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Dr Anis Muktiani:

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June 13rd, 2024

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Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

The energy needs of dairy cows during the lactation period increase significantly., and the essential mineral zinc is crucial for their metabolic, production, and reproductive processes, despite common deficiencies. This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The four treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 75% ZPOS + 25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from fistulated female dairy goats. The feed consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Our findings showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control ($P<0.05$). While DM, OM, and CP digestibility were unaffected by the treatments, digestibility of EE, CF, NDF, and ADF was significantly higher ($P<0.05$). ZPOS supplementation increased acetate and propionate levels but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1 and EPA compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and MUFA content in rumen liquid.

Keywords: Palm oil, soap, Zinc, fermentability, fatty acid ruminal.

INTRODUCTION

Adequate energy intake often becomes a challenge for high-producing dairy livestock, especially in early lactation. Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023).

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard

polyunsaturated fatty acids to preserve their biological roles, such as being structural components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In addition to Ca, Zinc (Zn) can also be used to protect the oil (Faizah et al., 2019; Muktiani et al., 2020).

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting

unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

The purpose of this study is to determine the effects of supplementing protected palm oil Zinc soap on feed fermentability, digestibility, and the profile of unsaturated fatty acids in the rumen in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation for enhancing energy supply and improving the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. The discovery of the most effective palm oil management method provides a practical alternative solution to energy and zinc deficiencies in dairy cattle, especially for small-scale farmers.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to (Cabatit, 1979). The palm oil used to make Zinc soap is commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by

adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with 4 treatments and 5 replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially ZPOS (75% ZPOS+25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from female goats with fistulas that belongs to the Faculty of Animal and Agricultural Sciences Diponegoro University. The goats were given a dietary trial following the ration for in vitro substrate for 1 week before rumen liquid was taken. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flasks under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley and Terry (1963). Into the fermenter tube, put 0.55 grams of feed samples from each treatment then added 40 ml of McDaugall's solution and 10 ml of rumen liquid. The fermentor cylinder

145 was flowed by CO2 gas and closed to anaerobic conditions. The fermentor tube was
146 incubated in a 39°C water temperature.
147
148 **Nutrien Digestibility**
149 The digestibility of nutrients including dry matter (DMD), organic matter (OMD),
150 crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through 2
151 stages of incubation, namely fermentative and enzymatic. First stage, the fermentor tube
152 was incubated in a 39°C water temperature for 2x24 hours, and process was stopped by
153 immersing the fermenter tube in ice water for 20 minutes. Then the tube was centrifuged
154 for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded. Second stage,
155 the HCL pensin solution was added 50 ml into the tube and reincubated for 2x24 hours
156 in a 39°C water temperature under aerobic conditions. The sample was filtered with
157 Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed by
158 DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility
159 values. Fermentation is also carried out without feed samples called blanks. Nutrien
160 digestibility samples are calculated by the formula :
161 $$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$
162
163 **pH value, VFA, NH3 and Methane production**
164 The process of measuring pH, VFA, and NH3 followed the same procedure as that
165 for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After
166 centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to
167 measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH
168 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample

was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). The production of partial VFAs (acetic acid, propionate, butyrate) was measured using gas chromatography, following the General Laboratory Procedures (1966). The VFA sample production was then calculated using the specified formula:

$$\text{Partial VFA (mM)} = \frac{\text{sample area x standard concentration x 1000}}{\text{(standard area x MW)}}$$

The methane gas concentration and Energy conversion efficiency was determined by calculating the VFA stockiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Asetate) - 0.25 (% Propionate) + 0.5 (% Butirate). Energy conversion efficiency was calculated based on the stockyometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\text{E (\%)} = \frac{(\ 0{,}622\ \text{pA} + 1{,}091\ \text{pP} + 1{,}558\ \text{pB}\ \text{pA} + \text{pP} + 2\ \text{pB}\ )}{\text{pA} + \text{pP} + 2\text{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde, 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein microbes using the method of Makkar et al. (1982) on the principle of gradual

centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SA), ranging from C4 to C15, and long-chain fatty acids (LA), which are greater than C16.

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p>0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability

The fermentability of feed is determined by various factors such as pH, total protozoa count, microbial protein content, NH3 concentration, and total VFA production in rumen liquid. These parameters are detailed in Table 2. The pH levels across all treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil supplementation had the most pronounced impact, notably reducing protozoa count and

microbial protein. When zinc soap palm oil protection (T2) was partially supplemented, it led to higher microbial protein and VFA production compared to feed supplemented with total zinc palm oil soap, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the palm oil zinc soap supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$) in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

Relative Proportion of VFA

Palm oil supplementation significantly influenced the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$), but did not impact the efficiency of hexose energy conversion into VFA. The relative proportions of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate, propionate, and butyrate

compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZSPO supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SA), with a notably higher concentration of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($P<0.05$). However, the proportion of stearate was significantly lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), and EPA (20:5), showed a significant increase in the 75% and 100% ZSPO treatments ($P<0.01$), while the increase in DHA was not statistically significant.

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 5, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), while it yielded a greater amount of LCFAs. SFAs showed no significant differences among all treatments, whereas ZSPO supplementation demonstrated an ability to elevate UFAs. Notably, the 100% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among

289 all treatments, while microbial protein production was the highest. This indicates that
290 NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).
291
292 **Feed Digestibility**
293 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
294 unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this
295 level of supplementation is safe for rumen microbial growth. These results are consistent
296 with previous research, which found that 6% supplementation with vegetable oils (olive
297 oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable
298 to the control, although linseed oil resulted in higher digestibility than sunflower oil
299 (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly
300 dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The
301 experimental feed in this study, characterized by high fiber content and low ADF
302 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
303 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
304 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
305 increased EED, supporting the conclusion that palm oil can be use d as an energy
306 supplement without compromising rumen feed fermentability.
307 A notable finding from this research is that both partial and total zinc soap
308 supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and
309 ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains
310 high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015).
311 Recent findings by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter
312 populations in response to palmitic acid supplementation. This suggests that cellulolytic

bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to the control, specifically 13.37 (T2) and 11.41 (T3) versus 8.01 in the control feed (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with

ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013).

The increase in propionate in the ZSPO supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with studies showing that ZSPO supplementation leads to an increase in protozoan populations (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio are advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

The introduction of oil supplementation led to a reduction in the proportion of short-chain fatty acids within rumen liquid, while concurrently increasing the presence of long-chain fatty acids. This transition is attributed to the predominant presence of linoleic acid (LA) in palm oil, constituting 98.3% of its composition, with the remaining 1.7% comprising short-chain fatty acids. Among the long-chain fatty acids, comprising 98.3% of the total, nearly half (49.9%) are unsaturated fatty acids (UFAs), comprising 39.2%

monounsaturated fatty acids (MUFAs) and 10.5% polyunsaturated fatty acids (PUFAs) (Mancini et al., 2015). The prevalence of saturated fatty acids (SFAs) in palm oil contributed to a consistent range of ruminal saturated fatty acid levels, varying insignificantly between 70.90% and 79.05% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, namely being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Mosley et al. (2002) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid. Both trans-11 18:1 and cis-9, trans-11 CLA found in milk fat have been shown to have health effects (Lock et al., 2006).

Consistent with our study's findings, Mosley et al. (2002) observed that supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of

palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed as much as 5% has beneficial effects, namely increasing fermentability, digestibility, MUFA and EPA. It was indicated that partial administration of ZPOS (75%) resulted in higher protozoa populations and fiber digestibility. So it can be considered in trials on dairy livestock.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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627 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

628 [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN = -17.2649 +
629 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according to (Wardeh, 1981).
630
631
632 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
633 rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment			
	T0	T1	T2	T3
pH	6,76	6,75	6,73	6,82
Protozoa (10^3 cel/ml)	$98,14^{a}$	$32,57^{c}$	$82,83^{ab}$	$69,31^{b}$
Microbial protein (mg/ml)	$13,01^{a}$	$9,37^{b}$	$13,37^{a}$	$11,41^{ab}$
NH^3 (mM)	$14,06^{a}$	$14,45^{a}$	$9,64^{b}$	$10,36^{b}$
VFA (mM)	$87,52^{b}$	$101,78^{b}$	$164,38^{a}$	$157,89^{a}$

