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Liquid Smoke as Fat Protector and Its Effect on Rumen Fermentation Characteristics and Microbial Activity

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ABSTRACT

This study was conducted to determine the effect of liquid smoke as a fat protector on unsaturated fatty acids (UFAs) and its effect on rumen fermentation characteristics and microbial activity. Crude palm oil (CPO) was mixed with Prosteo skim milk (1:2), then divided into three treatments i.e., crude palm oil without protection by liquid smoke as a control (P0), crude palm oil protected by 2.5% of liquid smoke (P1), and crude falm oil protected by 5.0% of liquid smoke (P2). For *in vitro* testing, 300 mg of the feed substrate (elephant grass and bran with the ratio of 60:40) was added with 5% of each crude palm oil preparation of P0, P1, and P2 and put in a fermentor syringe. Then, 30 mL of the mixture of rumen fluid and buffer-minerals solution (1:2) was added into each syringe fermentor and flushed with CO_2. The fermentor syringes were incubated in a water bath at 39°C for 48 hours. Variables measured were fatty acid composition, fermentation characteristics, and rumen microbial activity. The data were analyzed by the analysis of variance with a completely randomized design. The results showed that the protection of CPO with liquid smoke in P1 and P2 groups decreased saturated fatty acids (SFAs), but increased (p<0.01) monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and CMCase activity. Protection of CPO with 2.5% of liquid smoke (P1) significantly increased (p<0.01) fermentation characteristics (NH_3 content and pH). It can be concluded that the use of 2.5% of liquid smoke has a better effect on feed fats protection, because it can reduce hydrogenation, increase UFAs, and has no negative effects on fermentation characteristics and microbial activity.

Keywords: hydrogenation; liquid smoke; rumen fermentation characteristics; rumen fluid fatty acids; rumen microbial activity

INTRODUCTION

Feeding ruminants with fats that contain high unsaturated fatty acids (UFAs), the benefits of the UFAs will be disappeared, because most UFAs, especially polyunsaturated fatty acids (PUFAs) in the feed will be hydrogenated by rumen microbes (Abuelfatah *et al*., 2016) and converted into saturated fatty acids.

Microbial biohydrogenation is the process of converting unsaturated fatty acids into more saturated end products by rumen microbes (Li *et al*., 2012), especially stearic acid (C18:0) (Harfoot & Hazlewood, 1997). As a result, linoleic acid (C18: 2 n-6) and linolenic acid (C18: 3 n-3) in the feed are hydrogenated by rumen microbes (70%-95% and 85%-100%, respectively) into MUFA and SFA and only a small portion (about 10% of the feed consumed) enters the fat tissue (Nieto & Ros, 2012). High SFAs cause meat fat to be harder with high cholesterol levels, which causes people to avoid eating meat because meat with high SFA and cholesterol can trigger cardiovascular disease (CVD) (Soliman, 2018; Liu *et al*., 2019).

As a result of hydrogenation, the use of UFAs in ruminant feed is limited (Tran *et al*., 2017). The use of high fat in feed is toxic to rumen microbes that eventually inhibits fiber digestion and reduces feed intake (Behan *et al*., 2019). This condition is caused by the covering of the surface of the feed and wrapping of the rumen microbial cells by the UFAs that eventually inhibit the penetration of enzymes into the feed.

One way to reduce the hydrogenation of UFAs and maximize the use of high UFAs is to protect feed fat from hydrogenation with a technical formaldehyde. The mechanism is that the aldehyde from formaldehyde will join or form strong bonds with amino acids from a protein source, thus protecting the fat inside. These bonds that protect fats inside are resistant to neutral pH in the rumen (pH 6-7) but the bonds will be disrupted at the acidic pH in the omasum (pH 2-3) that eventually release the fat and UFA so that it can be

absorbed in the small intestine (Scott *et al*., 1970). The protection of crude palm oil (CPO) as a source of UFAs in sheep rations using formaldehyde can increase UFAs composition and microbial activity *in vitro* (Tiven *et al*., 2011a, 2011b). As much as 3% of CPO was protected by 2% formaldehyde and can reduce hydrogenation of UFA and increase UFAs concentrations in the blood and meat (Tiven *et al*., 2013), and gives more advantage to feed conversion aspect (Tiven *et al*., 2015). However, this good result cannot yet be applied because formaldehyde is a dangerous and carcinogenic material that can cause cancer (Swenberg, 2013).

