Research Article

# Effects of Encapsulated PUFAs-Antioxidant Supplements on Rumen Fermentation and Metabolomics in Goats: An *In Vitro* Study

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**Abstract** | The present study aimed to evaluate the encapsulated PUFAs-enriched natural antioxidant using in vitro rumen fermentability and metabolomic profile approaches. Three supplementation treatments were tested in vitro: Non-encapsulated PUFAs and natural antioxidant (R1), Calcium soap of PUFAs and natural antioxidant (R2), and Encapsulated PUFAs-enriched natural antioxidant (R3) using a completely randomized block design. The results showed that all supplementation forms did not show significant differences (P>0.05) in rumen pH, bacterial and protozoa populations, total VFA concentration, and ammonia concentration among treatments. However, R3 significantly decreased (P<0.05) the *iso*-valeric acid percentage. The encapsulation form reduced (P<0.05) ruminal dry matter and organic matter degradability, and post-ruminal dry matter digestibility compared to the others. The metabolites identified in the encapsulation treatment included talatizamine, as well as Leu-leu and Leu-phe, which are components involved in the formulation of the encapsulating material. The metabolomic analysis confirmed that encapsulation preserved bioactive compounds in the rumen and post-ruminal. In conclusion, the supplementation of encapsulated PUFAs-enriched natural antioxidant effectively protects PUFAs and natural antioxidant compounds from rumen microbial degradation, supporting their potential use as a high-energy supplement to support the reproductive phase in ruminants.

**Keywords** | Antioxidant, Encapsulation, Goat, Metabolomic, PUFAs, Rumen fermentability

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## INTRODUCTION

Polyunsaturated fatty acids (PUFAs) such as linoleic acid (LA (18:2n-6), alpha-linolenic acid (ALA (C18:3n-3), eicosapentaenoic acid (EPA (C20:5n-3), and docosahexaenoic acid (DHA (C22:5n-3) are essential nutrients for livestock reproduction, as they serve as precursors of prostaglandin which regulates the estrous cycle and fertility (Khotijah *et al*., 2014). According to Savoinin *et al*. (2010), PUFAs cannot be synthesized endogenously. Therefore, dietary PUFAs supplementation is required. A previous study reported that 4% PUFAs supplementation from sunflower seed oil and flaxseed oil increased estrus response, pregnancy rates, and embryo numbers, leading to greater lamb crops (Pujiawati *et al*., 2018). However, PUFAs supplementation during pregnancy and lactation may induce oxidative stress in livestock, leading to an increase in reactive oxygen species (ROS) that can impair

hormonal regulation and immune function. Antioxidants can neutralize ROS that can oxidize PUFAs, inhibit lipid peroxidation, interrupt propagation chains, and chelate metal ions. A compound with high antioxidant activity may better preserve the biological functions of PUFAs, allowing them to serve as an energy source and reproductive hormone precursors in livestock (Fassah *et al*., 2015; Zeng *et al*., 2023). Thus, supplementing high PUFAs diets with exogenous antioxidants is a promising strategy to mitigate the negative effects of PUFAs metabolism (Fassah and Khotijah, 2016). Antioxidants can reduce reactive oxygen species (ROS) that oxidize polyunsaturated fatty acids (PUFAs), suppress chain-carrying species involved in lipid peroxidation, interrupt chain propagation, and chelate metal ions. The higher the antioxidant activity of a compound, the more effective it is in protecting the biological functions of PUFAs (Fassah *et al*., 2015; Losano *et al*., 2018). One potential source of natural antioxidants is jackfruit leaves, active compounds such as flavonoids, terpenoids, saponins, tannins, and phenols. Flavonoids exhibit various biological functions, including antioxidant, anti-inflammatory, antifungal, anticancer, antiviral, and antibacterial activities (Darmawati *et al*., 2015; Prasad *et al*., 2014). The antioxidant content present in jackfruit leaves approximately 0.34% (Pujiawati *et al*., 2023).

In the ruminants, dietary PUFAs undergo extensive biohydrogenation in the rumen leading to the formation of saturated fatty acids and reducing their biological functions. This process commonly leads to stearic acid as the final product of biohydrogenation (Lashkari *et al*., 2019; Makmur *et al*., 2022). Similarly, natural antioxidant compounds, such as polyphenols, are vulnerable to rumen microbial degradation, limiting their protective roles against oxidative stress (Chedea *et al*., 2016). To address this, protection technologies such as calcium soap and encapsulation have been explored.

Protecting PUFAs through calcium soap (Ca-soap) has been proven to effectively reduce PUFAs from rumen degradation. This protection method allows higher fat levels to be included in the diet without negatively impacting rumen fermentation processes (Wahyuni *et al*., 2024), but the application may be limited due to practical application and its stability. Encapsulation offers a more controlled release mechanism and greater preservation by forming a physical barrier around the bioactive compounds, improve stability, and bioavailability of active ingredients (Wang *et al*., 2020; Du *et al*., 2022; Pawestri and Syahbanu, 2024). Additionally, the encapsulation method also improves the chemical stability and water dispersion ability of PUFAs (Venugopalan *et al*., 2021). This approach enables better utilization of encapsulated PUFAs and antioxidant compounds by the host livestock. The encapsulation using water-in-oil (W/O) emulsion technique is considered stable when the primary emulsion remains intact, without separating into free water and oil, during a storage period of 1–4 weeks (Wong *et al*., 2015).