634 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
635 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS(75%
636 ZPOS+25%PO); T3= basal diet+5% ZPOS
637
638
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Tabel 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment			
	T0	T1	T2	T3
Digestibility :				
Dry matter (%)	64.06	63.82	67.20	69.24
Organic matter (%)	69.16	65.89	69.65	69.98
Crude protein (%)	68.69	67.99	66.39	65.11
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}
Acid detergent fiber (%)	45,12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment			
	T0	T1	T2	T3
Acetate (mM)	41,17^{b}	51,10^{b}	59,31^{a}	65,80^{a}
Propionate (mM)	15,91^{c}	25,41^{bc}	28,73^{a}	29,89^{a}
Butyrate (mM)	8,73^{b}	10,28^{b}	12,55^{a}	14,20^{a}
A/P	2,59^{a}	2,01^{b}	2,06^{b}	2,20^{b}
Methan (mM)	31,87^{a}	28,04^{b}	28,58^{b}	29,57^{ab}
Efficiency of energy conversion (%)	73,05	73,19	74,74	74,47

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

668 **Table 5**. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm
669 Oil (ZPOS) In Vitro

Fatty Acids	Treatment			
	T0	T1	T2	T3
	----------% fat ----------			
Short chain Fatty Acids (SA)				
C4	7,34 [b]	5,34 [a]	5,27 [a]	2,36 [c]
C6	<0,1	<0,1	<0,1	<0,1
C8, kaprilat	<0,1	<0,1	<0,1	<0,1
C10, kaprat	<0,1	<0,1	<0,1	<0,1
C12, laurat	1,38	1,15	1,22	1,2
C13	0,3	0,17	<0,1	<0,1
C14, miristat	2,19	1,85	2,3	2,08
C14:1	1,00	0,5	0,57	0,64
C15,	0,51	0,75	0,36	0,43
C15:1	0,34	0,23	0,16	0,21
Long chain Fatty Acids (LA)				
C16, palmitat	22,03 [b]	29,34 [a]	29,23 [a]	31,14 [a]
C16:1, n7	1,02 [b]	1,04 [b]	1,36 [ab]	1,5 [a]
C17	0,39	0,23	0,23	0,28
C17:1	<0,1	<0,1	<0,1	<0,1
C18, stearate	20,13 [b]	30,74 [a]	23,17 [a]	21,5 [a]
trans 11 C18:1	12,55 [c]	17,84 [b]	26,16 [a]	28,05 [a]
cis 9 C18:1, oleat	1,09 [b]	1,43 [ab]	1,99 [a]	1,83 [a]
C18:2	1,04 [c]	1,31 [bc]	1,6 [ab]	1,86 [a]
C18:3	<0,1	<0,1	<0,1	<0,1
C18:3, omega 6	0,22[b]	0,26 [b]	0,45 [a]	0,53 [a]
C18:3,gamma linolenat	0,79	<0,1	0,38	0,41
C20	1,01	0,83	0,64	0,77
cis 11 C20:1	0,16	0,59	0,21	0,23
cis 11-14 C20:2	<0,1	<0,1	<0,1	<0,1
C20:3	<0,1	<0,1	<0,1	<0,1
C20:4, arakidonat	0,48 [a]	<0,1 [b]	<0,1 [b]	<0,1 [b]
C20:5, EPA	0,07 [c]	0,55 [b]	0,59 [b]	0,76 [ab]
C21	0,38	0,31	0,2	0,26
C22, behenate	0,4	0,23	0,23	0,25
C22:1	<0,1	<0,1	<0,1	<0,1
C22:6, DHA	3,19	4,81	4,57	4,52
C24	<0,1	<0,1	<0,1	<0,1
C24:1, nervonat omega 9	<0,1	<0,1	<0,1	<0,1

670 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
671 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75%
672 POZS+25%PO); T3= basal diet+5% ZPOS
673
674

675 **Table 6**. Proportion of short chain, long chain, saturated and unsaturated fatty acids in
676 rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro
677

Fatty Acids	Treatment			
	T0	T1	T2	T3
	----------% fat ----------			
Amount of fatty acids :				
SA	13,06[a]	9,99 [b]	9,88 [b]	6,92 [c]
LA	64,95	89,51[a]	81,01[a]	83,09 [a]
SFA	79,05	71,94	72,53	70,9
UFA	20,95 [b]	28,06 [a]	27,47 [a]	29,1 [a]
MUFA	15,16 [b]	17,13 [ab]	19,88 [a]	21,82 [a]
PUFA	5,79 [c]	6,93[bc]	7,59 [ab]	8,08 [a]

678 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
679 SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated
680 fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
681 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75%
682 POZS+25%PO); T3= basal diet+5% ZPOS
683

2. Permintaan revisi untuk draft yang dikirim ke jurnal

Gmail **Anis Muktiani <anis.muktiani@gmail.com>**

[TASJ] Revision Required of Your Manuscript

2 messages

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id> Tue, Jun 25, 2024 at 8:21 AM
To: Anis Muktiani <anismuktiani@gmail.com>

Dear Anis Muktiani:

It is my pleasure to inform you that your submission to Tropical Animal Science Journal, "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid" had been examined by peer reviewers. Please find the comments and suggestions:

Submission URL: https://journal.ipb.ac.id/index.php/tasj/authorDashboard/submission/56357
Username: {$authorUsername}

If you decide to revise the manuscript, please give a response or rebuttal against each point which are suggested by peer reviewers. The revised document should include revision note file in table form and revised manuscript in MS Word file. Please return back the documents to the editor within 14 days via OJS, we would be glad if you submit your revised manuscript as soon as possible.

If you have any questions, please contact me.

Prof. Dr. Komang G Wiryawan
Tropical Animal Science Journal
kgwiryawan@yahoo.com

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Anis Muktiani <anis.muktiani@gmail.com> Thu, Jun 27, 2024 at 8:45 AM
To: "Prof. Dr. Komang G Wiryawan" <jurnal@apps.ipb.ac.id>

Thank you, I will do that.
[Quoted text hidden]

FORM C1

Tropical Animal Science Journal

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PAPER EVALUATION

Paper Title: Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid

Comments (please use additional paper if more space is needed)

No	Comments	Author's response
A	**Editor**	
1	**Introduction** Please state the novelty clearly. The introduction should contain problems, previous research, novelty statements, and objectives.	
2	**References** a) Please check the writing of references from journals and books, also how to cite references in the text. b) Please ensure that every reference cited in the text is also present in the reference list (and vice versa). c) Please ensure that the number of journal publications published in the last 10 years is more than 80%.	
3	Tables 2-6: please provide the SEM data for all variables.	
B	**Reviewer I (MB1)**	
1	Title: Correct the title clearly	
2	Line 111-112, a dietary trial: Specify clearly the nutrient content of the diet	
3	Conclusion: Make it more compact, and do not repeat the statement of experimental result.	
C	**Reviewer II (MB2)**	
1	Please find the comments in the text.	

Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid

ABSTRACT

The energy needs of dairy cows during the lactation period increase significantly~~.~~, and the essential mineral zinc is crucial for their metabolic, production, and reproductive processes, despite common deficiencies. This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The four treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 75% ZPOS + 25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from fistulated female dairy goats. The feed consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). ~~Our findings~~Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control (P<0.05). While DM, OM, and CP digestibility were ~~unaffected by~~similar among the treatments, digestibility of EE, CF, NDF, and ADF was significantly higher (P<0.05). ZPOS supplementation increased acetate and propionate levels but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1 and EPA compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and MUFA content in rumen liquid.

Keywords: Palm oil, soap, Zinc, fermentability, fatty acid ruminal.

Commented [A1]: How many replicates?

Commented [A2]: From how many goats?

Commented [A3]: Compared to what? Please be more specific.

Commented [A4]: P-value?

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INTRODUCTION

Adequate energy intake often becomes a challenge for high-producing dairy livestock, especially in early lactation. Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023).

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Commented [A7]: Is it similar between tropical and temperate regions? Please address such difference.

Commented [A8]: The highest proportion is palmitic acid, which is SFA. Please also address here the palmitic acid in palm oil including its roles or effects in the rumen.

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In addition to Ca, Zinc (Zn) can also be used to protect the oil (Faizah et al., 2019; Muktiani et al., 2020).

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane

integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

The purpose of this study was~~is~~ to ~~determine~~ investigate the effects of supplementing protected palm oil Zinc soap on feed fermentability, digestibility, and the profile of unsaturated fatty acids in the rumen in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation for enhancing energy supply and improving the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. The discovery of the most effective palm oil management method provides a practical alternative solution to energy and zinc deficiencies in dairy cattle, especially for small-scale farmers.

