarta Regional Health Laboratory. The cashew nut shells was obtained from cashew plantation in Pati Regency, Central Java Province, Indonesia.

### Preparation of Cashew Nut Shells

The shells of cashew nuts that had been separated from the nuts were dried in an open sun drying. The dried shells were grinded into smaller particles using a laboratory blender and screened to obtain small size particles (2 mm diameter). Then, the small size cashew nut shells were processed into bioindustrial products namely oil (Biofat).

## Preparation of Biofat Product

The preparation of biofat products used the modified method of Simpen (2008). The method was described in Saenab *et al.* (2016).

### Isolation of Anacardic

Anacardic acid was isolated using modified method of Gandhi *et al.* (2012). Briefly, 10 g of biofat were dissolved in 120 mL of 5% methanol solution. While stirring the biofat solution on a stirred hot plate at 50°C, 10 g of Ca(OH)<sub>2</sub> (Calcium hydroxide) was added slowly with continuous stirring for 3 h until calcium anacardate was precipitated. Then, the precipitate was filtered and rinsed with 100 mL of methanol, and the precipitate was poured into 100 mL of water. Then, 15 mL of 11 N hydrochloric acid was added. The samples were then stirred for 1 h at room temperature. After that, the solution was extracted with hexane (2 × 100 mL) and shaken. Hexane layer was separated from the aqueous solution. Furthermore, the hexane extracts were evaporated with a rotary evaporator at 40°C until the remained was a thick dark brown fraction called anacardic acid. Characterization of the anacardic acid isolate was conducted qualitatively by TLC and GC-MS.

Aluminium plate used for TLC was a 10 x 10 cm Silica gel 60 F254. Eluent1 was a mixture of Diethyl ether (30) + Petroleum ether (70) + formic acid (1) and eluent 2: Diethyl ether (28) + Petroleum ether (70) + formic acid (2). The eluents were put into a TLC tank. The TLC plate was inserted after the tank became saturated. After being finished, the plate was removed from the tank. The plate was set aside to dry and then the plate was sprayed with phosphomolybdic acid solution (0.25 g dissolved in 10 mL of ethanol). The plate was then placed in the oven at temperature of 105°C for 15 min. Blue spot as bioactive compounds will appear with a light yellow background.

Concentration of anacardic acid was analysed using GCMS-QP2010, Shimadzu equipped with column Capiler Type Phase Rtx; 60 m x 0.25 mm LD. The carrier gas was helium. Temperature of column, SPL, MS interspace, pyrolysis were 50, 280, 280 and 400°C, respectively. Inlet Press was 101.0 kPa.

### Preparation of a Complete Feed for *In Vitro* Fermentation

Complete feed for cattle consisted of elephant grass, gliricidia leaves, yellow corn, copra meal, molasses, rice bran, urea, salt (NaCl), limestone (CaCO<sub>3</sub>), and premix. The nutrient composition of complete feed consisted of 15.63% CP, 3.83% CF, 3785 Gross Energy (kcal/kg), 6.85% Ash, 28.41% NDF, 15.02% ADF, 0.69% Ca, 0.29% P, and 69.7% TDN.

This animal feed was used in the *in vitro* test. The rumen fluid was taken from a fistulated *Friesian Holstein* crossbreed dairy cow (FH) at the IRIAP Ciawi Bogor. The cow was fed a mixture of napier grass and commercial concentrates. The rumen fluid was collected before the morning feeding and filtered using a nylon cloth,

then inserted into a thermos. Once collected, the rumen fluid was brought to the laboratory.

#### Analysis of *In Vitro* Fermentation

The rumen buffer liquid composition (in 1000 mL) consisted of 241 mL bicarbonate buffer solution, 121 mL macro-mineral solution, 61 mL micro-mineral solution, 0.61 mL resazurin, 362 mL distilled water, 23 mL reducing solution, and 253 mL rumen fluid (Makkar, 2003).

The samples were incubated following the methods of Theodorou *et al.* (1994). Substrates of about 750 mg were weighed and put into the incubation bottle. Complete animal feed, biofat, and anacardic acid were added to the tube according to the treatment. *In vitro* incubation used the mixture of rumen fluid and buffer solution as media. Prior to addition to the sample, the rumen-buffer solutions were flushed with  $\text{CO}_2$  gas. Then, 75 mL of rumen-buffer solutions were poured into each incubation bottle and immediately sealed with a rubber cap. Each bottle was incubated in the water bath (Lab master, Anax-Pty, Limited) at 39°C and incubated for 48 h. During the incubation period, the shaking process of the bottle was done manually. Gas production was then measured at 3, 6, 9, 12, 24, 30, and 48 h of incubation. The samples were taken to measure the levels of ammonia and fermented pH solutions at the 4<sup>th</sup> hour of the incubation process.