In addition to rumen fermentation analysis, metabolomics offers a powerful tool to evaluate biochemical changes in response to dietary interventions, allowing comprehensive profiling of rumen metabolites under different treatments (Belanche *et al*., 2016). Metabolomics approach enables the detection of a wide range of metabolites originating from both microorganisms and feed, and can be utilized to identify potential biomarkers associated with rumen fermentation status (de Almeida *et al*., 2018). Recently, studies investigating the effectiveness of encapsulated PUFAs and jackfruit leaf extract as an antioxidant using a metabolomic approach have remained scarce. Therefore, this study aimed to evaluate the effects of an encapsulated feed supplement containing PUFAs-enriched natural antioxidants on in vitro rumen fermentation characteristics and the metabolomic profiles compared to calcium soap and non-encapsulated forms.

## MATERIALS AND METHODS

### Antioxidant extraction

The extraction of jackfruit leaves was conducted based on a modified method described by Naijini *et al*. (2024). Jackfruit leaves were oven-dried at 60°C and ground into a fine powder. A total of 1 kg of the powder was macerated in 70% ethanol. Ensuring the solvent level was approximately 1 cm above the sample. The mixture was kept at room temperature for 24 hours. Then, the mixture was filtered using Whatman No. 41 filter paper and concentrated using a rotary evaporator to obtain a viscous extract.

### Calcium soap preparation

Calcium soap was prepared using a modified double decomposition method (Jenkins and Palmquist, 1984). Briefly, sunflower seed oil and flaxseed oil were heated at 80°C. A 10% NaOH solution was prepared using 67 g NaOH and 67 mL distilled water for sunflower oil, and 55.6 g NaOH with 55.6 mL distilled water for flaxseed oil. The NaOH solution (saponification values of 188.481 mg NaOH/g oil for sunflower oil and 192.185 mg NaOH/g oil for flaxseed oil, respectively) was added to the heated oils, and the mixture was stirred for 1 hour. Saponification value is defined as the amount of potassium hydroxide (KOH) required to saponify a given quantity of fat or oil as determined according to the method described by Jenkins and Palmquist (1984). Subsequently, a 30% $CaCl_2$ solution (prepared by dissolving 158.12 g $CaCl_2$ in 158.12 mL distilled water for sunflower oil and 131.22 g $CaCl_2$ in 131.22 mL distilled water for flaxseed oil) was added. The mixture was stirred for an additional 30 minutes until the

oil precipitated. The precipitate was homogenized and air-dried to produce calcium soap.

### Encapsulation preparation

Encapsulation was performed using a water-in-oil (W/O) emulsion method. This method was applied to combine the aqueous phase (natural antioxidants from jackfruit leaf extract) with the oil phase, which was present in a higher proportion. The emulsion was produced from two different ingredients using the emulsifier Tween 80. The resulting emulsion consisted of 10% Tween 80, 15% oil (sunflower seed oil and flaxseed oil), and 75% distilled water. The mixture was homogenized using an ultrasonic cell disruptor UCD-250 (Biobase Bioyu Co., Ltd) until thoroughly mixed. Chitosan (low molecular weight, shrimp-derived; Natura Chem Abadi, Indonesia) was used as a coating material via an ionic gelation method, following the method of Arfan *et al.* (2022) with modifications.

### *In vitro* fermentability and degradability measurement

A randomized complete block design with three dietary treatments was tested using the two-stage method described by Tilley and Terry (1963), namely: Non-encapsulated PUFAs and natural antioxidant (R1), Calcium soap of PUFAs and natural antioxidant (R2), and Encapsulated PUFAs-enriched natural antioxidant (R3). The basal diet consisted of a concentrate and grass mixture in a 70:30 ratio (Table 1). The nutrient content analysis was performed using Buchi NIRFlex N-500 Solids Cell (Flawil, Switzerland) following the method described by Zahera *et al.* (2022). The supplementation of the three feed additives contributed 2.69% to total digestible nutrients (TDN) and 2.54% to ether extract. Details of the supplement composition are provided in Table 2.