Commented [A9]: Were there any other studies that use Zn soap in the rumen? Please describe here.
Please also explain the novelty of this study in comparison to those relevant studies.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to ~~(~~Cabatit~~,~~ (1979). The palm oil used to make Zinc soap ~~is~~ was a commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and

stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

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The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with four~~4~~ treatments and ~~5~~ five replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially ZPOS (75% ZPOS+25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from female goats with fistulas that belongs to the Faculty of Animal and Agricultural Sciences Diponegoro University. The goats were given a dietary trial following the ration for in vitro substrate for one~~1~~ week before rumen liquid was taken. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flasks under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley and Terry (1963). Into the fermenter tube, put 0.55 grams of feed samples from each treatment then added 40 ml of McDaugall's solution and 10 ml of rumen liquid. The fermentor cylinder

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was flowed by CO2 gas and closed to anaerobic conditions. The fermentor tube was incubated in a 39°C water temperature.

Nutrient Digestibility

The digestibility of nutrients including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through two~~2~~ stages of incubation, namely fermentative and enzymatic. First stage, the fermentor tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then the tube was centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded. Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples called blanks. Nutrien digestibility samples are calculated by the formula :

$$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$

pH value, VFA, NH3 and Methane production

The process of measuring pH, VFA, and NH3 followed the same procedure as that for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample

was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). The production of partial VFAs (acetic acid, propionate, butyrate) was measured using gas chromatography, following the General Laboratory Procedures (1966). The VFA sample production was then calculated using the specified formula:

Commented [A12]: I don't think that GC measurement of rumen VFA is according to GLP, please check again.

$$\text{Partial VFA (mM)} = \frac{\text{sample area x standard concentration x 1000}}{\text{(standard area x MW)}}$$

The methane gas concentration and Energy conversion efficiency was determined by calculating the VFA stoichiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion efficiency was calculated based on the stockyometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\text{E (\%)} = \frac{(\ 0{,}622\ \text{pA} + 1{,}091\ \text{pP} + 1{,}558\ \text{pB}\ \text{pA} + \text{pP} + 2\ \text{pB}\ )}{\text{pA} + \text{pP} + 2\text{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde, 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein

microbes using the method of Makkar et al. (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SA), ranging from C4 to C15, and long-chain fatty acids (LA), which are greater than C16.

Commented [A13]: Should be categorized into SCFA, MCFA and LCFA.

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability

The fermentability of feed is determined by various factors such as pH, total protozoa count, microbial protein content, NH_3 concentration, and total VFA production in rumen liquid. These parameters are detailed in Table 2. The pH levels across all treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH_3 concentrations. Palm oil

Commented [A14]: Data on protozoa should be converted first into log scale, and then analyzed by using ANOVA.

supplementation had the most pronounced impact, notably reducing protozoa count and microbial protein. When zinc soap palm oil protection (T2) was partially supplemented, it led to higher microbial protein and VFA production compared to feed supplemented with total zinc palm oil soap, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the palm oil zinc soap supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$) in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

Relative Proportion of VFA

Palm oil supplementation significantly influenced the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$), but did not impact the efficiency of hexose energy conversion into VFA. The relative proportions of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate, propionate, and butyrate compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO

supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZSPO supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SA), with a notably higher concentration of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($P<0.05$). However, the proportion of stearate was significantly lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), and EPA (20:5), showed a significant increase in the 75% and 100% ZSPO treatments ($P<0.01$), while the increase in DHA was not statistically significant.

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 5, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), while it yielded a greater amount of LCFAs. SFAs showed no significant differences among all treatments, whereas ZSPO supplementation demonstrated an ability to elevate UFAs. Notably, the 100% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

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Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be use d as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total zinc soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015). Recent findings by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to the control, specifically 13.37 (T2) and 11.41 (T3) versus 8.01 in the control feed (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO_2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013).

311 The increase in propionate in the ZSPO supplementation treatment resulted from
312 the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted
313 into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated
314 fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids
315 from rumen bio-hydrogenation and increasing the duodenal flows of mono and
316 polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with
317 studies showing that ZSPO supplementation leads to an increase in protozoan populations
318 (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty
319 acid profiles, notably an increase in propionate and a reduction in the acetate-to-
320 propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane
321 production and a lower A/P ratio are advantageous, as higher propionate levels provide a
322 carbon framework and synthetic energy for livestock.

323 **Fatty Acids in Rumen Liquid**

324 The introduction of oil supplementation led to a reduction in the proportion of
325 short-chain fatty acids within rumen liquid, while concurrently increasing the presence of
326 long-chain fatty acids. This transition is attributed to the predominant presence of linoleic
327 acid (LA) in palm oil, constituting 98.3% of its composition, with the remaining 1.7%
328 comprising short-chain fatty acids. Among the long-chain fatty acids, comprising 98.3%
329 of the total, nearly half (49.9%) are unsaturated fatty acids (UFAs), comprising 39.2%
330 monounsaturated fatty acids (MUFAs) and 10.5% polyunsaturated fatty acids (PUFAs)
331 (Mancini et al., 2015). The prevalence of saturated fatty acids (SFAs) in palm oil
332 contributed to a consistent range of ruminal saturated fatty acid levels, varying
333 insignificantly between 70.90% and 79.05% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, namely being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Mosley et al. (2002) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid. Both trans-11 18:1 and cis-9, trans-11 CLA found in milk fat have been shown to have health effects (Lock et al., 2006).

Consistent with our study's findings, Mosley et al. (2002) observed that supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group

with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed as much as 5% has beneficial effects, namely increasing fermentability, digestibility, MUFA and EPA in the rumen. It was indicated that partial administration of ZPOS (75%) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS ~~So it can be considered in trials~~ on dairy ~~livestock~~cows in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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572 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

573 [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN =
574 -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according
575 to (Wardeh, 1981).
576
577
578 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
579 rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment			
	T0	T1	T2	T3
pH	6,76	6,75	6,73	6,82
Protozoa (10^3 cell/ml)	$98,14^{a}$	$32,57^{c}$	$82,83^{ab}$	$69,31^{b}$
Microbial protein (mg/ml)	$13,01^{a}$	$9,37^{b}$	$13,37^{a}$	$11,41^{ab}$
NH_3 (mM)	$14,06^{a}$	$14,45^{a}$	$9,64^{b}$	$10,36^{b}$
VFA (mM)	$87,52^{b}$	$101,78^{b}$	$164,38^{a}$	$157,89^{a}$

580 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
581 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
582 diet+partially ZPOS(75% ZPOS+25%PO); T3= basal diet+5% ZPOS
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Commented [A16]: Please provide SEM and P-values for all parameters.

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Table 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment			
	T0	T1	T2	T3
Digestibility :				
Dry matter (%)	64.06	63.82	67.20	69.24
Organic matter (%)	69.16	65.89	69.65	69.98
Crude protein (%)	68.69	67.99	66.39	65.11
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}
Acid detergent fiber (%)	45,12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

Commented [A17]: Please provide SEM and P-values for all parameters.

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment			
	T0	T1	T2	T3
Acetate (mM)	41,17^{b}	51,10^{b}	59,31^{a}	65,80^{a}
Propionate (mM)	15,91^{c}	25,41^{bc}	28,73^{a}	29,89^{a}
Butyrate (mM)	8,73^{b}	10,28^{b}	12,55^{a}	14,20^{a}
A/P	2,59^{a}	2,01^{b}	2,06^{b}	2,20^{b}
Methane (mM)	31,87^{a}	28,04^{b}	28,58^{b}	29,57^{ab}
Efficiency of energy conversion (%)	73,05	73,19	74,74	74,47

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

Commented [A18]: Please provide SEM and P-values for all parameters.