#### The Experimental Design

This study was arranged in a completely randomized block design. Three treatments were the control (substrate), biofat (substrate + biofat at the level of 0.75  $\mu\text{L}/\text{mL}$  buffer mixture), anacardic acid (substrate + anacardic acid at the level of 0.75  $\mu\text{L}/\text{mL}$ ). These three treatments were prepared in duplicate. The entire *in vitro* experiment were repeated 4 times.

Variables measured were total gas production, methane, pH, concentration of  $\text{NH}_3$ , DMD, OMD, and NDFD in the rumen, as well as anacardic acid degradation.

#### Determination of Anacardic Acid Degradation

Approximately 750 mg of elephant grass as substrates and 75 mL of rumen buffer solution were added into the 100 mL bottle. Anacardic acid was added to rumen-buffer solution to obtain the concentration of 0.75  $\mu\text{L}/\text{mL}$ . The bottles containing the mixture were incubated in a water bath at a temperature of 39°C for 48 h. At 3, 6, 12, 24, 48 h of incubations, 5 mL were taken from each sample and extracted twice with 5 mL hexane. Hexane fractions were collected and evaporated in a rotary evaporator, then the residues were redissolved in 2 mL ethyl acetate to be ready to be used for qualitative analyses by TLC with the same method as anacardic analysis.

## RESULTS

#### Isolation and Characteristics of Anacardic Acid

Eight grams of isolated anacardic acid were obtained from the initial weight of 10 g biofat sample. Qualitatively, the results from the TLC were shown in Figure 1 A&B. The biofat product showed three spots of different compounds each with  $R_f$  (0.53),  $R_f$  (0.37), and  $R_f$  (0.20) (Figure 1A). The isolated anacardic compounds showed the same spot with  $R_f$  value similar to the major spot of biofat, but in Figure 1B, the biofat product showed three different compounds with  $R_f$  (0.68),  $R_f$  (0.63), and  $R_f$  (0.47) and the anacardic acid compound produced two spots similar to the major spots in biofat.

The result of GC MS (Figure 2) showed that there was a very high peak at 35.317 minutes as the main peak. The compound had a value of 540 m/z. The fragmentations were: 355, 327, 302, 267, 230, 207, 175, 161, 147, 135, 121, 108, 97, 95, 81, 79, 67, and 55. The analysis showed that the concentration of anacardic acid compounds was 99.44%.

#### Effects of Anacardic Acid and Biofat on *In Vitro* Rumen Fermentation

Figures 3 and 4 showed that the addition of biofat and anacardic acid significantly decreased the production of methane and the total gas compared to the control. Addition of biofat and anacardic acid decreased methane productions by 51.21% and 39.62%, respectively. Meanwhile, additions of biofat and anacardic acid decreased total gas by 43.33 and 39.34%, respectively.

The additions of biofat and anacardic significantly decreased ( $P<0.01$ ) pH value compared to the control. However, the administration of biofat did not change ammonia level in the rumen. The results of the analysis (Table 1) showed that the dry matter degradation in the rumen significantly decreased ( $P<0.01$ ) and the degradation of organic matter and NDF also decreased

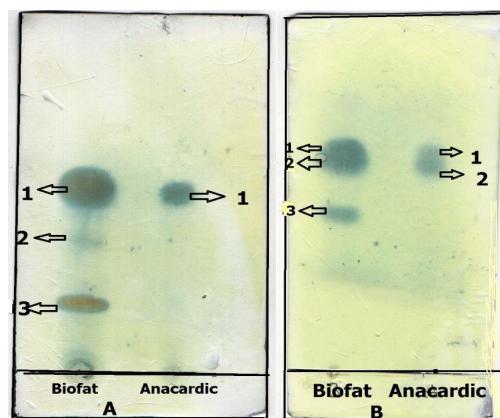


Figure 1. Anacardic and biofat separation on the TLC plate with eluent. Eluent A= a mixture of diethyl ether (30) + petroleum ether (70) + formic acid (1); Eluent B= a mixture of diethyl ether (28) + petroleum ether (70) + formic acid (2).

