RESEARCH ARTICLE Indian Journal of Animal Research, Volume 58 Issue 12: 2125-2131 (December 2024)

The Effects of Encapsulation and Double-layer Emulsion of Peanut Oil on *in vitro* Rumen Degradability Rates and Fermentation Profile in Sheep

A. Budiman[1], B. Nurhadi[2], H. Supratman[3], M.M. Rahman[4], Y.R. Yanza[3], I. Hernaman[3] 10.18805/IJAR.BF-1761

ABSTRACT

Background: Peanut oil is rich in polyunsaturated fatty acids (PUFAs) and plays an important role in human and animal health. PUFAs are generally undergo to the biohydrogenation (BH) process by the rumen microbes. The single-layer emulsion encapsulation has been used in sheep diet. However, a limited reference was found concerning double-layer techniques to protect the essential PUFAs from BH process in the rumen.

Methods: EDLEPO was made with a matrix of peanut oil, whey protein isolate (WPI), microcrystalline cellulose (MCC) and maltodextrin at 40: 2.5: 7.5: 55. The first layer was the binding of WPI with peanut oil and the second layer is the binding of WPI with MCC. Moreover, the in vitro experiment was designed with 5 treatments separated by CON group (0 g/kg EDLEPO) and experimental groups which are leveled by EDLEPO inclusion (25, 50, 75 and 100 g/kg levels of EDLEPO) in the buffered sheep's rumen.

Result: Increased levels of EDLEPO inclusion reduced rumen ammonia-N concentration ($P<0.05$) by a linear response. Hence, increased levels of EDLEPO inclusion increased rumen degradability rates expressed as IVDMD and IVOMD up to 12% and 9%, respectively, by a linear response ($P<0.05$). No significant differences were found between treatments on pH value, VFAs and total gas production. However, the increased levels of EDLEPO inclusion reduced CH_4 concentration expressed as CH_4/IVDMD and CH_4/IVOMD up to 9% and 17% ($P<0.05$). It can be concluded that the EDLEPO inclusion in the diet increased in vitro rumen degradability rates with no negative effects on the rumen fermentation profile.

Key words: Double-layer emulsion, Encapsulation, *In vitro*, Peanut oil, Rumen.

INTRODUCTION

Peanut oil is rich in polyunsaturated fatty acids (PUFAs) (Erdal and Seyfullah, 2018). In the rumen, PUFAs are generally authorized by the rumen microbes undergo to the biohydrogenation (BH) process (Suphrap *et al*., 2019; Makmur *et al.,* 2023). Most of the PUFAs will be hydrogenated into trans-UFAs, monounsaturated fatty acids (MUFAs) and mostly SFAs before being deposited in expected products of ruminant origins such as meat and milk (Yanza *et al.,* 2022). On the other hand, the BH process occurs due to the rumen microbial mode of action to protect themselves from the toxic effects of PUFAs (Carreno *et al.,* 2019). Such conditions may affect the composition of bacterial colonies and feed fermentation activities (Suharti *et al.,* 2019). However, the BH process indirectly influences the deposited fatty acids (FAs) composition in animal products, reduces the nutritional quality of animal products and may affect human health.

The encapsulation technique is a process of entrapment of an active agent into another biocompatible material to preserve its functionality (Sundar and Parikh, 2023). This process consists of a smaller amount of dispersed phase and a larger amount of dispersing phase than the emulsifier or surfactant so that the emulsification process can consist of o/w (oil (o) as the dispersed phase and water (w) as the dispersed phase or vice versa w/o, where mostly those process is single layered. The single-layer emulsion encapsulation is mainly used for curing and has been used in sheep diet (Fadhilah *et al.,* 2019). However, a limited reference was found concerning double-layered techniques for encapsulating the emulsified lipid, especially to protect the essential PUFAs of vegetable oil mixed in the

[1]Doctoral Programs at Faculty of Animal Husbandry, Universitas Padjadjaran Sumedang 45363, West Java, Indonesia.
[2]Department of Food Industrial Technology Faculty of Agricultural Industrial Technology, Universitas Padjadjaran, Sumedang 45363, West Java, Indonesia.
[3]Department of Animal Nutrition and Feed Technology, Faculty of Animal Husbandry, Universitas Padjadjaran Sumedang 45363, West Java, Indonesia.
[4]Faculty of Agro-Based Industry, University Malaysia Kelantan, Kelantan, Malaysia.