The research using natural aldehydes present in plants as fat protectors, including citronellal in Kaffir lime (*Citrus hystrix*) leaves powder increased UFAs *in vitro* (Tiven, 2017) and increased PUFAs, protein, and fat, but reduced cholesterol content in lamb meat (Tiven *et al*., 2016). These results cannot yet be applied to ruminants, because it requires a lot of cinnamon powder and kaffir lime leaves which are very numerous, making it less economical and polluting as well as changing the color of the ration.

The present research used liquid smoke as a protector of feed fat. Liquid smoke contains natural aldehydes in its carbonyl component (Montazeri *et al*., 2013) that can be used to replace technical formaldehyde. Liquid smoke can be used in animal feed because it is an antioxidant (Hadanu & Apituley, 2016), can prevent the growth of fungi and bacteria, prevent free radicals, so that nutrient contents of feed does not decrease (Hatta *et al*., 2018). Liquid smoke is safe for consumption, and it can affect the flavor and product storage time because carbonyl will react to protein with a relatively strong bond (Anggraini & Yuniningsih, 2017).

Protection of feed fats using liquid smoke has never been done before, so it has a very high novelty value. In line with that, this study was conducted to determine the effect of liquid smoke as a fat protector on unsaturated fatty acids (UFAs) and its effect on rumen fermentation characteristics and microbial activity. Protection of feed fats using liquid smoke is expected to reduce the hydrogenation of UFAs, increase UFAs, but do not negatively affect fermentation characteristics and rumen microbial activity so that it can be applied *in vivo*.

MATERIALS AND METHODS

Fat Protection by Liquid Smoke

Before being used, CPO as a source of feed fat was analyzed to get the fat profile (iodine value, saponification value, acid value, and fatty acid composition by following the protocols of Dymińska *et al*. (2017) and Nielsen (2019). CPO was mixed with Prosteo skim milk (at the ratio of 1:2) evenly manually. Then the liquid smoke was added into the mixture of CPO according the treatment: P0 (CPO without protection by liquid smoke); P1 (CPO protected by 2.5% of liquid smoke); and P2 (CPO protected by 5% of liquid smoke), and the mixture was then mixed evenly manually.

In Vitro Gas Test

As much as 300 mg of the feed substrate and 5% (15 mg) of CPO mixture with 0%, 2.5%, or 5% liquid smoke according to the treatments were put into fermentor syringes. Then, 30 mL of a mixture of rumen fluid and buffer-minerals solution (1:2 v/v) was added and the syringe was closed with a piston according to Tilley & Terry (1963). The rumen fluid, as the microbial source, was obtained from two local female sheep by the trocar method (Tiven, 2012). The syringe flowed with CO_2 gas so that the atmosphere becomes anaerobic, then the rubber pipe at the end of the syringe was clipped, then the syringe was put into water bath at the temperature of 39°C to be incubated for 48 hours. During the fermentation process, gas production was recorded at intervals of 0, 2, 4, 6, 8, 12, 24, and 48 hours (Menke & Steingass, 1988). After the fermentation process was stopped, the fermentor syringe containing fermentation fluid was divided into two groups, namely (1) for testing fatty acids and (2) for testing fermentation characteristics and microbial activity.

Fatty Acid Test

After the incubation process was completed, the fermentation fluid in the syringe was poured into a 100 mL Erlenmeyer for the measurement of fatty acids according to Park & Goins (1994), modified. The process of fat extraction in fermented liquid obtained from the fermentation fluid was conducted by adding 20 mL of a mixture of chloroform-methanol (2:1) and let stand until the formation of two layers. The top layer (supernatant) was removed, while the lower layer (sediment) was taken and filtered into a 50 mL test tube through filter paper. A total of 4 mL of 0.88% NaCl was added to the test tube and shaken out and then allowed to stand until two layers were formed again. The top layer was removed and the bottom layer was filtered again into a test tube through anhydrous Na_2SO_4 on filter paper to bind water that might still exist. The test tube was drained with N2 to remove chloroform.