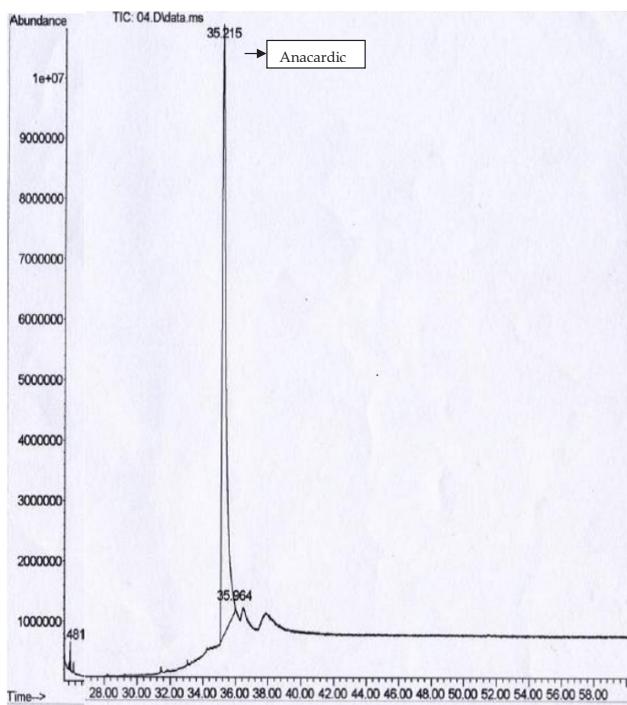
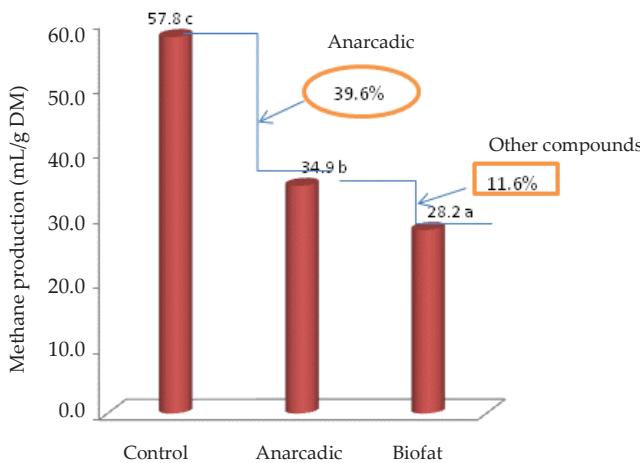


Figure 2. Chromatogram of anacardic acid analysed by GC-MS

Figure 3. Methane production at 48 hours of incubation in the *in vitro* rumen fermentation

significantly ( $P<0.05$ ) on the addition of anacardic acid compared to the control.

### Anacardic Acid Degradation During *In Vitro* Rumen Fermentation

The anacardic acid degradation in the *in vitro* rumen fermentation are shown in Figure 5. It was shown in the picture that the  $R_f$  value of anacardic acid before incubation was 0:55. After being incubated for 3-48 h, the spot with different intensities had the  $R_f$  value of 0.70. The strongest intensity of the spot was found at 12 h of incubation. After 24 hours of incubation, the spot was getting thinner and finally disappeared after 48 h of incubation.

## DISCUSSION

### Isolation of Anacardic Acid from Biofat

From the isolation process, the yield of anacardic acid amounted to 8 g from 10 g biofat or approximately 80%. The result were higher than the yield obtained by Gandhi *et al.* (2012) who isolated 50 g of CNSL and generated yield of 26 g, or about 52%. The differences in yield may be due to the different methods used to obtain biofat. The solution used in this experiment was 5% methanol, while Gandhi *et al.* (2012) used acetone that has different polarities than 5% methanol solution.

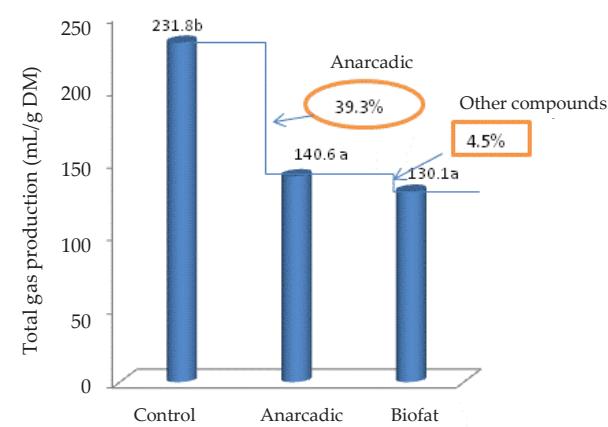
Figure 4. Total gas production at 48 hours of incubation in the *in vitro* rumen fermentation

Table 1. Ammonia and pH levels of rumen fluid (after 4-hours incubation), DMD, OMD, and NDFD of feed in control, biofat, and anacardic treatments