Table 5. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment			
	T0	T1	T2	T3
	----------% fat ----------			
Short chain Fatty Acids (SA)				
C4	7,34 [b]	5,34 [a]	5,27 [a]	2,36 [c]
C6	<0,1	<0,1	<0,1	<0,1
C8, kaprilat	<0,1	<0,1	<0,1	<0,1
C10, kaprat	<0,1	<0,1	<0,1	<0,1
C12, laurat	1,38	1,15	1,22	1,2
C13	0,3	0,17	<0,1	<0,1
C14, miristat	2,19	1,85	2,3	2,08
C14:1	1,00	0,5	0,57	0,64
C15,	0,51	0,75	0,36	0,43
C15:1	0,34	0,23	0,16	0,21
Long chain Fatty Acids (LA)				
C16, palmitat	22,03 [b]	29,34 [a]	29,23 [a]	31,14 [a]
C16:1, n7	1,02 [b]	1,04 [b]	1,36 [ab]	1,5 [a]
C17	0,39	0,23	0,23	0,28
C17:1	<0,1	<0,1	<0,1	<0,1
C18, stearate	20,13 [b]	30,74 [a]	23,17 [a]	21,5 [a]
trans 11 C18:1	12,55 [c]	17,84 [b]	26,16 [a]	28,05 [a]
cis 9 C18:1, oleat	1,09 [b]	1,43 [ab]	1,99 [a]	1,83 [a]
C18:2	1,04 [c]	1,31 [bc]	1,6 [ab]	1,86 [a]
C18:3	<0,1	<0,1	<0,1	<0,1
C18:3, omega 6	0,22[b]	0,26 [b]	0,45 [a]	0,53 [a]
C18:3,gamma linolenat	0,79	<0,1	0,38	0,41
C20	1,01	0,83	0,64	0,77
cis 11 C20:1	0,16	0,59	0,21	0,23
cis 11-14 C20:2	<0,1	<0,1	<0,1	<0,1
C20:3	<0,1	<0,1	<0,1	<0,1
C20:4, arakidonat	0,48 [a]	<0,1 [b]	<0,1 [b]	<0,1 [b]
C20:5, EPA	0,07 [c]	0,55 [b]	0,59 [b]	0,76 [ab]
C21	0,38	0,31	0,2	0,26
C22, behenate	0,4	0,23	0,23	0,25
C22:1	<0,1	<0,1	<0,1	<0,1
C22:6, DHA	3,19	4,81	4,57	4,52
C24	<0,1	<0,1	<0,1	<0,1
C24:1, nervonat omega 9	<0,1	<0,1	<0,1	<0,1

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

Commented [A19]: Please provide SEM and P-values for all parameters.

Commented [A20]: Please categorize the fatty acids into three:
1. Short chain FA (SCFA)
2. Medium chain FA (MCFA)
3. Long chain FA (LCFA)

Table 6. Proportion of short chain, long chain, saturated and unsaturated fatty acids in rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment			
	T0	T1	T2	T3
	----------% fat ----------			
Amount of fatty acids :				
SA	13,06[a]	9,99 [b]	9,88 [b]	6,92 [c]
LA	64,95	89,51[a]	81,01[a]	83,09 [a]
SFA	79,05	71,94	72,53	70,9
UFA	20,95 [b]	28,06 [a]	27,47 [a]	29,1 [a]
MUFA	15,16 [b]	17,13 [ab]	19,88 [a]	21,82 [a]
PUFA	5,79 [c]	6,93[bc]	7,59 [ab]	8,08 [a]

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

Commented [A21]: Please provide SEM and P-values for all parameters.

Commented [A22]: SCFA

Commented [A23]: LCFA

3. Perbaikan draft publikasi dari penulis

Gmail **Anis Muktiani <anis.muktiani@gmail.com>**

[TASJ] New notification from Tropical Animal Science Journal

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No	Comments	Author's response
A	**Editor**	
1	**Introduction** Please state the novelty clearly. The introduction should contain problems, previous research, novelty statements, and objectives.	The problem : Early lactation dairy cows experience negative energy balance due to decreased dry matter intake (DMI). Zinc deficiency is caused by increased zinc requirements at the beginning of lactation and low zinc content in feed ingredients. Previous research : Suplementation palm oil. Suplementation protected oil with Calcium. Suplementation Zinc soap (in vitro), however there has been no research that explores the contribution of long chain fatty acids, MUFA and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. Novelty : - The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock. - The results of this study provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock. Objectives : This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro.

FORM C1

Tropical Animal Science Journal

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No	Comments	Author's response
2	**References** a) Please check the writing of references from journals and books, also how to cite references in the text. b) Please ensure that every reference cited in the text is also present in the reference list (and vice versa). c) Please ensure that the number of journal publications published in the last 10 years is more than 80%.	a) Already did b) Already did, we used Mandeley. c) Amount of references 54 references Journal : 43 journal journal published 2014-2024 : 35 journal (81%)
3	Tables 2-6: please provide the SEM data for all variables.	a) Already did in Table 2, Table 3, Table 4, Table 5, and Table 6
B	**Reviewer I (MB1)**	
1	Title: Correct the title clearly	Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid
2	Line 111-112, a dietary trial: Specify clearly the nutrient content of the diet	The nutrient content of basal feed is listed in Table 1 (line 598)
3	Conclusion: Make it more compact, and do not repeat the statement of experimental result.	Already did
C	**Reviewer II (MB2)**	
1	Please find the comments in the text.	
	- Abstract a) Line 12 : - How many replicates? - From how many goats? b) Line 18 : - Compared to what? c) Line 19 : - P-value?	a) The study used a completely randomized design with 4 treatments and 5 replications. The inoculum source was rumen liquid from three fistulated female dairy goats, and were homogenized. b) ZPOS supplementation resulted higher acetate and propionate levels compared to the control and c) supplementation 5% palm oil (P<0.05)

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No	Comments	Author's response
	d) Line 21 : - Please describe. e) Line 23 : - Please describe.	d) eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) e) monounsaturated fatty acid (MUFA)
	- Introduction a) Line 27 - 32 : - Is it similar between tropical and temperate regions? Please address such difference. b) Line 37 – 38 : - The highest proportion is palmitic acid, which is SFA. Please also address here the palmitic acid in palm oil including its roles or effect in the rumen. c) Line 77 – 84 : - Were there any other studies that use Zn soap in the rumen? Please describe here. Please also explain the novelty of the study in comparison to those relevant studies.	a) Already did (line 34-43) b) High levels of palmitic acid are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears et al. (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. c) Already did (line 91-103). Novelty : - The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock. - The results of this study provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.
	- Material and Methods a) Line 98 – 99 : - Is there any proof that the saponification profess is really working to produce ZPOS?	a) The saponification reaction that occurs between palm oil (triglyceride,TG), KOH and ZnCl2 is as follows:

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No	Comments	Author's response
		$2\ TG + 6\ KOH \rightarrow 2\ Gly + 6\ R\text{-}C(=O)\text{-}OK$ $6\ R\text{-}C(=O)\text{-}OK + 3ZnCl_2 \rightarrow 3\ (R\text{-}COO)_2Zn + 6\ KCl$ $2\ TG + 6\ KOH + 3\ ZnCl_2 \rightarrow 2Gly + 3\ (R\text{-}COO)_2Zn + KCl$ A soap precipitate $(R\text{-}COO)_2Zn$ is formed. - The formation of ZPOS can also be seen from the type of rumen fatty acid (Table 5), oleic acid on ZPOS supplementation was higher than PO supplementation, indicating that the fatty acid did not undergo biohydrgenation.
	b) Line 118 – 119 : - Please pidaraphrase the sentence into passive voice.	b) Already did. (line 143-144) In the fermenter tube, 0.55 grams of feed samples from each treatment were added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid.
	c) Line 146 – 148 : - I don't think that GC measurement of rumen VFA is according to GLP, please check again.	c) Already did. (line 172-177) Gas chromatography, as reported by Cottyn and Boucque (1968) was used to quantify the generation of partial VFAs
	d) Line 176 – 177 : - Should be categorized into SCFA, MCFA, and LCFA.	d) Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).
	- Result a) Line 186 : - Data on protozoa should be converted first into log	a) Already did (file 3)

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No	Comments	Author's response
	scale, and then analyzed by using ANOVA.	
	- Table a) Table 2 : - Please provide SEM and P-values for all parameters. b) Table 3 : - Please provide SEM and P-values for all parameters. c) Table 4 : - Please provide SEM and P-values for all parameters. d) Table 5 : - Please provide SEM and P-values for all parameters. - Please categorize the fatty acids into three: 1. Short chain FA (SCFA) 2. Medium chain FA (MCFA) 3. Long chain FA (LCFA) e) Table 6 : - Please provide SEM and P-values for all parameters. - SCFA - LCFA	a) Already did b) Already did c) Already did d) Already did - Already did : 1. SCFA : C<6 2. MCFA : C6-C12 3. LCFA : C>12 e) Already did

Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid profiles in vitro. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated female dairy goats, and were homogenized. The goats were feed on consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control ($P<0.05$). Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher than another treatment ($P<0.05$). ZPOS supplementation resulted higher acetate and propionate levels compared to the control and supplementation 5% palm oil ($P<0.05$) but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content in rumen liquid.

Keywords: Palm oil soap, Zinc, fermentability, unsaturated fatty acid ruminal.