Corresponding Author: I. Hernaman, Department of Animal Nutrition and Feed Technology, Faculty of Animal Husbandry, Universitas Padjadjaran Sumedang 45363, West Java, Indonesia. Email: iman.hernaman@unpad.ac.id

How to cite this article: Budiman, A., Nurhadi, B., Supratman, H., Rahman, M.M., Yanza, Y.R. and Hernaman, I. (2024). The Effects of Encapsulation and Double-layer Emulsion of Peanut Oil on *in vitro* Rumen Degradability Rates and Fermentation Profile in Sheep. Indian Journal of Animal Research. 58(12): 2125-2131. doi: 10.18805/IJAR.BF-1761.

Submitted: 19-01-2024 **Accepted:** 09-03-2024 **Online:** 05-04-2024

feed diet from the BH process in the rumen. The present study aimed to determine the influence of PUFAs composition on the encapsulation of double-layer emulsified peanut oil (EDLEPO) and the effect of EDLEPO supplementation on *in vitro* rumen degradability and fermentation profile.

MATERIALS AND METHODS

Emulsification and encapsulation technique

The series of research was carried out in the period February-November 2022. Peanut oil was collected from the local industry at Paseh District, Sumedang Regency, West Java, Indonesia. The extracted peanut oil's double-layer emulsification and encapsulation process was done in several steps and solutions. This process was carried out at Food Chemistry Laboratory, Faculty of Agricultural Industrial Technology, Universitas Padjadjaran. First, the 2.5 g WPI was mixed with 100 g water where both liquids were later dissolved for 2-3 hours and set to a 3.5 pH value by adding HCl solution 0.1 M. Moreover, the 40 g oil was poured into the mixed solution and homogenized using a magnetic stirrer (solution 1). In the second step, 50 g maltodextrin was dissolved in 133 g water, stirred until clear and heated at 50°C while mixed with 7.5 g MCC, blended, before being adjusted to a 3.5 pH value by adding HCl solution 0.1 M (solution 2). Solution 1 and solution 2 were then mixed and homogenized with a colloid mill to become a double-layered emulsion peanut oil. Emulsified oil liquid was then dried using spray drying at an inlet temperature of 160°C and an exit temperature of 90°C. Thus, the ratio of peanut oil, WPI, MCC and maltodextrin is 40: 2.5: 7.5: 50, respectively. The pH of the mixture was set to pH 3.5 to make the electrical charge of WPI positive, whereas the electrical charge of MCC is negative. Thus, a strong electrical binding to encapsulate peanut oil could have occurred.

In vitro rumen fermentation

Experimental design

In vitro experiment was employed through batch culture technique following Tilley and Terry (1963) protocol using total mixed diet (TMR) as substrate followed with the supplementation of EDLEPO as experimental treatments. The *in vitro* experiment was performed in 5×5 (treatments × experimental bottle) where treatments were separated by CON treatment and 25, 50, 75 and 100 g/kg dry matter (DM) supplementation of EDLEPO. The TMR substrate is a combination of Elephant grass (Taiwan variants) and concentrates with a 60:40 ratio. Concerning the concentrate, it consisted of mixed copra meal, soy sauce dregs, soybean meal, corn distillers dried grains with solubles (DDGS), rice bran, pollard, cassava, palm oil and minerals (Table 1). CON group entirely used TMR substrate and for the experimental treatment groups, EDLEPO inclusion substituted the TMR substrate with about 25, 50, 75 and 100 g/kg dry matter (DM). *In vitro* experiment was carried out in the Laboratory of Ruminant Animal Nutrition and Feed Chemistry, Faculty of Animal Husbandry, Padjadjaran University.

Table 1: Ingredients and composition of experimental substrate as well as the fatty acids composition of EDLEPO.

