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# *In Vitro* Efficacy of Formulated Protected Fat from Used Cooking Oil (UCO) on Gas Production, Nutrient Digestibility and Rumen Fermentation

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This study was designed to determine *in vitro* gas production, nutrients digestibility, and rumen fermentation products of supplementary formulated dietary protected fat (PF) from used cooking oil (UCO). The dietary fat treatment was formulated from used cooking oil protected fat (UCOPF) and compared with other four dietary fat treatment were used cooking oil (UCO) and palm olein (PO which are active fat; palm olein protected fat (POPF) and commercial protected fat (CPF) which are inert fat added at 12 mg of dry matter (DM) in the 200 mg of basal diet (70:30 roughage to concentrate ratio). Control group contain basal diet without dietary fat supplement. Supplementation of dietary fat had no effect on gas production, pH, organic matter digestibility (OMD) and volatile fatty acid volatile fatty acids (VFA). The addition of fat in the form of oil and protected fat to *in vitro* setups up to 12 mg did not adversely affect the characteristics of rumen fermentation suggests that fat has a smaller impact on production of gas, digestibility of nutrients, and rumen fermentation products.

**Keywords:** animal supplement; dietary fat; inert fat; *in vitro*; rumen bypass fat

## I. INTRODUCTION

The incorporation of fermentable carbohydrates, such as cereal grains or lipids, into the diet of ruminants is one way to increase the energy density of animal diet (Naik, 2013; Palmquist, 1994). However, the inclusion of high quantities of cereal grains in the feed is limited because its affects rumen pH, which can lead to rumen acidosis (Naik *et al.*, 2009a). Depending on fat effects on rumen metabolism, supplementary fat can either be rumen active or rumen protected. Rumen active fats are rapidly degraded and may inhibit microbial fermentation in the rumen, whereas protected fat (PF) is resilient to hydrolysis by rumen microorganisms (Jenkins & Harvatine, 2014). Therefore, PF can increase the energy density of the ration without affecting ruminal fermentation. Calcium salts are well recognised PF, simple to synthesise, highly intestinally digestible, and a supply of calcium (Ca) among the numerous varieties and form of PF (Suksombat, 2009). Although PF are commercially accessible in developing nations, they are sometimes beyond of reach for small ruminant farmers because to their expensive price (Naik *et al.*, 2010).

Used cooking oil, often known as UCO, is a by-product of the utilisation of cooking oils like palm olein and not fitted for human consumption anymore (Orjuela & Clark, 2020; Teixeira *et al.*, 2018). UCO is an excellent source of fat for the feeding of animals due to its characteristics (Panadare & Rathod, 2015). UCO is having potential to satisfy the need for low-cost materials that cannot be fulfilled by other food crops (Panadare & Rathod, 2015). Hence, it was used in preparation of PF by a fusion method in this experiment.

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There is extremely limited research that explicitly highlights the effects of diets supplemented with formulated UCOPF on *in vitro* gas production, nutrient digestibility, and rumen fermented products. Consequently, the purpose of this study was to explore the *in vitro* gas production, nutrient digestibility, and rumen fermented products of diets supplemented with formulated PF derived from UCO. In addition, a comparison of the effects of other dietary fat supplement such as UCO, palm olein (PO), palm oil protected fat (POPF) and commercial protected fat (CPF) on *in vitro* fermentation has been conducted.

## II. MATERIALS AND METHOD

### *A. Ethical Approval*

This research did not involve the contribution of any living animals; hence, no ethical approval is necessary to be obtained and presented.

### *B. Preparation of Protected Fat*

Used cooking oil were acquired from a local UCO collection centre of Bangi Mosque and stored in airtight containers. The purifying procedure was followed according to Wei *et al.* (2011). The saponification procedure was performed through the utilisation of the modified fusion method followed by the drying and milling process (Pablos Pérez, 2008). The procedure is described in detail below.

Thirty grams of purified oil sample was heated to 80°C, then CaO powder with a 20% weight of oil was added. The mixture of oil and CaO was heated and stirred homogeneously. Immediately after homogeneous mixing was achieved, water equivalent to 20% of the oil weight at temperature 80°C was added to the mixture. The reaction mixture was stirred vigorously for about 10 min. After the saponification process was completed, the mixture was dried for about 24h at 80 °C in a forced air circulation oven (Memmert UF53) before being cooled at room temperature overnight. Then, the soap material was ground into flakes form.