Rumen fluid was collected from three goats using the stomach tube method before morning feeding (Shen *et al.*, 2012). The collected rumen fluid was then filtered using six-layer gauze and put into an insulated bottle at a temperature of 39 °C. Samples of 0.5 g basal diet and supplement were incubated in anaerobic conditions at 39 °C with 10 mL of rumen fluid and 40 mL McDougall buffer solution. Fermenters were duplicated for each treatment and incubated for 48 hours (stage 1) and 96 hours (stage 2). The experiment consisted of three treatments with three-time replications (n=6). After the first stage, samples were centrifuged at 3000 rpm for 10 minutes. The supernatant was collected to measure rumen pH, total and partial volatile fatty acid (VFA), ammonia concentration, and microbial populations, while the residue was used for analyzing rumen digestibility of dry matter (DMD) and organic matter (OMD). After enzymatic digestion using pepsin-HCl (2nd stage), the residue from the incubated sample was then filtered using Whatman 41 filter paper and placed in a porcelain dish, which was oven-dried at 105°C for 24 hours to measure the post-ruminal DMD, and burned in a muffle furnace at 600°C for 4 hours to measure post-ruminal OMD (AOAC, 1980). The filtered fluid was used for metabolomic profile analysis.

**Table 1:** Feed ingredients and nutrient composition of the basal diet.

| **Feed Ingredients** | **Percentage (%)** |
|---|---|
| Elephant grass | 30.00 |
| Cassava pulp | 15.51 |
| Copra meal | 20.68 |
| Soybean meal | 10.34 |
| Pollard | 17.17 |
| $CaCO_3$ | 0.72 |
| Premix | 0.72 |
| Salt | 0.72 |
| Molasses | 4.14 |
| **Nutrient** | **Content (%)[1]** |
| Dry Matter | 89.22 |
| Ash | 7.48 |
| Crude Protein | 15.02 |
| Ether extract | 1.99 |
| Crude Fiber | 15.13 |
| Nitrogen-Free Extract (NFE) | 60.38 |
| Total Digestible Nutrient (TDN) | 69.03 |

[1]NIRS result, NFE= 100 – Ash – Crude Fat – Crude Protein – Crude Fiber, TDN= 40.2625 – (0.1379 (Crude Fiber)) + (1.1903 (Crude Fat)) + 1.363 (NFE)) + (0.1969 (Crude Protein)) (Wardeh, 1981).

**Table 2:** Composition of the supplement in each treatment.

| **Feed Ingredients** | **Treatments** | | |
|---|---|---|---|
| | **R1** | **R2** | **R3** |
| | **%** | | |
| Sunflower seed oil | 1.42 | - | - |
| Flaxseed oil | 0.87 | - | - |
| Calcium soap of PUFAs | - | 2.29 | - |
| Antioxidant (Jack fruit leaf extract) | 0.02 | 0.02 | - |
| Encapsulated PUFAs-enriched natural antioxidants | - | - | 7.20 |

R1: Non-encapsulated PUFAs and natural antioxidant, R2: Calcium soap of PUFAs and natural antioxidant, R3: Encapsulated PUFAs-enriched natural antioxidant.

Rumen pH was measured using a pH meter (Mediatech). The ammonia concentration was analyzed using the Indophenol method as described by Chaney and Marbach (1962) with UV-Vis spectrophotometry at 630 nm. The total VFAs were determined using the steam distillation

method according to Despal *et al.* (2022). The molar proportion of VFA concentrations was determined using gas chromatography (GC FID Bruker).

### Rumen microbial population analysis

The rumen bacteria population was determined using the serial dilution method of rumen fluid (Ogimoto and Imai, 1981), followed by cultivation in Brain Heart Infusion (BHI) agar media. The bacterial population is calculated using the following formula:

$$Bacterial\ Population\ (CFU\ mL^{-1}) = \frac{Number\ of\ Colonies \times 10^{n}}{0.05 \times 0.1}$$

Where: n = Dilution series tube

The protozoa population was determined using a counting chamber with Trypan Blue Formalin Saline (TBFS) solution at a 1:1 ratio and observed using a microscope (Ogimoto and Imai, 1981). The protozoa population is calculated using the following formula:

$$Protozoa\ Population\ (cells\ mL^{-1}) \frac{100 \times DF \times C}{0.625 \times 0.25 \times 16 \times 16}$$

Where: C = Number of protozoa counted in the counting chamber; DF = Dilution Factor.

### Metabolomic analysis

The metabolomic profile is analyzed to assess the effectiveness of the encapsulation process. Duplicate filtered in vitro samples were extracted. After samples were thawed on ice and vortexed, 50 mg was weighed and diluted with 1 mL of methanol/water (1:1 vol/vol), vortexed, and centrifuged at 16,000 × g for 10 min at 4 °C. The supernatant and solid precipitate were separated into different vials for aqueous (supernatant) and organic (precipitate) extraction, respectively. For the aqueous extraction, the supernatant was transferred to a new vial, dried under a nitrogen stream, and re-suspended in 500 µL methanol/water (1:1 vol/vol). For the organic extraction, the solid precipitate was dissolved in 1 mL dichlormethane/methanol (3:1 vol/vol), centrifuged (16,000 × g, 10 min at 4 °C), dried under a nitrogen stream, and re-suspended in 500 µL of methanol/water (1:1 vol/vol).