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to decreased dry matter intake (DMI). Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023). To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessement of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera et al., 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (Bos taurus x Bos indicus) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (Bos taurus). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high

levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears et al. (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In

addition to Ca, NaOH can also be used to protect the oil (Wulandari et al., 2020). However, the use of Zn minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zinc soap supplementation research was conducted by Faizah et al., (2019), that compared between supplementation of 10% zinc soap from palm oil and 10% corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support

the synthesis of milk fatty acids in dairy cows, especially short chain fatty acids. However, there has been no research that explores the contribution of long chain fatty acids, MUFA and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. The results of this study was provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. Discovery of the most effective use of palm oil in providing an alternative solution to energy and zinc deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to (Cabatit, 1979). The palm oil used to make Zinc soap was a commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and

stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially ZPOS (3.75% ZPOS+1.25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belongs to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for in vitro substrate for one week before rumen liquid was taken. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flasks under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley and Terry (1963). In the fermenter tube, 0.55 grams of feed samples from each treatment were added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The

fermentor cylinder was flowed by CO2 gas and closed to anaerobic conditions. The fermentor tube was incubated in a 39°C water temperature.

Nutrient Digestibility

The digestibility of nutrients including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then the tube was centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded. Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples called blanks. Nutrien digestibility samples are calculated by the formula :

$$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$

pH value, VFA, NH3 and Methane production

The process of measuring pH, VFA, and NH3 followed the same procedure as that for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH

meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A mililiterl of 95–97% H_2SO_4 was combined with a 10 ml incubation sample. A mililiter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7^{o}C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency was determined by calculating the VFA stoichiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion efficiency was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\text{E (\%)} = \frac{(0{,}622\ \text{pA} + 1{,}091\ \text{pP} + 1{,}558\ \text{pB})}{\text{pA} + \text{pP} + 2\text{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde,

193 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated
194 by using a microscope at magnification 100 times. Measurement of rumen liquid protein
195 microbes using the method of Makkar et al. (1982) on the principle of gradual
196 centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas
197 chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were
198 stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty
199 acid composition was determined by converting oil to fatty acid methyl esters. This
200 involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The
201 peaks of the fatty acid methyl esters were identified by comparing their retention times
202 with those of authentic standards. The relative percentage of each fatty acid was
203 calculated based on its peak area relative to the total peak area of all fatty acids in the
204 sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer
205 than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and
206 long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).

207 **Statistical Analysis**

208 The data were analyzed using a completely randomized design in SPSS 16.
209 Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments
210 when a significant effect was observed.

211

212 **RESULTS**

213 **Feed Fermentability**

214 The fermentability of feed is determined by various factors such as pH, total
215 protozoa count, microbial protein content, NH3 concentration, and total VFA production
216 in rumen liquid. These parameters are detailed in Table 2. The pH levels across all

treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil supplementation had the most pronounced impact, notably reducing protozoa count and microbial protein. When zinc soap palm oil protection was partially supplemented (T2), it led to higher microbial protein and VFA production compared to feed supplemented with total zinc palm oil soap, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the palm oil zinc soap supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$) in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

Relative Proportion of VFA

Palm oil (PO and ZPOS) supplementation significantly influenced the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$), but did not impact the efficiency of hexose energy conversion into VFA. The relative persentation of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid,

and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate, propionate, and butyrate compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZSPO supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SCFA), with a notably higher concentration of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($P<0.05$). However, the proportion of stearate was significantly lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6) showed a significant increase in the 3.75% ZPOS (T2) and 5% ZSPO (T3) treatments ($P<0.05$).

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), but produced a lower amount of LCFA. SFAs showed higher in control and PO suplmentation, whereas ZSPO supplementation demonstrated an ability to elevate USFAs. Notably, the 3.75% ZPOS and 5% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among

all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be use d as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total zinc soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015). Research by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing

energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA

337 synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic
338 acid synthesis (Elamin et al., 2013).

339 The increase in propionate in the ZSPO supplementation treatment resulted from
340 the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted
341 into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated
342 fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids
343 from rumen bio-hydrogenation and increasing the duodenal flows of mono and
344 polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with
345 studies showing that ZSPO supplementation leads to an increase in protozoan populations
346 (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty
347 acid profiles, notably an increase in propionate and a reduction in the acetate-to-
348 propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane
349 production and a lower A/P ratio are advantageous, as higher propionate levels provide a
350 carbon framework and synthetic energy for livestock.

351 **Fatty Acids in Rumen Liquid**

352 Previous research showed that oil supplementation caused a decrease in the
353 proportion of short chain fatty acids in rumen fluid, while increasing the presence of long
354 chain fatty acids. Mancini et al. (2015) argue that this transition is caused by the large
355 number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
356 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which
357 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
358 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated
359 fatty acids (PUFA) . The prevalence of saturated fatty acids (SFA) in palm oil contributes
360 to the range of rumen saturated fatty acid levels, where PO supplementation treatment

(T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85% and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, it being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Harvatine et al. (2009) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification

involves bonding the carboxyl group with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed as much as 5% has beneficial effects, namely increasing fermentability, MUFA, EPA and DHA in the rumen . It was indicated that partial suplementation of ZPOS (3.75% ZPOS+1.25%PO) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS on dairy cow in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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609

610 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

611 [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN =
612 -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according
613 to (Wardeh, 1981).
614
615
616 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
617 rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6,76	6,75	6,73	6,82	0,018	0,224
Protozoa (10^3 cell/ml)	98,14^{a}	32,57^{c}	82,83^{ab}	69,31^{b}	0,044	0,000
Microbial protein (mg/ml)	13,01^{a}	9,37^{b}	13,37^{a}	11,41^{ab}	0,469	0,002
NH^3 (mM)	14,06^{a}	14,45^{a}	9,64^{b}	10,36^{b}	0,543	0,000
VFA (mM)	87,52^{b}	101,78^{b}	164,38^{a}	157,89^{a}	8,134	0,000

618 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
619 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
620 diet+partially ZPOS(3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
621
622
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Table 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Digestibility :						
Dry matter (%)	64.06	63.82	67.20	69.24	0,850	0,054
Organic matter (%)	69.16	65.89	69.65	69.98	0,632	0,068
Crude protein (%)	68.69	67.99	66.39	65.11	0,569	0,100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0,587	0,000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3,057	0,020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2,830	0,006
Acid detergent fiber (%)	45,12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2,704	0,006

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Acetate (mM)	41,17^{b}	51,10^{a}	59,31^{a}	65,80^{a}	2,579	0,000
Propionate (mM)	15,91^{c}	25,41^{b}	28,73^{a}	29,89^{a}	1,348	0,000
Butyrate (mM)	8,73^{b}	10,28^{b}	12,55^{a}	14,20^{a}	0,546	0,000
A/P	2,59^{a}	2,01^{b}	2,06^{b}	2,20^{b}	0,061	0,000
Methan (mM)	31,87^{a}	28,04^{b}	28,58^{b}	29,57^{ab}	0,489	0,014
Efficiency of energy conversion (%)	73,05	73,19	74,74	74,47	0,357	0,226

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

653 **Table 5**. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm
654 Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Short chain Fatty Acids (SCFA)						
C4	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,435	0,000
Medium chain Fatty Acids (SCFA)						
C6	<0,1	<0,1	<0,1	<0,1	-	-
C8, kaprilat	<0,1	<0,1	<0,1	<0,1	-	-
C10, kaprat	<0,1	<0,1	<0,1	<0,1	-	-
C12, laurat	1,38	1,15	1,22	1,2	0,044	0,299
Long chain Fatty Acids (LCFA)						
C13	0,3[a]	0,17[a]	<0,1[b]	<0,1[b]	0,030	0,000
C14, miristat	2,19	1,85	2,3	1,68	0,063	0,054
C14:1	1,00	0,5	0,57	0,44	0,072	0,053
C15,	0,51	0,75	0,36	0,33	0,056	0,055
C15:1	0,34	0,23	0,16	0,21	0,025	0,051
C16, palmitat	22,03[b]	29,34[a]	29,23[a]	31,14[a]	0,854	0,000
C16:1, n7	1,12 [b]	1,04 [b]	1,36 [ab]	1,5[a]	0,058	0,005
C17	0,39	0,23	0,23	0,28	0,025	0,052
C17:1	<0,1	<0,1	<0,1	<0,1	-	-
C18, stearate	40,13[a]	30,74[b]	23,17[b]	21,5[b]	2,198	0,002
trans 11 C18:1	12,55[c]	17,84[b]	26,16[a]	28,05[a]	1,476	0,834
cis 9 C18:1, oleat	1,09[b]	1,43[b]	1,99[a]	1,83[a]	0,099	0,000
C18:2	1,04[c]	1,31[bc]	1,6[ab]	1,86[a]	0,084	0,000
C18:3	<0,1	<0,1	<0,1	<0,1	-	-
C18:3, omega 6	0,22[b]	0,26[b]	0,45[a]	0,53[a]	0,032	0,000
C18:3,gamma linolenat	0,79[a]	<0,1[c]	0,38[b]	0,41[b]	0,064	0,000
C20	1,31	0,83	0,64	0,67	0,050	0,058
cis 11 C20:1	0,16[b]	0,59[a]	0,31[b]	0,23[b]	0,041	0,000
cis 11-14 C20:2	<0,1	<0,1	<0,1	<0,1	-	-
C20:3	<0,1	<0,1	<0,1	<0,1	-	-
C20:4, arakidonat	0,48[a]	<0,1[b]	<0,1[b]	<0,1[b]	0,048	0,000
C20:5, EPA	0,07[c]	0,55[b]	0,59[b]	0,76[ab]	0,069	0,000
C21	1,38	0,31	0,2	0,26	0,017	0,000
C22, behenate	0,94[a]	0,43[ab]	0,23[b]	0,25[b]	0,042	0,050
C22:1	<0,1	<0,1	<0,1	<0,1	-	-
C22:6, DHA	3,19[b]	4,81[a]	4,57[a]	4,42[a]	0,159	0,000
C24	<0,1	<0,1	<0,1	<0,1	-	-
C24:1, nervonat omega 9	<0,1	<0,1	<0,1	<0,1	-	-