The remaining fat in the test tube was methylated. A total of 1 µL of the supernatant (top layer) of the methylation result was injected into the gas chromatography Shimadzu GC 2010 Plus 2017, with Helium as a carrier gas with a flow rate of 40 mL/min and as a burning gases were hydrogen and oxygen. The column used was the capillary column Qtx-wax with a length of 30 meters and an inner diameter of 0.25 mm (Nielsen, 2019). The fatty acid composition was calculated with relative percentages (Nugraheni *et al*., 2015):

Fatty acid= [Area of fatty acid / (Total area of sample - Area of solvent)] x 100%

Fermentation Characteristics and Microbial Activity Test

Fermentation characteristics tested were NH_3, VFA, and rumen fluid pH, while microbial activity tested were CMC*ase* activity and microbial protein. After the

fermentation process was stopped, the fermentation fluid was centrifuged at a speed of 500 g for 15 minutes to separate the feed particles, then the filtrate is centrifuged again with a speed of 10,000 g for 15 minutes to separate microbial sediment. The filtrate was taken for the determination of CMC*ase* activity based on the amount of reducing sugar formed from the CMC substrate (Halliwel *et al*., 1985), expressed in the activity of specific enzymes with the measuring protein enzyme levels using the Lowry method, $N\text{-}NH_3$ (Weatherburn, 1967), total VFA by gas chromatography method (Jayanegara *et al*., 2016), and pH (Suharti *et al*., 2018). Microbial sediment was dissolved by adding 0.5 mL of 1N NaOH and then heated at 90°C for 10 minutes. After dissolving, distilled water was added to reach a total volume of 1 mL. The determination of the amount of biomass was based on the protein content of microbial cells by the Lowry method by using spectrophotometer.

The $N\text{-}NH_3$ test was carried out according to Weatherburn (1967). A total of 1-20 µL of the filtrate were entered into the test tube, added with 5.0 mL of reagent A (5 g of phenol and 25 mg of sodium nitroprusside) and reagent B (2.5 g of sodium hydroxide and 4.2 mL of sodium hypochloride). Fortex each test tube, then incubated for 30 minutes at room temperature and measured on a spectrophotometer with a wavelength of 625 nm. As a standard solution, 14.4 mg ammonium sulfate dissolved in distilled water to 100 mL volume, 1 µN solution contains 0.3 µg $N\text{-}NH_3$.

The VFA test was carried out according to Doreau *et al.* (1993). The filtrate was centrifuged 3000 g for 15 minutes, then 0.2 mL of filtrate was added with 1 mL of metaphosphoric acid. The filtrate was centrifuged again with 9000 g for 10 minutes to measure the levels of VFA using gas chromatography (GC) Shimadzu GC 2010 Plus 2017.

CMC*ase* activity was carried out according to Halliwel *et al*. (1985). The filtrate about 13,000 g was centrifuged for 15 minutes for determination of CMC-*ase* activity. All tubes were incubated at 39°C for 45 minutes. The enzyme activity was stopped by adding a solution consisting of 1 mL of carbonate cyanide, 0.2 mL of sodium carbonate, and 2 mL of 0.05% potassium ferricyanide. The contents of the tube are homogenized with vortices. All tubes were heated in boiling water for 30 minutes, then the results obtained were read by a spectrophotometer at a wavelength of 420 nm. The standard graph was made using glucose levels of 1.39-19.44 mg/mL in a sample of 1.8 mL. The tube containing glucose was treated the same as the steps above so that absorbance data were obtained. The CMC-*ase* activity was calculated based on a formula: The absorbance unit= (BL-ES) - (BL-E) - (BL-S), where ES was enzyme and sample, E was enzyme, S was sample, and BL was blank. The result from the absorbance unit (Y) is entered into the regression equation of the standard glucose graph equation, Y= 0.0303x + 0.0076 to determine the level of CMC-acid.

Microbial protein was carried out according to Plummer (1987). The precipitate from the centrifugation of 13,000 g was used to measure the microbial protein content using the Lowry method. A sample of 0.5 mL was put into a test tube then added with 2.5 mL of Lowry I solution (2% Na_2CO_3 in 0.1 NaOH, 2% sodium tartrate, 1% $CuSO_4 5H_2O$, then mixed with a ratio of 100: 1:1) and let stand at room temperature for 10 minutes. The sample was then added with 0.25 mL of Lowry II solution (1 N Folin ciocalteceau, H_2O, mixed with a ratio of 1:1), and let stand at room temperature for 30 minutes, then read using a spectrophotometer at a wavelength of 750 nm. Standard charts were made using Bovine Serum Albumin (BSA) with levels of 0.00-0.27 mg/mL. From the absorbance (Y) of the sample obtained, it will be known the dissolved protein content (X) by entering into the regression equation from the albumin standard graph equation: Y= 2.1706x + 0.03281.