Treatments	$\text{NH}_3$ (mM)	pH	DMD (%)	OMD (%)	NDFD (%)
Control	5.71	6.73 <sup>b</sup>	67.80 <sup>b</sup>	69.23 <sup>b</sup>	68.70 <sup>b</sup>
Biofat	5.93	6.68 <sup>a</sup>	58.78 <sup>a</sup>	60.28 <sup>a</sup>	51.80 <sup>a</sup>
Anacardic	6.01	6.69 <sup>ab</sup>	63.73 <sup>b</sup>	66.35 <sup>ab</sup>	55.12 <sup>a</sup>
SE	0.51	0.01	1.24	2.15	3.42
P Value	0.871	0.044	0.006	0.064	0.028

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

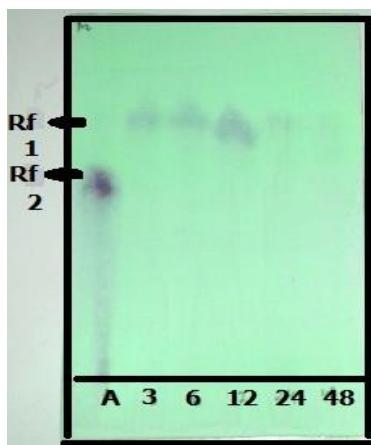


Figure 5. TLC separation of anacardic acid degradation during 48-hours incubation in the *in vitro* rumen fermentation. Note: A (anacardic acid); 3, 6, 12, 24, 48 (incubation hours).

The isolated compound was analyzed using TLC that showed different results depend on the eluent used. Both eluents were non-polar mixture and the addition of formic acid was more in the second eluent. This caused the second eluent to be slightly more polar. For the first eluent, the flow rate of eluent was faster, so it was unable to separate the spot with the Rf that was almost the same, while the second eluent which was slightly more polar, the eluent flow rate was slower so it could be separated into two spots. Njuku *et al.* (2014) stated that there were 4 types of anacardic acid compounds with different concentration levels, namely  $C_{15}:0$  (saturated) with a concentration of 3%,  $C_{15}:1$  with a concentration of 34%-36%,  $C_{15}:2$  with a concentration of 21%-22%, and  $C_{15}:3$  with a concentration of 40%-41%. It is possible that anacardic acids with higher concentrations were appeared on this TLC plate. There was a possibility that saturated anacardic acid did not appear because the concentration was very small.

Chromatogram results of GC-MS analysis (Figure 2) showed that the anacardic acid isolation process of the biofat product succeeded to obtain a pure compound (99.44%). Fragments obtained from the GC-MS were equal to those reported by Silva *et al.* (2008) and Saenab *et al.* (2016) who showed that the obtained compound was anacardic acid. In addition, the content of anacardic acid compounds in biofat from the cashew nut shells amounted to 78.16% and with the isolation process, the amount obtained increased to 99.44%. Therefore, the other compounds such as cardol, cardanol, and methyl cardol can be separated from anacardic acid compounds during the isolation process (Gandhi *et al.*, 2012). Furthermore, the results of this isolation were used to test the effectiveness and degradability of anacardic acid in the rumen fluid *in vitro*.

#### *In Vitro* Methane and Gas Production

The production of methane and total gas (Figures 3 and 4) was lower by the addition of anacardic acid

and biofat compared to the control. The inhibition of methane production by anacardic acid was lower than biofat. However, as part of bioactive compounds present inside the biofat, anacardic acid contribution to the total reduction in methane amounted to 77.36% while the contribution of other compounds amounted to 22.64%. This showed the dominant role of anacardic acid to suppress methane, which was 3 times higher than the role of other bioactive compounds in the biofat. The other compounds besides anacardic acid are cardol, cardanol, dimethyl cardol (Rodrigues *et al.*, 2011; Gandhi *et al.*, 2012; and Njuku *et al.*, 2014), 2-(800Z-eicosenoyl)-6-(80Z-pentadecenyl) salicylic acid, 2-(900Z-hexadecenoyl)-6-(80Z, 110 Z-pentadecadienyl) methyl salicylate, 2-(1000Z, 1300Z-nonadecadienoyl)-6-(80Z, 110Z-pentadecadienyl) salicylic acid, 2-(1600Z-pentacosenoyl)-6-(80Z-pentadecenyl) salicylic acid, and 2-(900Z-octadecenoyl)-6-(80Z, 110 Z-pentadecadienyl) methyl salicylate (Suo *et al.*, 2012).