Feed ingredients	CON	EDLEPO in TMR (g/kg DM)			
		25	50	75	100
Elephant grass cv taiwan (%)	60	60	60	60	60
Concentrate (g/kg DM))	40	40	40	40	40
Soybean meal (%)	0.56	1.01	1.46	1.91	2.36
Palm meal (%)	6.77	6.38	5.99	5.61	5.22
Soy sauce dregs (%)	6.44	6.03	5.62	5.21	4.8
DDGS (%)	3.79	3.8	3.81	3.82	3.83
Pollard (%)	7.67	7.13	6.59	6.05	5.51
Cassava pulp (%)	11.22	10.22	9.23	8.23	7.24
Palm oil (%)	2.55	1.92	1.29	0.67	0.04
EDLEPO (g/kg DM)	0	2.5	5	7.5	10
Mineral mix (%)	1	1	1	1	1
Total	100	100	100	100	100
Nutrients (%)					
Moisture (%)	22.60	22.57	22.54	22.51	22.48
Crude protein (%)	13.84	13.84	13.84	13.84	13.84
Crude fat (%)	7.47	7.82	8.16	8.50	8.84
Crude fiber (%)	26.25	25.91	25.58	25.23	24.89
NFE (%)	44.34	44.59	44.84	45.09	45.33
Ash (%)	8.09	7.85	7.60	7.34	7.10
TDN (%)	66.17	66.81	67.43	68.06	68.69

*TDN was calculated using the Sutardi equation as described in Hernaman *et al.* (2022): (70.6 + 0.259% Crude Protein + 1.01% Crude Fat - 0.76% Crude Fiber + 0.0991% Nitrogen Free Extract).

In vitro batch culture preparation

All experimental fermenter tubes were prepared and sterilized a few days before the experiment with a total of 27, 25 tubes for samples and 2 for blanks. Previously, samples were weighed about 0.5 g and poured into fermenter tubes and all fermenter tubes were put into a water bath (39-40°C temperature). On the other hand, fresh ruminal fluid was collected at the slaughterhouse from 6 sheep (two sheep in each run) and was filtered through a four-layered cheesecloth into two glass bottles that were maintained at 39°C with the anaerobic condition. Rumen fluids were immediately transported to the laboratory, put into the water bath at 39°C and rumen fluid was then mixed in equal volume basis (100 mL rumen fluid per each sheep) in a beaker before being diluted with an artificial buffer solution (Yanza *et al.,* 2018).

A mixture of artificial saliva following McDougall's (1948) protocol and sheep rumen fluid were mixed in a (4:1 ratio) and flowed with CO_2 gas to maintain an aerobic condition of mixed rumen fluid. Each fermenter tube that already consisted of 0.5 g experimental substrate was then poured with 50 mL of mixed rumen fluid and closed with a rubber and incubated anaerobically at 39-40°C for 24 h (first repetition) and 48 h (second and third repetition). Samples of rumen fluid were collected and 24 h samples were centrifuged at 10,000 rpm for 10 minutes, the supernatant was taken and stored in a tube to measure VFAs, ammonia and pH of rumen fluid. Second and third repetition samples were collected for their *in vitro* dry matter degradability (IVDMD), *in vitro* organic matter degradability (IVOMD) and total gas production.

Analysis of incubated rumen fluid samples

All collected rumen fluid samples after incubation were determined with their pH values (Hanna instruments, USA) before being stored in the fridge, at a 4°C temperature. The collected rumen fluid samples that were previously stored in a coolbox were analyzed for volatile fatty acids (VFAs) profile at The Chemical Services Laboratory, Standards Center for Poultry and Various Livestock Instruments. The sample was added with sulfonic acid in an Eppendorf tube to provide acidic properties to the rumen fluid, which tends to be acidic. Then, vortexed and centrifuged for 15 minutes at 12,000 rpm. After that, the liquid was separated into small vials and carried out using GC (Bruker 336-GC Scion, USA) with an FID (flame ionization detector) column. The ammonia of rumen fluid samples was analyzed using the micro diffusion method of Conway (1962) protocol.

Substrate degradability was determined after 48 hours of incubation where all fermenter tubes were added with 0.25 mL of Hg_2Cl_2 to kill the microbes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was separated and added to the precipitate in a fermenter 5 mL of a 0.2% pepsin-HCl solution in an acid atmosphere with a pepsin activity of 1:10,000. the precipitate supernatant was filtered through filter paper (Whatman No. 41) and then analyzed for DM and organic matter (OM) content. As a blank, untreated sheep rumen fluid was also analyzed to correct the total degraded substrate. In vitro dry matter degradability (IVDMD) and *in vitro* organic matter digestibility (IVOMD) following (Tilley and Terry, 1963) procedure.