Statistical Analysis

The data obtained were statistically tested using analysis of variance used completely randomized design, with three treatments, namely CPO without protection by liquid smoke (P0), CPO protected by 2.5% of liquid smoke (P1), and CPO protected by 5.0% of liquid smoke (P2) with five replications. Significant treatment was tested further by Duncan's New Multiple Range Test (Gupta *et al*., 2016).

RESULTS

Lipid Profile and Fatty Acid Composition of Crude Palm Oil (CPO) before and after being Protected by Liquid Smoke

The qualities of CPO were determined by measuring the fat profile (iodine value, saponification value, acid value), which are presented in Table 1. The iodine value, saponification value, acid value, and peroxide value of CPO used in this research were 36.27 g I_2/100 g, 182.84 mg KOH/g, 6.98 mg KOH/g, and 0.30 mg KOH/g, respectively. The fatty acids composition of CPO used in this study with the contents of SFA, MUFA, and PUFA were 60.42%, 30.21%, and 8.41%, respectively.

Fatty acid composition of CPO before and after being protected by liquid smoke (before fermentation) are presented in Table 2. The results shows that there is no significant effect (increase or decrease) of the treatments to the fatty acis contents.

The Fatty Acid Composition of Rumen Fluid After Fermentation of Crude Palm Oil (CPO) Protected by Liquid Smoke

The results of the statistical tests shows that there are significant effects ($p<0.01$) of CPO protection by liquid smoke on SFAs, MUFAs, and PUFAs in rumen fluid after *in vitro* fermentation (Table 3). When compared between treatments, the SFAs in the CPO protected by 2.5% and 5.0% of liquid smoke, P1 and P2, were lower, while MUFA and PUFA were higher compared to the unprotected CPO (P0).

When compared to CPO without protection by liquid smoke (P0), after being fermented, CPO protected with 2.5% (P1) and 5% (P2) of liquid smoke had lower

Table 1. The average fat profiles of crude palm oil (CPO) used in the study

Fat profiles	Units	Compositions
Iodium value	g I2/100g	36.27±0.03
Saponification value	mg KOH/g	182.84±0.11
Acid value	mg KOH/g	6.98±0.03
Peroxide value	mg KOH/g	0.30±0.01
Fatty acid :		
Caprylic	%	0.08±0.05
Capric	%	0.07±0.01
Lauric	%	0.63±0.04
Myristic	%	2.20±0.11
Palmitic	%	54.71±0.49
Palmitoleic	%	0.23±0.01
Stearic	%	2.74±0.10
Oleic	%	29.98±0.66
Linoleic	%	8.09±0.35
Linolenic	%	0.17±0.01
Arachidic	%	0.15±0.00
SFAs	%	60.42±0.43
MUFAs	%	30.21±0.65
PUFAs	%	8.41±0.37
Total	%	99.03±0.34

Note: SFAs= Saturated fatty acids, was calculated from Caprylic, Capric, Lauric, Myristic, Palmitic, Stearic; MUFAs= Monounsaturated fatty acids, was calculated from Palmitoleic, Oleic; PUFAs= Polyunsaturated fatty acids, was calculated from Linoleic, Linolenic, Arachidic; The total was calculated from the number of SFAs, MUFAs, PUFAs.

(p<0.01) SFAs by 10.47% and 2.61%, respectively. CPO protected by 2.5% of liquid smoke (P1) had higher (p<0.01) MUFAs and PUFAs, by 5.81% and 5.27%, respectively, while CPO protected by 5.0% of liquid smoke (P2) had higher (p<0.01) MUFAs and PUFAs, by 2.37% and 1.51%, respectively.

Fermentation Characteristics and Rumen Microbial Activities

The fermentation characteristics and rumen microbial activities of rumen fluid after fermentation of CPO protected by various concentrations of liquid smoke are presented in Table 4. The results of the statistical tests showed that there were significant effects (p<0.01) of CPO protection by liquid smoke on NH_3, pH, and CMC-ase, but there was no significant effects on total VFA and microbial protein in rumen fluid during *in vitro* fermentation. CPO protected by 2.5% of liquid smoke had significantly higher NH_3, pH, and CMC-ase compared to control CPO without protection by liquid smoke and CPO protected by 5.0% liquid smoke.