### *C. Dietary Fat and Fermentation Substrate*

The dietary fat treatment was formulated used cooking oil protected fat (UCOPF) and compared with four dietary fat treatment were used cooking oil (UCO) and palm olein (PO) which are active fat; palm olein protected fat (POPF) and commercial protected fat (CPF) which are inert fat added at 12 mg of DM in the 200 mg of basal diet. The substrate consists of 70:30 roughage to concentrate ratio. Guinea grass was utilised as a roughage source, while commercial concentrates were used as a source for concentrated nutrients.

### *D. In Vitro Fermentation*

The protocol for *in vitro* incubation experiment was explained in detail by Menke and Steingass (1988). In brief, each of the five dietary fats (12 mg) and the substrate (200 mg) were placed in a 100 mL glass syringe and was made in triplicated. The control group was without dietary fat inclusion but added with substrate only. Rumen liquid for each *in vitro* gas run was acquired at a slaughterhouse from slaughtered bulls (~3 bulls per rumen inoculum). The thermos bottles were pre-warm at 39 °C before used. Rumen contents were obtained from squeezing 500 ml of rumen liquid into a 1 L of thermos before filled completely with unsqueezed rumen substrate. The rumen content was then transported to the laboratory within half an hour in airtight and sealed thermos bottles.

Liquid and solid components were combined and mix together in the laboratory for 1 min and constantly infuse with $CO_2$ gas. The mixture was strained through at least four layers of cheesecloth The filtrate was then combined with buffer (1:2 ratio) pre-warmed at 39 °C. A total 30 mL of inoculum was added to each syringe containing incubation medium and filtered rumen fluid. A 39 °C water bath was employed to accommodate the syringes, which were vertically positioned. Three syringes were loaded with standard hay (University of Hohenheim in Stuttgart, Germany) were used for calibration and three syringes were left blank with no substrate were used to correct cumulative gas production.

After 0, 3, 6, 9, 12, 18, 24, 48, and 74h of incubation, the amount of gas produced was manually recorded. At each reading, the syringes were gently swirled, and the gas output

was adjusted for the amount of gas produced by the blanks. Each measured value was subtracted by the blank values, which calculated net gas production. Gas production data were fitted to the non-linear model of exponential equation of Ørskov and McDonald (1979) with NEWAY Excel Version 5.0 package (Chen, 1997).

$$y = a + b\,(1 - e^{-ct}) \quad (1)$$

Where y = gas generated at time "t", a = the gas generated by the instantly soluble part, b = the gas generated from the insoluble part, c = the insoluble fraction's gas generation rate constant (b), (a+b) = the magnitude of potential gas production and, t = incubation time.

The pH of the rumen fluid was determined immediately after the fermentation was completed and the syringe contents were removed. The residues were filtered using a sintered glass filter and was analysed for the *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter degradability (IVOMD) using the method describe by Menke and Steingass (1988). The rumen fluid samples were then placed in a freezer at -20 °C until further analyses. The ammonia ($NH_3$) was analysed as described by Parsons *et al.* (1984), and volatile fatty acid (VFA) was analysed using gas chromatography (GS) (Cottyn & Boucque, 1968).

### *E. Chemical Analysis*

Chemical analyses for DM, ash, crude protein (CP), and ether extract (EE) were described by AOAC (2005). The neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were quantified using the protocol described by Van Soest *et al.* (1991). Table 1 lists the components and chemical compositions of the guinea grass and concentrate utilised in the *in vitro* experiment.

Table 1. Chemical composition (g/kg dry matter) of substrates

| | **Guinea Grass** | **Concentrate** |
|---|---|---|
| Dry matter | 886.80 | 948.8 |
| ash | 54.44 | 47.78 |
| Crude protein | 96.20 | 183.57 |
| Ether extract | 13.33 | 60.00 |
| Neutral detergent fibre | 726.33 | 652.00 |
| Acid detergent fibre | 427.77 | 343.00 |
| Lignin | 24.67 | 86.67 |

### *F. Statistical Analysis*

Data were analysed using General Linear Model (GLM) of the Statistical Analysis System (SAS) version 9.4 software program (Sas Institute Inc, 2017) for a randomised complete block design (RCBD) with repeated measurement for the gas production. Differences between treatment means were differentiate and asserted significant at $P<0.05$ by Tukey post-hoc procedure.