The metabolomic profile was analyzed following Artegoitia *et al.* (2017) using Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS). The column used was a Thermo Scientific™ Accucore™ Phenyl-Hexyl analytical column (100 mm × 2.1 mm ID × 2.6 µm). A gradient technique was applied with a flow rate of 0.3 mL/min. The column temperature was maintained at 40 °C. A total of 200 µL of the sample was injected into the LC-HRMS system. The results of the analysis are processed using chemometric analysis. Sample differentiation and classification are carried out using Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) in the metabolomic analysis of the encapsulation effects on PUFA and antioxidants. Chemometric analysis is performed using MetaboAnalyst 6.0 (Ametaj *et al.*, 2010). Classification of each metabolite was annotated using chemical databases, National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), and Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/).

### Data analysis

Data was analyzed by a completely randomized complete block design with 3 treatments and 3-time replications (Steel and Torrie, 1993). Any significant differences were determined using Duncan's Multiple Range Test. The analysis was carried out using the General Linear Model in IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA). Data was considered statistically significant at $P<0.05$, and a trend was indicated at $0.05<P \leq 0.10$.

## RESULT

### *In vitro* fermentability and degradability

The supplementation of PUFAs-enriched natural antioxidant compounds in different forms did not significantly alter ($P>0.05$) rumen pH, total bacteria and protozoa population, total VFA and ammonia concentrations (Table 3). However, the encapsulated form resulted in the lowest ($P < 0.05$) *iso*-valeric acid molar proportion compared to other treatments. In the present study, encapsulated treatment (R3) significantly reduced ($P<0.05$) DMD in the rumen compared to the non-protected one (Table 4). Similarly, the ruminal OMD tends to be lower ($P=0.05$) in encapsulated form than in non-protected form, indicating improved protection of supplement compounds from rumen degradation. The post-ruminal DMD tended to decrease ($P=0.06$) compared to the non-protected treatment. In addition, post-ruminal OMD was lower ($P<0.05$) in encapsulated treatment compared to others. These findings suggest that encapsulation may enhance the rumen bypass potential of bioactive compounds in supplements.

**Table 3:** *In vitro* rumen fermentation characteristics with different supplementation forms of PUFAs-enriched natural antioxidant in goat rations.

| Parameters | Treatments | | | SEM | P value |
|---|---|---|---|---|---|
| | R1 | R2 | R3 | | |
| Rumen pH | 7.18 | 7.14 | 7.04 | 0.09 | 0.31 |
| Total bacteria (log CFU $mL^1$) | 6.52 | 6.34 | 6.22 | 0.20 | 0.35 |
| Total protozoa (log sel $mL^{-1}$) | 6.21 | 6.02 | 6.19 | 0.04 | 0.23 |
| Total VFA (mM) | 100.03 | 77.82 | 113.93 | 32.42 | 0.59 |
| **Molar proportion of VFA (mM/100 mM)** | | | | | |
| Acetate | 46.47 | 42.43 | 54.17 | 2.38 | 0.22 |
| Propionate | 35.26 | 39.47 | 32.90 | 1.40 | 0.17 |
| *Iso*-Butyrate | 1.94 | 1.87 | 1.28 | 0.15 | 0.22 |
| *n*-Butyrate | 10.36 | 10.85 | 8.17 | 0.75 | 0.40 |
| *Iso*-Valerate | $4.04^{b}$ | $3.30^{ab}$ | $2.25^{a}$ | 0.30 | 0.04 |
| *n*-Valerate | 1.91 | 2.04 | 1.20 | 0.23 | 0.46 |
| Acetat/Propionate | 1.32 | 1.09 | 1.66 | 0.11 | 0.20 |
| $NH_3$ mM | 8.07 | 9.81 | 9.56 | 0.42 | 0.22 |
| Total VFA/$NH_3$ | 12.94 | 7.89 | 11.96 | 3.64 | 0.43 |

R1: Non-encapsulated of PUFAs and natural antioxidant, R2: Calcium soap of PUFAs and natural antioxidant, R3: Encapsulated of PUFAs-enriched natural antioxidant; VFA= Volatile Fatty Acid; $NH_3$= Ammonia; SEM= Standard Error of the Mean; $^{a,b}$ Values with different superscript letters in the same row differ ($P < 0.05$).

**Table 4:** *In vitro* degradability with different supplementation forms of PUFAs-enriched natural antioxidant in goat rations

| Parameters | Treatments | | | SEM | P value |
|---|---|---|---|---|---|
| | R1 | R2 | R3 | | |
| | % | | | | |
| **DM degradability** | | | | | |
| Rumen | $76.16^{b}$ | $65.57^{a}$ | $63.16^{a}$ | 3.34 | 0.02 |
| Post-rumen | $83.91^{b}$ | $82.29^{ab}$ | $78.93^{a}$ | 2.07 | 0.06 |
| **OM degradability** | | | | | |
| Rumen | $77.28^{b}$ | $68.86^{ab}$ | $63.75^{a}$ | 3.12 | 0.05 |
| Post-Rumen | $80.09^{b}$ | $78.44^{b}$ | $74.25^{a}$ | 3.03 | 0.02 |

R1: Non-encapsulated of PUFAs and natural antioxidants, R2: Calcium soap of PUFAs and natural antioxidants, R3: Encapsulated of PUFAs-enriched natural antioxidants; DM = Dry matter; OM = Organic matter; SEM = Standard Error of the Mean; $^{a,b}$ Values with different superscript letters in the same row differ ($P < 0.05$).