Statistical analysis

All data were analyzed using one-way ANOVA where levels of the EDLEPO used in the diet were dependent factors using IBM SPSS Statistic (26th Version) software. Significant data were tested with Tukey's post hoc test to determine the significant differences among groups and the polynomial contrasts to determine the linear responses of different levels of EDLEPO included in the diet. Different treatments are considered significant differences at $P<0.05$.

RESULTS AND DISCUSSION

The IVDMD and IVOMD were increased by a linear response ($P<0.05$) by the increased level of EDLEPO inclusion in the diet (Table 2). The EDLEPO inclusion increased the *in vitro* rumen DM and OM degradability rate by about 4-12% and 5-9%, respectively. Increased IVDMD and IVOMD reached their optimum rate at the highest level of EDLEPO inclusion in the diet. However, Ferreira *et al.* (2016) found that supplementation of soybean oil and sunflower oil had no certain effect on dry matter and organic digestibility in ruminants. Perhaps the increased *in vitro* degradability rate relates to the experimental substrate composition in the present study. Because the substrate degradability rate depends on the physical, nutrient, composition ratio between feed sources and feed processing (Mayulu *et al.,* 2020; Hernaman *et al.,* 2022). The substrate of experimental groups was included with EDLEPO, in which the major materials for the encapsulation and emulsification process are maltodextrin and MCC. Those components are known as carbohydrates, acceptable to rumen microbes and would be degraded into volatile fatty acids (Zhang *et al.,* 2020). These compounds contribute carbohydrates in crude fiber and NFE, hence, the substrate in each treatment had an identical carbohydrate composition. Double encapsulation can trap peanut oil, so it does not affect digestibility. Vegetable oils also that are known to have a negative effect on cellulolytic bacteria which could show inhibitory effects of the fibre digestion (Ibrahim *et al.,* 2021).

This condition will allow sufficient time (slow release) for the first layer of emulsion (WPI and peanut oils) to pass into post-rumen. It can be confirmed with the decreased ammonia-N concentration by the increased level of EDLEPO inclusion, significantly lowered by a linear response ($P<0.05$) (Table 2). However, ammonia-N concentration for all treatments remained within the range of rumen microbial requirements. Ammonia-N is an essential nutrient for microbial growth. The rumen microbial needs 5-11 mmol/L ammonia to maximize microbial protein (Shen *et al.*, 2023). It is suspected that WPI is a source of protein that acts as an emulsifier with peanut oil as the first layer in EDLEPO and was protected from rumen microbial during fermentation.

Moreover, ammonia is a source of N for microbial protein synthesis derived from the fermentation of proteins, peptides, or amino acids (Kim *et al.,* 2017; Yu *et al.,* 2022). Fortunately, the double-layer emulsion has the advantage of carrying hydrophilic active components protecting the inner material from disturbance. The trapping of active components in the internal phase through the coating on the external oil and water phases will slow the rate of release and degradation of active components for a particular duration (Sapei *et al.,* 2012). Such a condition might indicate that the WPI of the EDLEPO layer was not degraded and fermented by rumen proteolytic microbes. Then at this stage, added pepsin-HCl produces a pH of 2 to 3, which is the same as conditions in the abomasum (post-rumen) (Hildebrandt *et al.,* 2017). However, the WPI emulsion may be unstable due to the peanut oil's molecular bonds. Abdolmaleki *et al.* (2016) stated that emulsion was very unstable at pH 2.5, with the emulsion stability index decreasing almost three times more than other emulsions during storage time. As a result, protein in WPI will be digested by pepsin which increased the EDLEPO experimental group's degradability rate more than the CON group. This mechanism is expected when it occurs in ruminants, to inhibit the BH process where PUFAs pass to the small intestine and can be absorbed optimally in the targeted ruminant products, such as milk and/or meat (Barroso *et al.,* 2014).