## III. RESULT AND DISCUSSION

### *A. The Effect of Used Cooking Oil Protected Fat on Gas Production*

The *in vitro* gas production value and gas production kinetic for the UCOPF and other dietary fat treatment are shown in Table 2.

Observation showed (Table 2) that the cumulative gas production between dietary treatments during the incubation period was not significantly different ($P>0.05$) between each other. The gas production kinetic characteristics, such as the fermentation of the soluble fraction (a), the insoluble fraction (b), and the total amount of gas produced (a + b) were similar ($P>0.05$) for all dietary treatment groups compared with control group. However, the fermentation of soluble fraction (a) was significantly higher for UCOPF treatment group compared to POPF group. The increase of a soluble fraction (a) for a UCOPF shows that the fat was readily available for digestion by the rumen microbial population (Carriquiry *et al.*, 2008). Accordingly, our findings suggest that UCOPF offered less overall protection compared to POPF against the rumen microorganisms' metabolism of fatty acids.

Table 2. Effect of formulated used cooking oil protected fat (UCOPF) with comparison of different fat supplement on the gas production

| | C | UCO | PO | UCOPF | POPF | CPF | SEM | P-values |
|---|---|---|---|---|---|---|---|---|
| Net gas production (mL/200 mg DM) | | | | | | | | |
| Incubation time (h) | | | | | | | | |
| 3 | 26.67 | 27.08 | 25.42 | 28.75 | 27.50 | 26.67 | 4.05 | 0.81 |
| 6 | 57.50 | 57.50 | 56.25 | 59.08 | 58.33 | 57.50 | 6.01 | 0.98 |
| 9 | 82.08 | 83.33 | 83.33 | 81.67 | 84.17 | 83.75 | 7.35 | 0.99 |
| 12 | 95.83 | 97.92 | 97.08 | 95.83 | 97.92 | 98.33 | 8.61 | 0.99 |
| 18 | 122.08 | 124.58 | 126.25 | 123.33 | 123.75 | 127.92 | 11.03 | 0.95 |
| 24 | 122.92 | 125.83 | 124.58 | 123.33 | 125.83 | 128.75 | 11.96 | 0.96 |
| 48 | 150.42 | 152.92 | 150.41 | 150.00 | 152.92 | 155.42 | 12.96 | 0.97 |
| 72 | 155.00 | 158.75 | 155.00 | 156.66 | 160.42 | 161.67 | 13.76 | 0.94 |
| Kinetic gas production | | | | | | | | |
| a (ml) | $-9.93^{ab}$ | $-7.86^{ab}$ | $-13.18^{a}$ | $-3.50^{b}$ | $-7.22^{ab}$ | $-8.77^{ab}$ | 4.00 | 0.01 |
| b (ml) | 165.71 | 166.13 | 162.69 | 157.48 | 162.37 | 165.89 | 11.94 | 0.80 |
| c (ml/h) | 0.09 | 0.09 | 0.09 | 0.08 | 0.08 | 0.09 | 0.005 | 0.16 |
| a+b | 155.78 | 158.27 | 149.51 | 153.98 | 155.15 | 157.12 | 11.70 | 0.83 |

C – control; UCO – used cooking oil; PO – palm olein; UCOPF – used cooking oil protected fat; POPF – palm olein protected fat; CPF – commercial protected fat; a - volume of gas produced from immediate soluble fraction; b - a volume of gas produced from insoluble fraction; c -gas production rate constant from insoluble fraction; a+b - the potential extent of gas production; IVDMD – *in vitro* dry matter digestibility; OMD - organic matter digestibility; DM - dry matter; SEM - standard error of the mean. $^{a,b}$ Different superscript in each row are significantly differ ($P<0.05$);

The amount of gas generated during *in vitro* incubation may be indicative of the degree to which a substrate is degradable and fermentable. Additionally, a substantial rumen microorganism population helps accelerate the fermentation of dietary components to create VFA, biomass and certain gases such as $CH_4$ and $CO_2$, which serve as the primary supply of energy for rumen microorganisms (Trotta *et al.*, 2018). Measuring *in vitro* gas production offers beneficial insight on the decomposition dynamics of both soluble and insoluble dietary components (Getachew *et al.*, 1998). Cumulative and kinetic gas production was unaffected by the addition of UCOPF and other dietary fat. The UCO and PO dietary treatment were predicted to inhibit rumen fermentation since they are in unprotected forms of fat compared to UCOPF, POPF and CPF. However, the addition of 6% of UCO and PO is not enough to inhibit rumen fermentation. Getachew *et al.* (2001) observed that inclusion of tallow and yellow grease had no effect on *in vitro* gas generation. Vargas *et al.* (2017) found that inclusion 2% of sunflower oil to the substrate had no obvious effect on total gas release. Jenkins (1987) observed that the incorporation of maize oil had no deleterious effect on rumen fermentation. In contrast, Getachew *et al.* (2001) reported of increase in total gas production of corn oil inclusion and potassium soap negatively affect *in vitro* rumen fermentation.