## Metabolomic profiling

### Principal component analysis and partial least squares discriminant analysis of post-rumen fluid metabolites

The PCA score plot showed that the three supplementation forms had some similar metabolites (Figure 1a). However, PLS-DA analysis showed that the three supplementation forms were distinctly separated (Figure 1b), suggesting a significant difference in post-rumen metabolites. This result also indicates a unique metabolomic signature resulting from enhancing the protection of bioactive compounds. The encapsulation process appeared to influence both the quantity and quality of bioactive compounds detected in the post-rumen.

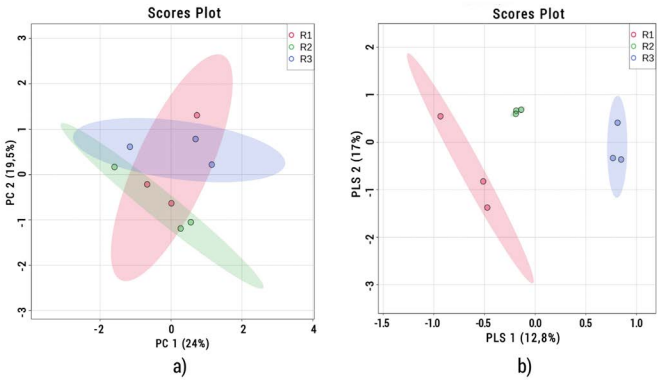


**Figure 1:** (a) Principal Component Analysis (PCA) based on the composition of metabolite compounds in post-rumen fluid subjected to different treatments. (b) Partial Least Squares Discriminant Analysis (PLS-DA) based on identified compounds from LC-HRMS analysis of post-rumen fluid, with treatment-based projections on the scores plot. PCA and PLS-DA analyses were presented separately due to their distinct analytical objectives. PCA was employed to examine natural patterns and the variance structure of metabolite data without considering treatment groups. In contrast, PLS-DA served as a supervised method to evaluate and confirm separation based on predefined treatment groups.

## PLS-DA VIP scores

The PLS-DA with VIP score > 1 identified several key metabolites contributing to the separation between the three supplementation forms (Figure 2). The identified potential metabolites comprised a range of chemical classes, including amino acids, short- and medium-fatty acids, benzene derivatives, and other rumen fermentation by-products. The relative abundance of each compound was visualized by a different color. Semagacestat showed the highest VIP score and was the most abundant in the non-protected treatment, which also exhibited greater levels of the synthetic derivative (3S)-3-(aminomethyl)-5-methylhexanoic acid. Calcium soap treatment showed higher levels of dipeptide derivative, Val-Leu and Leu-Phe, 1(E)-1-(4-methoxyphenyl)-4-methyl-penten-3-one and 2-(Hydroxymethyl)-1-methylpiperidine-3,4,5-triol. In contrast, encapsulated form was characterized by a high abundance of Leu-Leu and 2-phenyl-4-pentenal. The variation in metabolite profiles among the three treatment

groups reflects the distinct impacts of lipid protection strategies on rumen fermentation dynamics and post-ruminal biochemical transformation.

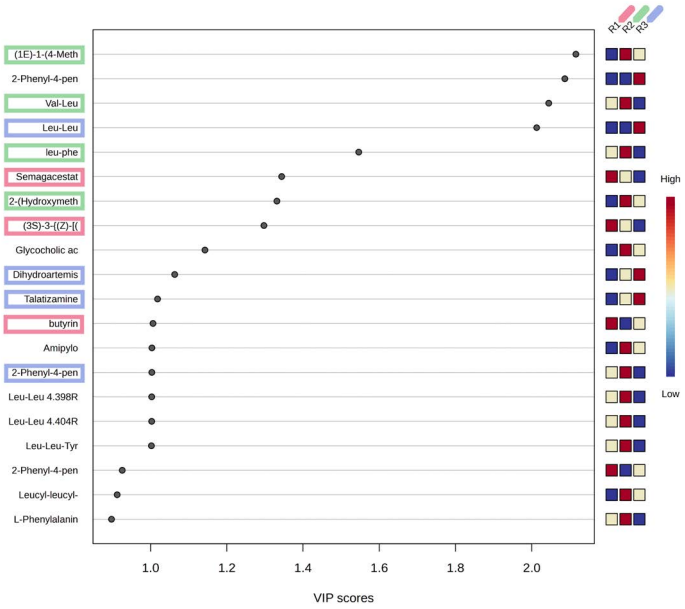


**Figure 2:** Variable Importance in Projection (VIP) scores of secondary metabolite compounds in post-rumen fluid under three different treatments, based on the feature projection of partial least squares discriminant analysis (PLS-DA) model.