655 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
656 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
657 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
658

Table 6. Proportion of short chain, long chain, saturated and unsaturated fatty acids in rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Amount of fatty acids :						
SCFA	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,453	0,000
MCFA	1,38[a]	1,15[b]	1,22[b]	1,2[b]	0,028	0,009
LCFA	91,23[c]	93,41[b]	93,50[b]	96,35[a]	0,443	0,000
SFA	77,9[a]	71,14[b]	61,85[c]	59,67[c]	1,766	0,000
USFA	22,05[c]	28,76[b]	38,14[a]	40,24[a]	1,722	0,000
MUFA	16,26[c]	21,63[b]	30,55[a]	32,26[a]	1,531	0,000
PUFA	5,79[c]	7,13[b]	7,59[a]	7,98[a]	0,201	0,000

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

4. Permintaan revisi dari editor terhadap hasil perbaikan pertama

Gmail **Anis Muktiani <anis.muktiani@gmail.com>**

[TASJ] Revision Required of Your Manuscript

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id> Fri, Jul 19, 2024 at 1:55 PM
To: Anis Muktiani <anismuktiani@gmail.com>

Dear Anis Muktiani:

It is my pleasure to inform you that your submission to Tropical Animal Science Journal, "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid" had been examined by Editor. Please find the comments and suggestions:

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If you have any questions, please contact me.

Prof. Dr. Komang G Wiryawan
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PAPER EVALUATION

Paper Title: Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid

Comments (please use additional paper if more space is needed)

No	Comments	Author's response
1	Generally, the authors have revised the manuscript following the reviewer suggestions. However, the references writing need to be revised.	
2	**References** a) Please provide link for the references below: **Elamin, K. M., N. A. Dafalla, K. A. Abdel Atti, & A. A. Tameem Eldar**. 2013. Effects of Zinc Supplementation on Growth Performance and Some Blood Parameters of Goat Kids in Sudan. International Journal of Pure and Applied Biological Research and Sciences,. 1(1):1–8. **Koushki, M., M. Nahidi, & F. Cheraghali**. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. Journal of Paramedical Sciences (JPS). 6(3):117–34. **Wang, Siyu, Yu Wang, Fereidoon Shahidi, & Chi-Tang Ho**. 2020. Health Effects of Short-Chain, Medium-Chain, and Long-Chain Fatty Acids, Saturated Vs Unsaturated and Omega-6 Vs Omega-3 Fatty Acids and Trans Fats. Pp. 1–22 in Bailey's Industrial Oil and Fat Products. **Wulandari, B.P. Widyobroto, & A. Agus**. 2020. In Vitro Digestibility and Ruminal Fermentation Profile of Pangola Grass (Digitaria Decumbens) Supplemented with Crude Palm Oil Protected by Sodium Hydroxide. Livestock Research for Rural Development. 32. b) ***Please check the writing of author's names***. They must be written following on the journal's guidelines c) Cottyn (1968): write all authors	

Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid profiles in vitro. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated female dairy goats, and were homogenized. The goats were feed on consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control ($P<0.05$). Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher than another treatment ($P<0.05$). ZPOS supplementation resulted higher acetate and propionate levels compared to the control and supplementation 5% palm oil ($P<0.05$) but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content in rumen liquid.

Keywords: Palm oil soap, Zinc, fermentability, unsaturated fatty acid ruminal.

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to decreased dry matter intake (DMI). Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023). To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessement of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera et al., 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (Bos taurus x Bos indicus) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (Bos taurus). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high

levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears et al. (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In

addition to Ca, NaOH can also be used to protect the oil (Wulandari et al., 2020). However, the use of Zn minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zinc soap supplementation research was conducted by Faizah et al., (2019), that compared between supplementation of 10% zinc soap from palm oil and 10% corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support

the synthesis of milk fatty acids in dairy cows, especially short chain fatty acids. However, there has been no research that explores the contribution of long chain fatty acids, MUFA and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. The results of this study was provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. Discovery of the most effective use of palm oil in providing an alternative solution to energy and zinc deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to (Cabatit, 1979). The palm oil used to make Zinc soap was a commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and

stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially ZPOS (3.75% ZPOS+1.25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belongs to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for in vitro substrate for one week before rumen liquid was taken. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flasks under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley and Terry (1963). In the fermenter tube, 0.55 grams of feed samples from each treatment were added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The

fermentor cylinder was flowed by CO2 gas and closed to anaerobic conditions. The fermentor tube was incubated in a 39°C water temperature.

Nutrient Digestibility

The digestibility of nutrients including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then the tube was centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded. Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples called blanks. Nutrien digestibility samples are calculated by the formula :

$$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$

pH value, VFA, NH3 and Methane production

The process of measuring pH, VFA, and NH3 followed the same procedure as that for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH

meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A mililiterl of 95–97% H_2SO_4 was combined with a 10 ml incubation sample. A mililiter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7^{o}C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency was determined by calculating the VFA stoichiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion efficiency was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\text{E (\%)} = \frac{(0{,}622\ \text{pA} + 1{,}091\ \text{pP} + 1{,}558\ \text{pB})}{\text{pA} + \text{pP} + 2\text{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde,

2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein microbes using the method of Makkar et al. (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability

The fermentability of feed is determined by various factors such as pH, total protozoa count, microbial protein content, NH3 concentration, and total VFA production in rumen liquid. These parameters are detailed in Table 2. The pH levels across all

treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil supplementation had the most pronounced impact, notably reducing protozoa count and microbial protein. When zinc soap palm oil protection was partially supplemented (T2), it led to higher microbial protein and VFA production compared to feed supplemented with total zinc palm oil soap, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the palm oil zinc soap supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$) in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

Relative Proportion of VFA

Palm oil (PO and ZPOS) supplementation significantly influenced the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$), but did not impact the efficiency of hexose energy conversion into VFA. The relative persentation of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid,

and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate, propionate, and butyrate compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZSPO supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SCFA), with a notably higher concentration of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($P<0.05$). However, the proportion of stearate was significantly lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6) showed a significant increase in the 3.75% ZPOS (T2) and 5% ZSPO (T3) treatments ($P<0.05$).

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), but produced a lower amount of LCFA. SFAs showed higher in control and PO suplmentation, whereas ZSPO supplementation demonstrated an ability to elevate USFAs. Notably, the 3.75% ZPOS and 5% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among

all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be use d as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total zinc soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015). Research by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing

energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA

337 synthesis, growth, CO2 transport, essential fatty acid metabolism, and protein and nucleic
338 acid synthesis (Elamin et al., 2013).

339 The increase in propionate in the ZSPO supplementation treatment resulted from
340 the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted
341 into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated
342 fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids
343 from rumen bio-hydrogenation and increasing the duodenal flows of mono and
344 polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with
345 studies showing that ZSPO supplementation leads to an increase in protozoan populations
346 (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty
347 acid profiles, notably an increase in propionate and a reduction in the acetate-to-
348 propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane
349 production and a lower A/P ratio are advantageous, as higher propionate levels provide a
350 carbon framework and synthetic energy for livestock.