### *B. The Effect of Used Cooking Oil Protected Fat on Nutrient Digestibility*

Evaluating the quality of animal feed components is insufficient for determining the importance of the food ingredients present within; however, the significance of these elements for livestock can be measured after digestion, absorption, and metabolism in the rumen and post-rumen organs (Jarmuji *et al.*, 2021).

Table 3. Effect of formulated used cooking oil protected fat (UCOPF) with comparison of different fat supplement on the digestibility of nutrient

| | C | UCO | PO | UCOPF | POPF | CPF | SEM | P-values |
|---|---|---|---|---|---|---|---|---|
| Dry matter digestibility | 52.67 | 58.25 | 47.54 | 56.81 | 52.58 | 49.37 | 10.06 | 0.43 |
| Organic matter digestibility | 57.92 | 62.78 | 53.17 | 60.92 | 58.37 | 54.25 | 9.15 | 0.44 |

C – control; UCO – used cooking oil; PO – palm olein; UCOPF – used cooking oil protected fat; POPF – palm olein protected fat; CPF – commercial protected fat; SEM - standard error of the mean. There is no significant difference ($p>0.05$) between groups in each row.

Incorporating UCOPF and other dietary fat that supplemented into the substrate showed no influence ($P>0.05$) on the IVDMD or IVOMD shown in Table 3. The increased digestibility of feed substrate can be indicated by increased gas production (Calabrò *et al.*, 2012). Based on our findings, digestibility of fatty acid was not differ between treatment group similar trend with net gas production that also same among treatment group. Reddy *et al.* (2001) observed that the addition of PF in the form of calcium salts from red palm oil had no impact on IVDMD. Naik *et al.* (2009b) reported that calcium salts of long-chain fatty acid (Ca-LCFA) inclusion at 10 g/kg, 20 g/kg, and 30g/kg of DM did not adversely affect the IVDMD. In contrast to another study, calcium salts of red palm oil supplementation at 5, 7.5, and 10% lowered the IVDMD (Kumar *et al.*, 2017).

### *C. The Effect of Used Cooking Oil Protected Fat on Rumen Fermented Products*

The pH, concentration of $NH_3$, and VFA concentrations in the rumen are summarised in Table 4. The addition of all dietary fat treatments did not affect on the mean of ruminal pH levels ($P>0.05$). The concentration of $NH_3$ was not altered ($P>0.05$) by the addition of UCOPF and other dietary fat supplement to the mixture of the substrate. When the different kinds of dietary fat supplementation were compared, the individual VFA, total VFA, and acetic acid to propionic acid (A/P) ratios were not significantly different ($P>0.05$).

At 72h, ruminal pH was unaffected by a substrate containing UCOPF and other dietary fat, varying from 6.39 to 6.57. The rumen pH was within the acceptable range of 6.0-7.0, which is optimal for microbial fibre and protein digestion (Wales *et al.*, 2004). Since the fibre component offers an ideal habitat for rumen bacteria to hydrolyse the dietary oil, sufficient roughage content in the diet minimises dietary oil's detrimental influence on rumen fermentation (Atikah *et al.*, 2018; Jenkins, 1993). PF characteristics to avoid the rumen microbe's digestion, particularly lipolysis and biohydrogenation, reduced negative effect on the rumen fermentation (Carriquiry *et al.*, 2008). The current report is similar to those of Anantasook *et al.* (2013), who found that palm oil supplementation had no deleterious impact on the ruminal pH of dairy cows. Additionally, research evaluating incorporation of PF in ruminants has yielded comparable outcomes (Bayourthe *et al.*, 1993; GÜMÜŞ *et al.*, 2021; Kang *et al.*, 2019). Rumen pH is the most influential factor on the production of VFA and $NH_3$ by rumen microorganisms (Huyen *et al.*, 2016).