### Heatmaps clustering analysis (HCA)

Heatmap Clustering Analysis (HCA of the post-rumen metabolite data visually highlighted the differences in degradation patterns (Figure 3). In non-protected forms, dominant compounds included Dibenzylamine, Navenone A, Butyrin, Semagacestat, and N-Desmethyldiphenhydramine. Calcium soap forms showed a higher abundance of several metabolites including bicine, 2-amino-1,3,4-octadecanetriol, dodecylamine, Leucyl-leucyl-norleucine, Leu-leu-Tyr, and (3S)-3-{(Z)-[(3S)-3-{(Z)-[(3R)-3-Amino-1-hydroxy-4-methylpentylidene] amino}-1-hydroxybutylidene]amino}-5-methylhexanoic acid. Meanwhile, the encapsulated forms was characterized by a high abundance of metabolites such talatizamine, dihydroartemisinin, 2-Phenyl-4-pentenal and leu-leu. These results showed that different protection strategies influence the stability and bioavailability of metabolites across the digestive phase.

## DISCUSSION

### *In vitro* fermentability and degradability

The inclusion of PUFA-enriched natural antioxidants in ruminant diets may exert certain effects in digestive and metabolic processes. In the rumen, microbial biohydrogenation and enzymatic hydrolysis rapidly change the chemical structure of unsaturated fatty acids and polyphenol compounds, compromising their functional benefits. In this study, different supplementation forms did not alter the ruminal pH value. Ruminal pH is closely associated with rumen microbial growth and serves as a key indicator of feed fermentability within the rumen (Tanggela *et al*., 2024; Usboko *et al*., 2024). The pH values observed in all treatments were relatively attributed to the utilization of high PUFAs oil in the ration. Similarly, Hartanto *et al*. (2019) reported high goat's rumen pH (6.96–7.14) when their diets were supplemented with sunflower seed oil.

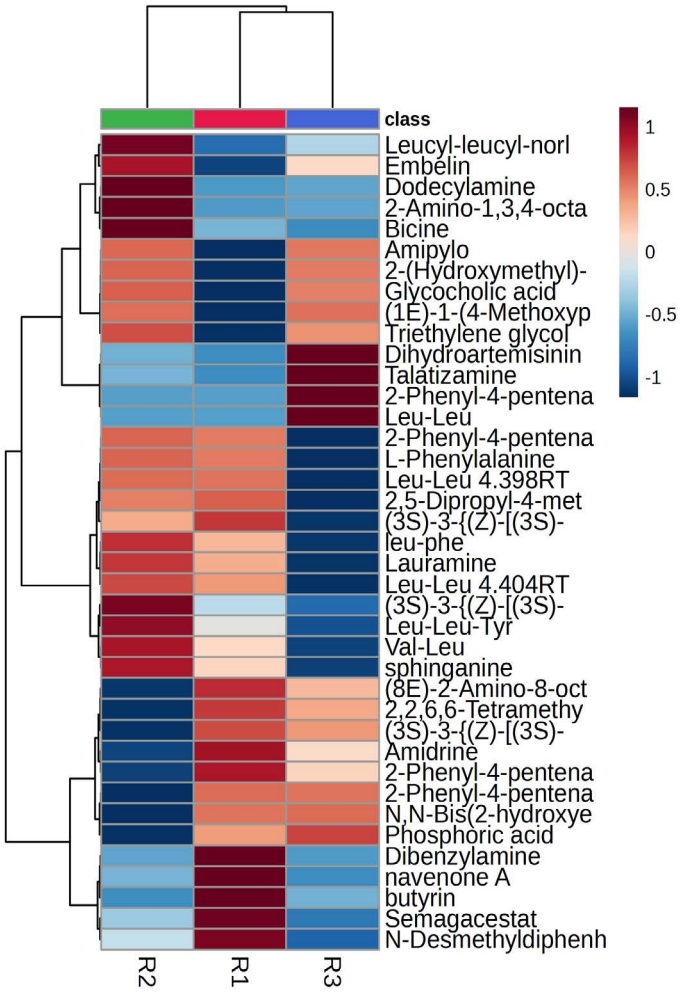


**Figure 3:** Heatmap profiles combined with cluster analysis of secondary metabolites identified by LC-HRMS in post-rumen fluid across the three treatment groups. The distinct clustering patterns and color intensities reflect the variations in metabolite abundance suggesting treatment-specific biochemical transformation with red indicating high abundance and blue indicating low abundance. Data were log transformed and normalized before plotting.

The population of bacteria and protozoa remained stable across different supplementation forms. These results were aligned with Khotijah *et al*. (2016) and Ebrahimi et al. (2017), who reported that dietary inclusion of PUFAs does not necessarily affect total bacteria and protozoa

populations, since the fat level inclusion does not exceed the threshold (Suharti *et al*., 2018). These findings also indicate that both protection methods maintained a stable rumen environment, as indicated by no change in rumen pH and microbial populations. An appropriate pH condition is necessary for supporting optimal microbial growth (Pramono *et al*., 2013). In addition, all treatments used the same basal diet, which met the nutritional requirements for microbial growth.