In the previous study, Watanabe *et al.* (2010) reported that anacardic acid reduced methane production. However, they did not conduct the anacardic acid isolation process. They only changed the composition of CNSL (biofat) by heating process at high temperature so that the anacardic acid turned into cardanol. They reported that the heated CNSL which contained cardanol (73.9%) had no effect in lowering methane, whereas the unheated CNSL which contained anacardic acid (62.3%) can reduce methane production.

Biofat can decrease methane because biofat contains several bioactive compounds, one of which is the anacardic acid. The content of anacardic acid in biofat is very high and as the major bioactive compound, it gives a dominant influence of lowering methane. This can be seen from the results of the study (Figure 3) where the acid decreased methane (39.6%). These anacardic acids are antibacterial and can suppress methanogen activity thereby decreasing methane.

The inhibition of the total gas formation by anacardic acid was amounted to 39.34% and by biofat was amounted to 43.33%. However, inhibition by other compounds in biofat was only 4.52%. This research showed the dominant role of anacardic acid to suppress total gas was 8 times higher than other bioactive compounds. This result showed that anacardic acid did not only inhibit the formation of methane but also inhibited the formation of other gases that were the results of the activities of other microbes in the rumen. Inhibitions of the activities of rumen microbe were indicated by the decreased degradabilities of dry matter, organic matter, and neutral detergent fiber of the feed in this experiment. It was confirmed by the study of Watanabe *et al.* (2010) who reported that the population of some bacteria in the rumen were reduced due to the addition of unheated CNSL, namely *Prevotella ruminocola*, *Prevotellabryantii*, *Treponemabryantii*, *Ruminococcus flavigravies*, and *Fibrobacter succinogenes*. These bacteria have the ability to break down fiber and capable of forming propionate and succinate.

Anacardic acid showed negative influences on the activity and the population of methanogenic microbes and the other bacteria. This may be caused by the ana-

cardic acid structures which have phenolic cluster and unsaturated fatty acids. It was reported that phenolic had negative effect on rumen bacteria (Jayanegara *et al.*, 2011) and unsaturated fatty acids (PUFAs) were also toxic to rumen bacteria (Kim *et al.*, 2009).

The mechanism for reducing methane may also be similar to the mechanism by other phenolic compounds which inhibit the methanogenic microbes and fiber degrading bacteria that eventually decrease the degradability. In contrast to tannin which inhibited protein degrading bacteria, the anacardic acids in this experiment did not inhibit protein degrading bacteria and also did not inhibit the formation of  $\text{NH}_3$ . Besides, anacardic acid is a powerful amphiphilic lipid phenolic that can interact with the cell's wall or membrane structure and the hydrophobic domain of the protein (Kim *et al.*, 2009 and Watanabe *et al.*, 2010).

### Anacardic Acid Degradation in the *In vitro* Rumen Fermentation

The anacardic acid degradation in the rumen was analyzed by using TLC (Figure 4) showing a shift of Rf value of anacardic acid compounds after being incubated in the rumen. It was assumed that before incubation, the thick spot (Rf 0.55) were the unsaturated anacardic acid compounds. It was reported by Tyman *et al.* (1996) that the fatty acids of anacardic acid compound in the saturated form have higher Rf value than those in the unsaturated form. This result implied that during the incubation in the rumen, there was a change in the chemical structure of anacardic acid. After 12 hours of incubation, a thicker spot was visible, showing that the entire unsaturated anacardic acid became all saturated. This process of structure change from unsaturated to become saturated form is called hydrogenation process which is carried out by the rumen bacteria (Kim *et al.*, 2009 and Lejonklev *et al.*, 2013). The hydrogenation process is bacteria's strategy to prevent itself because unsaturated fatty acid is toxic to rumen bacteria (Kim *et al.*, 2009). After 24-48 h, the spot was disappeared. This result was probably due to the degradation of anacardic acid into simple phenol and free fatty acids. A complete disappearance of anacardic acid in the *in vitro* rumen took rather long time (24 hour of incubation) suggesting that anacardic acid was not easily degraded in the rumen. Furthermore, phenolic acids may be degraded by enzymes into simpler compounds (Cao *et al.*, 2015). Some fatty acids are absorbed by the rumen and some are used by the microbes itself (Jenkins *et al.*, 2008).

### CONCLUSION

Anacardic acid isolated from biofat has a dominant role in the biofat product to decrease methane production in the rumen fermentation. As it is slowly degraded by rumen bacteria, anacardic acid is very potential as a methane reducing agent. Considering its effect to reduce degradability in the rumen, the level of anacardic acid to be used as a methane reducing agent should be further evaluated *in vivo*.

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