When compared to CPO without protection by liquid smoke (P0), after fermentation, CPO protected by 2.5% liquid smoke (P1) had higher NH_3 and CMC-ase activity by 10.43 mg/100 mL and 6.98 U/g, respectively, compared to control CPO without protection of liquid smoke. When the level of liquid smoke increased to 5% (P2), NH_3 content decreased by 0.72 mg/100 mL, whereas CMC-ase activity only increased by 1.68 U/g.

Table 2. Fatty acid composition (%) of crude palm oil (CPO) before and after being protected by liquid smoke (before fermentation)

Fatty acids	Treatments		
	P0	P1	P2
Caprylic	nd	nd	nd
Capric	0.09±0.00	0.10±0.00	0.10±0.01
Lauric	0.68±0.04	0.69±0.01	0.71±0.07
Myristic	2.26±0.13	2.64±0.05	2.96±0.16
Palmitic	57.28±0.86	56.67±0.14	57.48±0.42
Palmitoleic	0.21±0.00	0.92±0.08	0.34±0.10
Stearic	2.53±0.11	2.61±0.01	2.52±0.10
Oleic	28.82±0.71	27.68±0.44	27.78±0.28
Linoleic	6.93±0.08	6.51±0.03	6.54±0.22
Linolenic	0.16±0.00	0.24±0.01	0.18±0.02
Arachidic	0.12±0.01	0.11±0.01	0.11±0.01
SFAs	62.83±0.91	62.71±0.22	63.76±0.57
MUFAs	29.03±0.71	28.60±0.35	28.12±0.38
PUFAs	7.20±0.08	6.86±0.04	6.82±0.25
Total	99.06±0.11	98.16±0.10	98.70±0.06

Note: P0= CPO without protection by liquid smoke; P1= CPO protected by 2.5% of liquid smoke; P2= CPO protected by 5.0% of liquid smoke; nd= not detected. SFAs= Saturated fatty acids, was calculated from Caprylic, Capric, Lauric, Myristic, Palmitic, Stearic; MUFAs= Monounsaturated fatty acids, was calculated from Palmitoleic, Oleic; PUFAs= Polyunsaturated fatty acids, was calculated from Linoleic, Linolenic, Arachidic; The total was calculated from the number of SFAs, MUFAs, PUFAs.

DISCUSSION

Lipid Profile and Fatty Acid Composition of Crude Palm Oil (CPO) before and after being Protected by Liquid Smoke

The determination of fat profile (iodine number, saponification number, acid number, peroxide number, and fatty acid) of CPO reflects the quality of CPO used as a source of unsaturated fatty acids. Iodine value (or Iodine number or Iodine absorption value) is an essential characteristic of fat or oil, expressed by the number of grams of I_2 that will react with double bonds in 100 grams of fat, oil, and wax (Yildiz *et al*., 2019). This variable is used to measure the degree of unsaturated bond in fats or oils (Famobuwa *et al*., 2016). The iodine value of CPO used in this research was 36.27 g I2/100 g. The Iodine value of CPO, according to Malaysian standards, is 50.4–53.7 g I_2/100 g (Japir *et al*., 2017). The lower iodine value found in this study could be due to the lower content of UFAs, i.e., oleic (29.98%), linoleic (8.09%), and linolenic (0.17%).

Saponification values represent the number of milligrams of potassium hydroxide, or sodium hydroxide needed to saponify 1 g of fat or oil, which indicates the molecular weight or length of the fat or oil chain that is present (Sharma *et al*., 2013). Oils or fats that contain fatty acids with short carbon chains have a relatively small molecular weight, so they have large saponification values and vice versa. The saponification value of CPO used in this research was 182.84 mg KOH/g. The saponification value of CPO, according to Malaysian

Table 3. The fatty acid composition (%) of rumen fluid after fermentation of crude palm oil (CPO) protected by liquid smoke