Volatile Fatty Acids (VFA) are the primary products of ruminal fermentation and serve as the primary energy sources for ruminants. The total VFA concentrations in all treatments were aligned with Pi *et al*. (2019), who reported total rumen VFA of a cow fed with a diet supplemented with flaxseed oil is around 105.71 mM. According to Wang *et al*. (2012), the variability in total VFA concentrations is presumed to be influenced by the distribution of rumen bacterial species present in each sample. This suggests that the supplementation of PUFAs-enriched natural antioxidant compounds, regardless of the protection method, did not negatively affect rumen fermentation. Morsy *et al*. (2015) emphasized that the total rumen VFA concentration is influenced by feed digestibility, microbial population, and dietary composition. The molar proportion of individual VFA is influenced by specific microbial groups. Acetate is typically produced by fibrolytic bacteria through fermentation of dietary fiber and resistant starch, while propionate arises from fermentation of non-structural carbohydrate by amylolytic bacteria (Sutrisno *et al*., 2021; Ahmad *et al*., 2024). No difference in major VFAs such as acetate, propionate, and butyrate molar proportions among treatments may reflect the stability of microbial ecosystems, supported by comparable pH and microbial population. In addition, minimal variation on major VFAs molar proportion suggests similar substrate availability and fermentation duration (Oematan *et al*., 2024).

In this study, PUFAs-enriched natural antioxidant were supplemented in different forms. A decrease in *iso*-valerate molar proportion in calcium soap and encapsulated treatments was likely attributed to a reduction of microbial access to lipid substrates**.** Similarly, PUFAs protection through saponification inhibits the hydrogenation of unsaturated fatty acids into saturated fatty acids by rumen microbes (Muktiani *et al*., 2022). Non-protected supplementation is more susceptible to the rumen biohydrogenation process, and it may alter the composition of rumen microbes, which may lead to the production of various intermediates such as *iso*-valerate (Mirzaei-Alamouti et al., 2021). The encapsulation of PUFAs-enriched natural antioxidant compounds with chitosan likely introduced additional structural resistance to microbial hydrolysis that prevents lipolysis and biohydrogenation process (Budiman *et al*., 2024), contributing to reducing *iso*-valerate formation and altering VFA distribution. Protection technologies such as encapsulation and calcium salts formation prevent biohydrogenation in the rumen, thereby increasing their availability for absorption (Gadeyne *et al*., 2017). The acetate to propionate (C2:C3) ratio reflects the efficiency of energy utilization and the quality of fermentation product (Hambakodu *et al*., 2019). Our result was consistent with a previous study using palm oil in goat ration, which showed a C2:C3 ratio ranging from 1.63 to 1.77 (Chanjula *et al*., 2022).

Comparable ammonia concentrations across the treatments may reflect the uniformity of basal diet composition and similar rumen pH conditions. Ammonia concentration in the rumen is determined by several factors, including rumen pH, feed retention time, and the protein content of the diet (Usboko *et al*., 2024). The result of this study implies that the use of different supplementation forms did not interfere with protein degradability and microbial nitrogen assimilation. This result is also supported by similar microbial populations among treatments, as bacteria and protozoa were involved in degrading dietary protein and recycling of nitrogenous compounds (Suharti *et al*., 2018). All treatments resulted in normal range ammonia concentrations of 8.5–30 mg/100 mL as reported by McDonald *et al*. (2002).

Both protected PUFAs-enriched antioxidant forms resulted in lower rumen DMD and post-rumen OMD, suggesting a protective effect that limits microbial access to bioactive compounds. Calcium soap provides protection by forming stable fatty acid-calcium salt complexes, which prevent microbial hydrolysis at neutral pH. The hydrophilic and hydrophobic tails formed in calcium soap molecules protect them from water, thus reducing the availability of PUFAs and natural antioxidant for microbial metabolism (Pramono *et al*., 2013).

In encapsulated treatment, chitosan-based encapsulation provides physical protection through the matrix, which restricts the diffusion of microbial digestive enzymes to degrade the nutrient contents and potentially delays the nutrient release. Additionally, the antimicrobial properties of chitosan may suppress microbial activity, thereby reducing nutrient degradation (Besharati *et al*., 2022). The low tendency of post-rumen OMD in the encapsulated form may also be influenced by chitosan, which serves as a matrix for encapsulation. Shrimp-derived chitosan contains 81.39% carbohydrates, reflecting its nature as a natural biopolymer derived from carbohydrate-based compounds present in the chitin biomaterial (Cahyono, 2018). In this

study, chitosan was employed as an encapsulating agent, thereby reducing the extent of microbial degradation in the rumen. Despite the reductions, DMD and OMD were in line with the previous study that reported the utilization of protected vegetable oils, which produce DMD and OMD from within the ranges of 70.9%–72% and 74.31%–75.28%, respectively (Bain *et al*., 2018). Taken together, these findings demonstrate that the PUFAs protection method successfully modulates the degradability profile without compromising overall rumen fermentability. The reduction in degradability may serve as an effective strategy to improve rumen bypass nutrient availability and target intestinal nutrient absorption.