Table 4. Effect of formulated used cooking oil protected fat (UCOPF) with comparison of different fat supplement on the ruminal fermentation products

| | C | UCO | PO | UCOPF | POPF | CPF | SEM | P-values |
|---|---|---|---|---|---|---|---|---|
| pH | 6.36 | 6.41 | 6.57 | 6.47 | 6.55 | 6.40 | 0.16 | 0.15 |
| $NH_3$ (mM) | 7.38 | 10.44 | 7.02 | 8.54 | 8.85 | 9.05 | 2.41 | 0.19 |
| Acetic (mM) | 5.77 | 7.79 | 7.86 | 4.54 | 4.02 | 5.00 | 3.07 | 0.40 |
| Propionic (mM) | 3.98 | 5.46 | 5.63 | 3.48 | 2.83 | 3.57 | 2.18 | 0.43 |
| Butyric (mM) | 1.99 | 4.93 | 3.27 | 2.22 | 1.80 | 1.98 | 5.47 | 0.25 |
| Iso-butyric (mM) | 0.61 | 0.85 | 0.67 | 0.52 | 0.43 | 0.55 | 0.31 | 0.48 |
| Total VFA (mM) | 12.35 | 19.03 | 17.43 | 10.76 | 9.08 | 10.23 | 7.12 | 0.35 |
| Acetic:propionic | 1.47 | 1.44 | 1.39 | 1.32 | 1.42 | 1.45 | 0.10 | 0.48 |

C – control; UCO – used cooking oil; PO – palm olein; UCOPF – used cooking oil protected fat; POPF – palm olein protected fat; CPF – commercial protected fat; SEM - standard error of the mean. There is no significant difference ($p>0.05$) between groups in each row.

The addition of fat in ruminant feed has consistently been observed to decrease rumen $NH_3$ production (Hristov *et al.*, 2009; Vargas *et al.*, 2020). However, contradictory outcomes in terms of $NH_3$ content have been observed in previous ruminant research incorporating fat supplementation (Ivan *et al.*, 2001; Szumacher-Strabel *et al.*, 2009). According to the results of the present research, the $NH_3$ concentrations in all dietary fat groups were within the standard level, which exceeded 5-8 mg/dl for microbial protein production (Ørskov and MacLeod, 1982; Satter and Slyter, 1974). Earlier study Atikah *et al.* (2018) observed that $NH_3$ concentrations were similar with and without fat addition. Naik *et al.* (2009a) reported concentrations of $NH_3$ similarly in the substrates supplemented with both unprotected and PF.

VFA, including its acetic, propionic, and butyric acids, and a lesser concentration of other component, such as isobutyrate, valerate, isovalerate are produced during fermentation in the rumen and can be absorbed by the ruminant (Kowalczyk, 1989). Dietary fat treatments did not affect the total VFA, acetic, propionic, or butyric concentrations in the rumen in this study. However, non-statistically total VFA was slightly higher in dietary fat treatment of UCO and PO than in the control group. Unsaturated fatty acid (USFA) has greater antimicrobial properties and therefore suppress ruminal fermentation more effectively proposed by Jenkins (1993). Although UCO and PO have unprotected fatty acid (FA) compared to the PF, UCO and PO did not influence on individual and overall VFA yield. This was due to the triglyceride characteristics of UCO and PO, which are more stable than FFA (Getachew *et al.*, 2001). Atikah *et al.* (2018) found a similar result, in which PO supplementation had a negligible effect on total VFA production. In the rumen, calcium salts are known to be difficult to digest (Chalupa *et al.*, 1984). According to Chalupa *et al.* (1984), adding calcium salts of tallow to *in vitro* incubations did not influence on total VFA generation, consistent with the current findings. Nonetheless, contrasting results were obtained in the research of Getachew *et al.* (2001), involving a reduction in the content of VFA supplemented with tallow FA.

## IV. CONCLUSION

While it is often considered that dietary fats and PF are harmful to rumen microbial development and fibre digestion, our findings contradict this assumption. This study conducted *in vitro* rumen incubations found that the addition of UCOPF had no effect on *in vitro* gas production, nutrient digestibility, or total VFA output. The lack of detrimental impacts on rumen fermentation characteristics from UCOPF shows that supplementation of UCOPF at 6% have a considerably lower impact on rumen fermentation.

## V. ACKNOWLEDGEMENT

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## VI. PATENT

A part of current data has been patented in Malaysia entitled "Method for Producing a Safe Cattle Lipid Supplement from Used Cooking Oil" PI 2022004648.

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