Increased levels of EDLEPO inclusion in the diet had no effects on rumen total gas production. According to Olfaz *et al.* (2018), there is a positive relationship between gas production and the degradability of the organic matter of feed. Unfortunately, the present study results on degradability and gas production are not coherent with Olfaz *et al.* (2018) findings. However, Besharati *et al.* (2022) reported that the encapsulation of flaxseed oil with calcium alginate increased the dry matter degradability of substrate with a fluctuation effect on gas production during 96 h incubation. Some researchers reported that vegetable oil supplementation in the diet negatively affects rumen fermentation indicating reduced degradability rates and a lower microbial population (Yanza *et al.,* 2021). The contrary results between the present study and previous findings concerning rumen degradability rates by encapsulated oils supplementation may be affected by several factors such as microcapsule membrane materials, treatments of internal matter in the microcapsule (double-layer oil emulsion) and feed diet. Rumen microbial can bind the microcapsule layer of EDLEPO made from WPI and bind with MCC and maltodextrin as the bilayer of emulsified peanut oil. However, the protected peanut oil may be unattached by rumen microbes due to the exhaustion period to bind with the EDLEPO microcapsule membrane and emulsified layers. Besharati *et al.* (2022) also confirmed the effects of fat coating and the type of microcapsule sources may explain the rumen degradability rates on the substrate, which can be due to differences in the amount of oil released from different microcapsules per unit of time and the total amount of oil released from the microcapsules.

Total VFA concentration and composition such as acetate, propionate and butyrate proportion, as well as the acetate: propionate ratio of experimental treatments were similar. It may suggest that the EDLEPO supplementation had no negative effects on rumen fermentation. The present study results were also confirmed by Suharti *et al.* (2019), which trialed a single layer of canola/sesame oil emulsion encapsulation treatment combined with *Sapindus rarak* extract. Nevertheless, although EDLEPO supplementation had no significant effects on rumen total gas production, gas in the form of H_2, CO_2 and CH_4 was consequentially produced (Moss *et al.,* 2000). Enteric CH_4 is produced by rumen microbes called methanogenic archaea, involved in converting the free hydrogen (H_2) and carbon dioxide (CO_2)

Table 2: The effect of EDLEPO on *in vitro* rumen fermentation profile.

Variable	CON	EDLEPO in TMR (g/kg DM)				SEM	P value	Contrast
		25	50	75	100			linear
IVDMD (%)	67.71^{c}	70.75^{b}	74.45^{a}	75.98^{a}	75.45^{a}	0.68	<0.01	<0.01
IVOMD (%)	71.99^{b}	76.12^{a}	76.46^{a}	78.49^{a}	77.53^{a}	0.56	<0.01	<0.01
pH	7.00	7.04	7.08	7.00	6.98	0.02	0.25	0.45
NH_3 (mM)	9.59^{a}	9.79^{a}	7.90^{b}	7.28^{bc}	6.96^{c}	0.25	<0.01	<0.01
VFA (mM)	148.00	151.96	166.90	166.93	176.47	4.84	0.36	0.95
C_2 (%)	72.16	71.92	73.07	73.37	74.38	0.41	0.35	0.06
C_3 (%)	20.43	20.73	20.42	19.96	19.80	0.24	0.77	0.27
C_4 (%)	7.41	7.35	6.52	6.67	5.82	0.30	0.45	0.08
C_2:C_3 ratio	3.55	3.48	3.60	3.68	3.56	0.07	0.93	0.68
VFA/IVDMD (mM/g)	436.98	429.98	447.99	436.87	471.47	12.21	0.91	0.91
VFA/IVOMD (mM/g)	607.07	565.61	591.27	557.89	613.83	16.29	0.81	0.68

IVDMD: *In vitro* dry matter degradability, IVOMD: *In vitro* organic matter degradability, NH_3: Ammonia concentration, VFA: Volatile fatty acids, C_2: Acetate, C_3: Propionate, C_4: Butyrate.

Different letters within rows represented significant differences ($p<0.05$).

Table 3: The effect of EDLEPO on *in vitro* methane and total gas production in the rumen.