Fatty acids (%)	Level of liquid smoke		
	P0	P1	P2
Caprylic	nd	nd	nd
Capric	nd	nd	nd
Lauric	1.71±0.44^{a}	0.01±0.02^{b}	0.52±0.51^{b}
Myristic	6.25±0.76^{a}	5.08±0.35^{b}	4.65±0.47^{b}
Palmitic	56.42±0.87^{b}	53.61±1.85^{c}	59.95±1.39^{a}
Palmitoleic	0.16±0.16^{b}	2.72±0.31^{a}	0.13±0.23^{b}
Stearic	10.60±0.72^{a}	5.81±1.07^{c}	7.25±0.27^{b}
Oleic	11.79±1.20^{b}	15.03±0.68^{a}	14.17±1.03^{a}
Linoleic	5.55±0.52^{b}	8.70±0.65^{a}	5.77±0.52^{b}
Linolenic	1.15±0.42^{c}	3.27±0.45^{a}	2.44±0.47^{b}
Arachidic	nd	nd	nd
SFAs	74.98±1.13^{a}	64.51±2.79^{c}	72.37±1.20^{b}
MUFAs	11.94±1.33^{c}	17.75±0.38^{a}	14.31±1.02^{b}
PUFAs	6.70±0.27^{c}	11.97±1.03^{a}	8.21±0.78^{b}
Total	93.63±1.17	94.23±3.95	94.89±1.72

Note: P0= CPO without protection by liquid smoke; P1= CPO protected by 2.5% of liquid smoke; P2= CPO protected by 5.0% of liquid smoke; nd= not detected. Means in the same row with different superscripts differ significantly ($p<0.01$). SFAs= Saturated fatty acids, was calculated from Caprylic, Capric, Lauric, Myristic, Palmitic, Stearic; MUFAs= Monounsaturated fatty acids, was calculated from Palmitoleic, Oleic; PUFAs= Polyunsaturated fatty acids, was calculated from Linoleic, Linolenic, Arachidic; The total was calculated from the number of SFAs, MUFAs, PUFAs.

Table 4. Fermentation characteristics and microbial activity of rumen fluid after fermentation of crude palm oil (CPO) protected by liquid smoke

Items	Level of liquid smoke		
	P0	P1	P2
Fermentation characteristics:			
NH_3 (mM)	34.92±1.06^{b}	45.35±1.68^{a}	34.20±2.24^{b}
VFA (mM)			
- Acetic	13.80±1.58	15.74±1.94	14.45±1.07
- Propionic	3.48±0.40	4.15±0.84	4.00±0.66
- Butyric	1.37±0.29	1.84±0.56	1.70±0.45
- T-VFA	18.65±1.70	21.72±2.68	20.15±1.29
- Ratio C2:C3	4.00±0.66	3.87±0.61	3.66±0.36
pH	7.04±0.01^{b}	7.06±0.01^{a}	7.03±0.01^{b}
Microbial activity :			
CMC-ase activity (U/g)	14.00±1.51^{c}	20.98±1.15^{a}	15.68±0.82^{b}
Microbial protein (mg/mL)	0.47±0.03	0.50±0.07	0.47±0.13

Note: P0= CPO without protection by liquid smoke; P1= CPO protected by 2.5% of liquid smoke; P2= CPO protected by 5.0% of liquid smoke; nd= not detected. Means in the same row with different superscripts differ significantly ($p<0.01$).

standards, is 194 to 205 mg KOH/g (Japir *et al*., 2017). The lower saponification number was caused by the high levels of MUFAs and PUFAs.

The acid value is the number (mg) of KOH required to neutralize free fatty acids present in 1.0 g of oil or fat. The acid value of CPO used in this research was 6.98 mg KOH/g. The acid value of CPO, according to Malaysian standards, is less or equal to 10.95 mg NaOH/g (Japir *et al*., 2017). According to Chinedu *et al*. (2017), acid value of different palm oils in local factories in Imo State, Nigeria ranged from 20.76 to 37.59 mg KOH/g with a mean of 28.30 mg KOH/g, while according to Abdulkadir & Jimoh (2013), acid value of reference sample with the mean and standard deviation of its collected samples, respectively were 4.23 mg KOH/g and 11.82±4.54 mg KOH/g. It shows that the CPO used in this study is still in the high quality.

Peroxide value is an index of the amount of fat or oil that has undergone oxidation. This peroxide value is significant for the identification of the level of fat or oil oxidation (Dermis *et al*., 2012). The results showed that the peroxide value of the CPO used in this study was 0.30 mg KOH/g. High peroxide numbers indicate fat or oil has been oxidized, which may be caused by the exposure to oxygen, light, and high temperatures.