### Metabolomic profiles

Metabolomic approaches provide comprehensive insight into biochemical transformations occurring during digestion. When PUFAs and antioxidant compounds are protected, either in calcium soap or encapsulation, their accessibility to rumen microbes is limited, resulting in alterations in nutrient metabolism in both rumen and post-rumen stages. This modulation is evident in the distinct clustering of metabolite profiles observed through PCA, where the two components, PC1 and PC2, account for 24% and 19.5% of the total variation, respectively. The PCA visualization allows for the assessment of sample relationships based on shared metabolic abundance, with grouping patterns indicating underlying biochemical similarity (Roy *et al*., 2021). The sample metabolite distributions revealed clear separation between treatments, highlighting the influence of protection strategies on metabolite fate. Elliptical clustering structures suggest that specific compound groups are dominant within each treatment, while the intersecting ellipses indicate the abundance of similar compounds within the treatments. The PLS-DA further strengthens the separation observed in PCA by maximizing group discrimination based on metabolite variance. In this study, the calcium soap and encapsulated form presented distinct metabolites, suggesting that the protection methods alter the fate of bioactive compounds by delaying microbial access to nutrient degradation.

The VIP scores identified key metabolites responsible for treatment differentiation, while heatmaps clustering was performed to observe the expected separation of classes from the treatments and features with high clustering coefficients (Figures 2 and 3). Among them, butyrin (tributyrin) and talatizamine were notable. Butyrin or tributyrin is a triglyceride formed from the esterification of butyric acid and glycerol, which have anticancer properties (Kumar *et al*., 2014; Palma *et al*., 2022). Butyrin or tributyrin can be hydrolyzed in the animal intestine and subsequently absorbed and utilized by the intestinal tract. This compound functions to reduce the activity of enzymes such as alanine aminotransferase, aspartate transferase, and alkaline phosphatase, thereby potentially lowering stress levels in animals. The butyrate released from tributyrin/butyrin serves as an energy source for intestinal mucosal cells, contributing to tissue repair and maintaining intestinal health (Guo *et al*., 2021). Higher levels of butyrin were observed in non-protected treatment, which likely reflects increased lipolytic and fermentative activity of rumen microbes since the fat sources are readily available for access (Possente *et al*., 2022).

In contrast, talatizamine appeared more abundant in the encapsulated treatment. This compound may come from jackfruit leaf extract, containing flavonoids, alkaloids, tannins, saponins, and phenols that have anti-inflammatory, analgesic, and antiarrhythmic properties (Wong *et al*., 2021; Tandi *et al*., 2020), and its preservation suggests effective protection by the encapsulation matrix.

Dipeptide derivative such as Leu-Leu and Leu-Phe, known for their assembly and gel-forming ability in aqueous systems, since both of them were aliphatic hydrophobic amino acids, (Scarel *et al*., 2023). These compounds were predominantly found in protected treatments, which presumably derived from either protein breakdown products or the interaction with encapsulation materials by ionic gelation method. Its high abundance of Leu-leu in the encapsulated group suggests that the chitosan matrix effectively preserved the integrity of PUFAs and antioxidant compounds, allowing their release in the intestinal environment. Overall, the protection form, particularly the encapsulation method, not only preserves the structural integrity of bioactive compounds but also modulates their metabolic fate, resulting in an increase in nutrient bioavailability post-rumen.

## CONCLUSIONS

Both encapsulation and calcium soap forms may maintain rumen fermentation stability while offering protection of bioactive compounds. Encapsulation is more effective in reducing dry matter and organic matter degradability compared to calcium soap, suggesting enhanced rumen bypass potential. Metabolomic profiling confirms its ability to preserve key metabolites and altered post-rumen biochemical profile. Encapsulated PUFA-enriched natural antioxidant supplements may be used as a functional supplementation strategy to improve nutrient stability and utilization in the reproductive phase of ruminants.

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## NOVELTY STATEMENT

This study evaluates the effectiveness of encapsulated PUFAs-enriched natural antioxidant compounds on rumen fermentability and metabolomic analyses. The protective effect of encapsulation preserves the active compounds from ruminal degradation. To date, no studies have investigated the protective effect of such encapsulation through in vitro rumen fermentability and metabolomic profile. This approach provides new insights into biochemical modulation relevant to ruminant reproductive nutrition.

## AUTHOR'S CONTRIBUTION

Fassah DM, Khotijah L, Pujiawati Y: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing-review and editing. Damayanti SP and Fassah DM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing original draft. Azka SM and Alfiyyah MN: Investigation and Methodology.

### Ethical approval

The *in vitro* study using rumen fluids from goat in this experiment was approved by the Animal Ethics Committee of Directorate of Research and Innovation Licensing Governance, and Scientific Authority (BRIN) (No. 123/KE.02/SK/06/2023).

### Conflict of interest

The authors have declared no conflict of interest.

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