Variable	CON	EDLEPO in TMR (g/kg DM)				SEM	P value	Contrast
		25	50	75	100			linear
Gas production (mL)	75.83	77.75	62.86	91.15	89.15	5.19	0.41	0.41
Gas/IVDMD (mL/g)	223.94	219.28	168.22	241.61	238.41	13.92	0.43	0.32
Gas/IVOMD (mL/g)	311.19	289.48	218.24	304.69	310.08	17.98	0.39	0.27
CH_4 (mM)*	29.82	29.60	29.88	30.19	30.18	0.18	0.82	0.32
CH_4/IVDMD (mM/g)	88.06^{a}	83.68^{ab}	80.28^{b}	79.49^{b}	80.05^{b}	0.83	<0.01	<0.01
CH_4/IVOMD (mM/g)	122.32^{a}	109.93^{b}	105.02^{bc}	101.33^{c}	103.40^{bc}	1.72	<0.01	<0.01

CH_4: Methane production, IVDMD: *In vitro* dry matter degradability, IVOMD: *In vitro* organic matter degradability. *Calculated by using the formula: 0.45(C2) - 0.275(C3) + 0.4(C4) (Moss *et al.,* 2000).

Values are expressed as mean (n=5) and different letters within rows represented significant differences (p<0.05).

by protozoa, bacteria and anaerobic fungi communities to formed CH_4 (Patra *et al.,* 2017). Moreover, acetic and butyric acids promote CH_4 production, while propionic acid can be considered a competitive pathway for hydrogen uptake in the rumen. Hence, a specific stoichiometry model can be adapted to estimate the rumen enteric methane production from the partial VFA results (Moss, 2000).

Estimated methane concentration between treatments had a similar result. It can be concluded unchanged CH_4 concentration occurred due to the similar proportion of acetic, butyric and propionic acid production among treatments (Table 3). However, when the CH_4 concentration was expressed as CH_4/IVDMD (mM/ g degraded DM), CH_4 was significantly reduced by the increased level of EDLEPO supplementation by about 5 to 9.7% by a linear response (P<0.05). The CH_4 concentration expressed as CH_4/IVOMD (mM/ g degraded OM), was also significantly reduced by the increased level of EDLEPO supplementation by about 10 to 17% by a linear response (P<0.05). It can be suggested that supplementation of EDLEPO may increase feed efficiency by the increased substrate degradability rates. The formation of CH_4 resulted in no direct relationship with the presence of EDLEPO in the diet. In the EDLEPO, the proportion of peanut oil was 41.66%, with the highest level of EDLEPO inclusion in the present study being 100 g/kg DM so the peanut oil content was 41.66 g/kg DM. Meanwhile, the encapsulation efficiency level was 30.14%, which means that only 12.55 g/kg DM of peanut oil was trapped in 100 g/kg DM EDLEPO, while the remaining 29.11 g/kg was the surface oil. This amount is not expected to interfere with the degradability and fermentability of diets in rumen fluid because oil supplementation can affect the microbial community and fermentation process in the rumen.

However, it is known that the rumen produces less methane when there is more fat in the diet because dietary fat decreases the amount of hydrogen that is accumulated through the process of fatty acid biohydrogenation, the amount of fermentable organic matter that is consumed, the rate of fiber digestion and the count and activity of the ruminal bacteria (El-Sherbiny *et al.,* 2023). Hence, although the EDLEPO supplementation may not directly alter microbial activity, rumen microbes were not observed in the present study. Nevertheless, rumen microbes can synthesize the encapsulated and emulsified layers of peanut oil due to the material used for making EDLEPO, hence increased degradability rates indirectly reduced the enteric CH_4 concentration in the rumen.

CONCLUSION

The EDLEPO inclusion in the diet increased in vitro rumen degradability rates but reduced ammonia concentration in the rumen. However, there are no negative effects on other rumen fermentation parameters, such as VFAs and pH and total gas production. However, the increased levels of EDLEPO inclusion in the diet reduced CH_4 concentration expressed as CH_4/IVDMD and CH_4/IVOMD by about 5 to 9.75% and 10 to 17%, respectively. It can be concluded that the EDLEPO inclusion in the diet increased in vitro rumen degradability rates with no negative effects on the rumen fermentation profile.

ACKNOWLEDGEMENT

Acknowledgment to the Rector of Universitas Padjadjaran who has allowed the author to attend school in the animal science doctoral study program through the Universitas Padjadjaran Lecturer Dissertation Research Scheme.

Conflict of interest

There is no conflict of interest.

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