From the results of this fat profile test, it can be seen that the CPO can be protected by liquid smoke because it has a relatively good quality, although it has a slight decrease in quality, especially in PUFA, which only decreased by ±2.09%. According to Mancini *et al*. (2015), the fatty acid composition of palm oil is 0.2% lauric, 1.1% myristic, 44.0% palmitic, 4.5% stearic, 39.2% oleic, 10.1% linoleic, 0.4% linolenic, and 0.1% arachidic, with total SFAs, MUFAs, and PUFAs of 49.9%, 39.2%, and 10.5%, respectively. Based on this composition, especially for unsaturated fatty acids, the CPO used in this study had a lower content of oleic, linoleic, and linolenic by 9.22%, 2.01%, and 0.23%, respectively, which caused the content of MUFAs and PUFAs were also lower, by 8.99% and 2.09%, respectively. It shows that before being protected by liquid smoke, the PUFAs of CPO used in this study decrease slightly, only 2.09%. This decrease may occur during the storage process. According to Frank *et al*. (2011), deteriorative or decreasing quality in palm oil during storage are caused by the type of storage material, light, air, and autocatalytic hydrolysis by lipolytic microorganisms, and water content. According to Sampaio *et al*. (2011), CPO qualities are influenced by several factors, namely water and impurities, free fatty acids, peroxide value, saponification value, bleaching power, melting points, and heavy metal content. After being protected by liquid smoke before being fermented, SFAs in P0, P1, and P2 were increased, while MUFAs and PUFAs were decreased. When compared to P0, MUFAs in P1 and P2 decreased by only 0.43% and 0.91%, while PUFAs in P1 and P2 decreased by only 0.34% and 0.38%. The decreases of MUFAs and PUFAs during protection (before fermentation) are not essential; the most important thing is the effectiveness of the results of this protection during fermentation. The decrease of MUFAs and PUFAs might be caused by the addition of Prosteo skim milk in CPO, thereby increasing SFAs (palmitic), but decreasing MUFAs (oleic) and PUFAs (linoleic). This condition may also be caused by water content in liquid smoke, which donates H+ and

is bound by double bonds in UFAs so that it becomes SFAs. This condition can be occurred before fermentation in the rumen, so it is not related to the process of lipolysis and hydrogenation of fatty acids. According to Baltes *et al*. (1981), the water content in liquid smoke is relatively high, around 20.3%.

The Fatty Acid Composition of Rumen Fluid after Fermentation of Crude Palm Oil (CPO) Protected by Liquid Smoke

When compared among treatments, CPO protected by 2.5% of liquid smoke (P1) has lower SFAs with higher MUFAs and PUFAs compared to CPO without protection by liquid smoke (P0). The low SFAs may be caused by aldehydes (natural formaldehyde) in liquid smoke that bind very strongly to proteins in skim milk so that they protect fatty acids from the hydrogenation by rumen microbes. Decreased rumen microbial hydrogenation causes SFAs to decrease, due to the decreased lauric, myristic, palmitic, and stearic fatty acids concentrations. The CPO without protection by liquid smoke will be hydrogenated by rumen microbes into SFAs, especially stearic acid. As a result, most of MUFAs (70% to 95%) and PUFAs (85% to 100%) are hydrogenated to SFAs (stearic), so that stearic will increase, while oleic, linoleic, and linolenic acids will decrease in the rumen. The CPO protected by liquid smoke, is a source of UFAs and can reduce the hydrogenation process on oleic, linoleic, and linolenic, thus increasing MUFAs and PUFAs.

These results indicate that the use of liquid smoke containing aldehydes (natural formaldehyde) as a protector can protect CPO as a source of UFA. Aldehydes in the carbonyl group of liquid smoke will bind to amino acids in skim proteins, form strong bonds in the form of coagulation to protect fats, so it can reduce hydrogenation of UFA by rumen microbes, thereby increasing UFA in the rumen fluid. This result is in line with the result reported by Tiven *et al*. (2011b) that CPO protected with formaldehyde can reduce rumen microbial hydrogenation so increase oleic and linoleic acids. Formaldehyde and protein will form chemical bonds that are stable at neutral pH in the rumen but become unstable at acidic pH in the abomasum.

Fermentation Characteristics and Rumen Microbial Activity after Fermentation of Crude Palm Oil (CPO) Protected by Liquid Smoke

After fermentation of CPO protected by liquid smoke, fermentation characteristics and rumen microbial activity in P1 were higher compared to those in P0 and P2 indicating that rumen fermentation activity is going well. The high concentration of NH_3 in the rumen is affected by protein content in the feed and the solubility of feed protein. In P1 and P2, the availability of the same feed protein is derived from skim milk, which is used as a protein source for the protection of feed fat. When the level of liquid smoke rises that binds to the protein, it causes the solubility of the protein to decrease. The protection of CPO with 2.5% of liquid smoke provides the optimal protein solubility for protein degradation, thereby increasing NH_3 content. This condition is also thought to be caused by a high protein content that are easily degraded, resulting in a higher NH_3 content in the rumen. High NH_3 content in P1 causes the availability of Nitrogen (N) in P1 to be higher compared to those in P0 and P2. The availability of N sources and C framework of T-VFA (which tends to be higher in P1) will be used by rumen microbes for the synthesis of microbial proteins. Therefore, microbial proteins tend to be higher in P1 (0.03 mg/mL) compared to those in P0 and P2. The higher availability of NH_3 is also used for the growth and development of microbial cells so that CMC-ase activity in P1 is higher compared to those in P0 and P2.

When the liquid smoke level increases to 5% (P2), protein solubility decreases that eventually decreases the NH_3 content. The decrease in NH_3 content causes the decrease in the availabilities of N and C framework of T-VFA as an energy source, thereby reduce protein synthesis and CMC-ase activity in P2. This decrease may also be due to the anti-microbial properties of liquid smoke because it contains formaldehyde, thus affecting microbial activity in the rumen. This result is in line with the result reported by Tiven *et al*. (2012) that treatment of feed with formaldehyde significantly reduces NH_3 content in the rumen fluid.

CPO protected by 2.5% of liquid smoke (P1) tends to increase VFA, namely acetate, propionate, butyrate, and total VFA (although they are not statistically significant). However, the increased in liquid smoke level to 5% in P2 tended to decrease VFA. According to Hartati (2015), fat protection using formaldehyde did not significantly affect VFA content. This decrease may be caused by the increase in the liquid smoke concentration, thereby reducing microbial activity in degrading feed.

The NH_3 content obtained in this study ranged from 342 to 453 mg/L (34.20 to 45.35 mg/100 mL). According to Tiven *et al.* (2011a), the protection of CPO using technical formaldehyde (with different levels) produced NH_3 content in the range of 134 to 227 mg/L (13.44 to 22.79 mg/100 mL). The NH_3 content in this study was also higher than that reported by Seradj *et al*. (2019) who tested alfalfa *in vitro* i.e., 307 mg/L. The high NH3 concentration in CPO protected by 2.5% of liquid smoke (P1) might be due to the high protein content in skim milk with a good solubility that eventually increased feed degradation. According to McDonald *et al*. (2010), the range of optimum concentration of NH_3 in the rumen is 85-300 mg/L and this range is highly depended on the degree of feed degradation.

The content of T-VFA in this study ranges between 18.65 to 21.72 mM/L, while CMC-ase activity ranges between 14.00 to 20.98 U/g. This result is in line with the results reported by Tiven *et al.* (2011a) who protect CPO using technical formaldehyde (with different levels) and obtain an average T-VFA in the range of 19.53 to 32.77 mM/L, while CMC-ase activity is in the range of 14.42 to 20.74 U/g. According to McDonald *et al*. (2010), the optimum VFA content in the rumen are between 10 to 70 mM/L, so it can be said that the NH_3 content and T-VFA in this study (P0, P1, and P2) are within the nor-

mal range. It shows that CPO, as a source of unsaturated fatty acids which is protected by liquid smoke, has no negative effect on fermentation characteristics and rumen microbial activity after fermentation.

CONCLUSION

It can be concluded that the use of 2.5% liquid smoke is better in the protection of feed fats, because it can reduce hydrogenation, thereby increasing UFAs and has no adverse effect on fermentation characteristics and microbial activity *in vitro*. This research needs to be further tested *in vivo* to determine the effectiveness of liquid smoke as a fat protector on unsaturated fatty acids, fermentation parameters, and microbial activity.

CONFLICT OF INTEREST

We declare that there is no conflict of interest with any financial, personal, or relationships with other people or organizations related to the material used and discussed in the manuscript.

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