351 **Fatty Acids in Rumen Liquid**

352 Previous research showed that oil supplementation caused a decrease in the
353 proportion of short chain fatty acids in rumen fluid, while increasing the presence of long
354 chain fatty acids. Mancini et al. (2015) argue that this transition is caused by the large
355 number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
356 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which
357 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
358 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated
359 fatty acids (PUFA) . The prevalence of saturated fatty acids (SFA) in palm oil contributes
360 to the range of rumen saturated fatty acid levels, where PO supplementation treatment

(T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85% and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, it being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Harvatine et al. (2009) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification

involves bonding the carboxyl group with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed as much as 5% has beneficial effects, namely increasing fermentability, MUFA, EPA and DHA in the rumen . It was indicated that partial suplementation of ZPOS (3.75% ZPOS+1.25%PO) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS on dairy cow in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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609

610 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

611 [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN =
612 -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according
613 to (Wardeh, 1981).
614
615
616 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
617 rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6,76	6,75	6,73	6,82	0,018	0,224
Protozoa (10^3 cell/ml)	98,14^{a}	32,57^{c}	82,83^{ab}	69,31^{b}	0,044	0,000
Microbial protein (mg/ml)	13,01^{a}	9,37^{b}	13,37^{a}	11,41^{ab}	0,469	0,002
NH^3 (mM)	14,06^{a}	14,45^{a}	9,64^{b}	10,36^{b}	0,543	0,000
VFA (mM)	87,52^{b}	101,78^{b}	164,38^{a}	157,89^{a}	8,134	0,000

618 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
619 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
620 diet+partially ZPOS(3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
621
622
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Table 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Digestibility :						
Dry matter (%)	64.06	63.82	67.20	69.24	0,850	0,054
Organic matter (%)	69.16	65.89	69.65	69.98	0,632	0,068
Crude protein (%)	68.69	67.99	66.39	65.11	0,569	0,100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0,587	0,000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3,057	0,020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2,830	0,006
Acid detergent fiber (%)	45,12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2,704	0,006

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Acetate (mM)	41,17^{b}	51,10^{a}	59,31^{a}	65,80^{a}	2,579	0,000
Propionate (mM)	15,91^{c}	25,41^{b}	28,73^{a}	29,89^{a}	1,348	0,000
Butyrate (mM)	8,73^{b}	10,28^{b}	12,55^{a}	14,20^{a}	0,546	0,000
A/P	2,59^{a}	2,01^{b}	2,06^{b}	2,20^{b}	0,061	0,000
Methan (mM)	31,87^{a}	28,04^{b}	28,58^{b}	29,57^{ab}	0,489	0,014
Efficiency of energy conversion (%)	73,05	73,19	74,74	74,47	0,357	0,226

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

653 **Table 5**. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm
654 Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Short chain Fatty Acids (SCFA)						
C4	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,435	0,000
Medium chain Fatty Acids (SCFA)						
C6	<0,1	<0,1	<0,1	<0,1	-	-
C8, kaprilat	<0,1	<0,1	<0,1	<0,1	-	-
C10, kaprat	<0,1	<0,1	<0,1	<0,1	-	-
C12, laurat	1,38	1,15	1,22	1,2	0,044	0,299
Long chain Fatty Acids (LCFA)						
C13	0,3[a]	0,17[a]	<0,1[b]	<0,1[b]	0,030	0,000
C14, miristat	2,19	1,85	2,3	1,68	0,063	0,054
C14:1	1,00	0,5	0,57	0,44	0,072	0,053
C15,	0,51	0,75	0,36	0,33	0,056	0,055
C15:1	0,34	0,23	0,16	0,21	0,025	0,051
C16, palmitat	22,03[b]	29,34[a]	29,23[a]	31,14[a]	0,854	0,000
C16:1, n7	1,12 [b]	1,04 [b]	1,36 [ab]	1,5[a]	0,058	0,005
C17	0,39	0,23	0,23	0,28	0,025	0,052
C17:1	<0,1	<0,1	<0,1	<0,1	-	-
C18, stearate	40,13[a]	30,74[b]	23,17[b]	21,5[b]	2,198	0,002
trans 11 C18:1	12,55[c]	17,84[b]	26,16[a]	28,05[a]	1,476	0,834
cis 9 C18:1, oleat	1,09[b]	1,43[b]	1,99[a]	1,83[a]	0,099	0,000
C18:2	1,04[c]	1,31[bc]	1,6[ab]	1,86[a]	0,084	0,000
C18:3	<0,1	<0,1	<0,1	<0,1	-	-
C18:3, omega 6	0,22[b]	0,26[b]	0,45[a]	0,53[a]	0,032	0,000
C18:3,gamma linolenat	0,79[a]	<0,1[c]	0,38[b]	0,41[b]	0,064	0,000
C20	1,31	0,83	0,64	0,67	0,050	0,058
cis 11 C20:1	0,16[b]	0,59[a]	0,31[b]	0,23[b]	0,041	0,000
cis 11-14 C20:2	<0,1	<0,1	<0,1	<0,1	-	-
C20:3	<0,1	<0,1	<0,1	<0,1	-	-
C20:4, arakidonat	0,48[a]	<0,1[b]	<0,1[b]	<0,1[b]	0,048	0,000
C20:5, EPA	0,07[c]	0,55[b]	0,59[b]	0,76[ab]	0,069	0,000
C21	1,38	0,31	0,2	0,26	0,017	0,000
C22, behenate	0,94[a]	0,43[ab]	0,23[b]	0,25[b]	0,042	0,050
C22:1	<0,1	<0,1	<0,1	<0,1	-	-
C22:6, DHA	3,19[b]	4,81[a]	4,57[a]	4,42[a]	0,159	0,000
C24	<0,1	<0,1	<0,1	<0,1	-	-
C24:1, nervonat omega 9	<0,1	<0,1	<0,1	<0,1	-	-

655 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
656 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
657 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
658

Table 6. Proportion of short chain, long chain, saturated and unsaturated fatty acids in rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Amount of fatty acids :						
SCFA	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,453	0,000
MCFA	1,38[a]	1,15[b]	1,22[b]	1,2[b]	0,028	0,009
LCFA	91,23[c]	93,41[b]	93,50[b]	96,35[a]	0,443	0,000
SFA	77,9[a]	71,14[b]	61,85[c]	59,67[c]	1,766	0,000
USFA	22,05[c]	28,76[b]	38,14[a]	40,24[a]	1,722	0,000
MUFA	16,26[c]	21,63[b]	30,55[a]	32,26[a]	1,531	0,000
PUFA	5,79[c]	7,13[b]	7,59[a]	7,98[a]	0,201	0,000

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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1	Generally, the authors have revised the manuscript following the reviewer suggestions. However, the references writing need to be revised.	Thank you very much. I will revise it.
2	**References** a) Please provide link for the references below: **Elamin, K. M., N. A. Dafalla, K. A. Abdel Atti, & A. A. Tameem Eldar**. 2013. Effects of Zinc Supplementation on Growth Performance and Some Blood Parameters of Goat Kids in Sudan. International Journal of Pure and Applied Biological Research and Sciences,. 1(1):1–8. **Koushki, M., M. Nahidi, & F. Cheraghali**. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. Journal of Paramedical Sciences (JPS). 6(3):117–34. **Wang, Siyu, Yu Wang, Fereidoon Shahidi, & Chi-Tang Ho**. 2020. Health Effects of Short-Chain, Medium-Chain, and Long-Chain Fatty Acids, Saturated Vs Unsaturated and Omega-6 Vs Omega-3 Fatty Acids and Trans Fats. Pp. 1–22 in Bailey's Industrial Oil and Fat Products.	a) Link not found, replaced with another reference. Lines 75-79 Lines 341-343 (written in red ink) **Koushki, M., M. Nahidi, & F. Cheraghali**. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. Journal of Paramedical Sciences (JPS).6:117–34. https://doi.org/10.22037/jps.v6i3.9772 . **Wang, S., Y. Wang, F. Shahidi, & C. Ho**. 2020. Health Effects of Short-Chain, Medium-Chain, and Long-Chain Fatty Acids, Saturated vs Unsaturated and Omega-6 vs Omega-3 Fatty Acids and Trans Fats. In : F. Shahidi (Ed). Bailey's Industrial Oil and Fat Products: 7th ed. John Wiley & Sons, Ltd. https://doi.org/10.1002/047167849X.bio100.

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	b) ***Please check the writing of author's names***. They must be written following on the journal's guidelines	b) Already did. Line 568 Lines 587-588 Line 592
	c) Cottyn (1968): write all authors	c) Already did. **Cottyn, B. G., & C. V. Boucque**. 1968. Rapid Method for the Gas-Chromatographic Determination of Volatile Fatty Acids in Rumen Fluid. J.Agric.Food Chem. 16:105–7. https://doi.org/10.1021/jf60155a002.

Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid profiles in vitro. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated female dairy goats, and were homogenized. The goats were feed on consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control ($P<0.05$). Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher than another treatment ($P<0.05$). ZPOS supplementation resulted higher acetate and propionate levels compared to the control and supplementation 5% palm oil ($P<0.05$) but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content in rumen liquid.

Keywords: Palm oil soap, Zinc, fermentability, unsaturated fatty acid ruminal.

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to decreased dry matter intake (DMI). Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023). To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessement of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera et al., 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (Bos taurus x Bos indicus) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (Bos taurus). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high

levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears et al. (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In

addition to Ca, NaOH can also be used to protect the oil (Wulandari et al., 2020). However, the use of Zn minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, acts as a structural component in gene expression and signal transduction (Franco et al., 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase which play a role in protein synthesis (Sloup et al., 2017). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zinc soap supplementation research was conducted by Faizah et al., (2019), that compared between supplementation of 10% zinc soap from palm oil and 10% corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility

of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short chain fatty acids. However, there has been no research that explores the contribution of long chain fatty acids, MUFA and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. The results of this study was provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. Discovery of the most effective use of palm oil in providing an alternative solution to energy and zinc deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to (Cabatit, 1979). The palm oil used to make Zinc soap was a commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an

amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially ZPOS (3.75% ZPOS+1.25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belongs to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for in vitro substrate for one week before rumen liquid was taken. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flasks under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley and Terry (1963). In the fermenter tube, 0.55 grams of feed samples from each treatment were added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The fermentor cylinder was flowed by CO2 gas and closed to anaerobic conditions. The fermentor tube was incubated in a 39°C water temperature.

Nutrient Digestibility

The digestibility of nutrients including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then the tube was centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded. Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples called blanks. Nutrien digestibility samples are calculated by the formula :

$$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$

pH value, VFA, NH3 and Methane production

The process of measuring pH, VFA, and NH3 followed the same procedure as that for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After

centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A mililiterl of 95–97% H_2SO_4 was combined with a 10 ml incubation sample. A mililiter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7^{o}C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency was determined by calculating the VFA stoichiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion efficiency was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\mathrm{E}\ (\%)\ = \frac{(0{,}622\ \mathrm{pA}\ +\ 1{,}091\ \mathrm{pP}\ +\ 1{,}558\ \mathrm{pB})}{\mathrm{pA} + \mathrm{pP} + 2\mathrm{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa

were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde, 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein microbes using the method of Makkar et al. (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability

The fermentability of feed is determined by various factors such as pH, total protozoa count, microbial protein content, NH3 concentration, and total VFA production in rumen liquid. These parameters are detailed in Table 2. The pH levels across all treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil supplementation had the most pronounced impact, notably reducing protozoa count and microbial protein. When zinc soap palm oil protection was partially supplemented (T2), it led to higher microbial protein and VFA production compared to feed supplemented with total zinc palm oil soap, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the palm oil zinc soap supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$) in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

241 **Relative Proportion of VFA**

242 Palm oil (PO and ZPOS) supplementation significantly influenced the partial
243 VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$),
244 but did not impact the efficiency of hexose energy conversion into VFA. The relative
245 persentation of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid,
246 and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both
247 partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate,
248 propionate, and butyrate compared to PO supplementation (T1) and the control (T0).
249 Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated
250 methane production also decreased with PO and ZSPO supplementation.

251 **Fatty Acids in Rumen Liquid**

252 Table 5 outlines the fatty acid composition in rumen liquid. The control diet
253 primarily contained short-chain fatty acids (SCFA), with a notably higher concentration
254 of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and
255 ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1)
256 relative to the control ($P<0.05$). However, the proportion of stearate was significantly
257 lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9
258 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6)
259 showed a significant increase in the 3.75% ZPOS (T2) and 5% ZSPO (T3) treatments
260 ($P<0.05$).

261 These fatty acids are classified into various categories, including short-chain fatty
262 acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs),
263 saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty
264 acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the

control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), but produced a lower amount of LCFA. SFAs showed higher in control and PO suplmentation, whereas ZSPO supplementation demonstrated an ability to elevate USFAs. Notably, the 3.75% ZPOS and 5% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially

(T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be use d as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total zinc soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015). Research by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of

propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, including DNA and RNA polymerase which play a role in protein synthesis (Franco et al., 2024; Sloup et al., 2017).

The increase in propionate in the ZSPO supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with studies showing that ZSPO supplementation leads to an increase in protozoan populations (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio are advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short chain fatty acids in rumen fluid, while increasing the presence of long chain fatty acids. Mancini et al. (2015) argue that this transition is caused by the large

number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated fatty acids (PUFA) . The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85% and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, it being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Harvatine et al. (2009) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished

stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation

in feed as much as 5% has beneficial effects, namely increasing fermentability, MUFA, EPA and DHA in the rumen . It was indicated that partial suplementation of ZPOS (3.75% ZPOS+1.25%PO) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS on dairy cow in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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611 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

612 [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN =
613 -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according
614 to (Wardeh, 1981).
615
616
617 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
618 rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6,76	6,75	6,73	6,82	0,018	0,224
Protozoa (10^3 cell/ml)	98,14^{a}	32,57^{c}	82,83^{ab}	69,31^{b}	0,044	0,000
Microbial protein (mg/ml)	13,01^{a}	9,37^{b}	13,37^{a}	11,41^{ab}	0,469	0,002
NH^3 (mM)	14,06^{a}	14,45^{a}	9,64^{b}	10,36^{b}	0,543	0,000
VFA (mM)	87,52^{b}	101,78^{b}	164,38^{a}	157,89^{a}	8,134	0,000

619 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
620 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
621 diet+partially ZPOS(3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
622
623
624
625
626

Table 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Digestibility :						
Dry matter (%)	64.06	63.82	67.20	69.24	0,850	0,054
Organic matter (%)	69.16	65.89	69.65	69.98	0,632	0,068
Crude protein (%)	68.69	67.99	66.39	65.11	0,569	0,100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0,587	0,000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3,057	0,020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2,830	0,006
Acid detergent fiber (%)	45,12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2,704	0,006

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Acetate (mM)	41,17^{b}	51,10^{a}	59,31^{a}	65,80^{a}	2,579	0,000
Propionate (mM)	15,91^{c}	25,41^{b}	28,73^{a}	29,89^{a}	1,348	0,000
Butyrate (mM)	8,73^{b}	10,28^{b}	12,55^{a}	14,20^{a}	0,546	0,000
A/P	2,59^{a}	2,01^{b}	2,06^{b}	2,20^{b}	0,061	0,000
Methan (mM)	31,87^{a}	28,04^{b}	28,58^{b}	29,57^{ab}	0,489	0,014
Efficiency of energy conversion (%)	73,05	73,19	74,74	74,47	0,357	0,226

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

654 **Table 5**. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm
655 Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Short chain Fatty Acids (SCFA)						
C4	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,435	0,000
Medium chain Fatty Acids (SCFA)						
C6	<0,1	<0,1	<0,1	<0,1	-	-
C8, kaprilat	<0,1	<0,1	<0,1	<0,1	-	-
C10, kaprat	<0,1	<0,1	<0,1	<0,1	-	-
C12, laurat	1,38	1,15	1,22	1,2	0,044	0,299
Long chain Fatty Acids (LCFA)						
C13	0,3[a]	0,17[a]	<0,1[b]	<0,1[b]	0,030	0,000
C14, miristat	2,19	1,85	2,3	1,68	0,063	0,054
C14:1	1,00	0,5	0,57	0,44	0,072	0,053
C15,	0,51	0,75	0,36	0,33	0,056	0,055
C15:1	0,34	0,23	0,16	0,21	0,025	0,051
C16, palmitat	22,03[b]	29,34[a]	29,23[a]	31,14[a]	0,854	0,000
C16:1, n7	1,12 [b]	1,04 [b]	1,36 [ab]	1,5[a]	0,058	0,005
C17	0,39	0,23	0,23	0,28	0,025	0,052
C17:1	<0,1	<0,1	<0,1	<0,1	-	-
C18, stearate	40,13[a]	30,74[b]	23,17[b]	21,5[b]	2,198	0,002
trans 11 C18:1	12,55[c]	17,84[b]	26,16[a]	28,05[a]	1,476	0,834
cis 9 C18:1, oleat	1,09[b]	1,43[b]	1,99[a]	1,83[a]	0,099	0,000
C18:2	1,04[c]	1,31[bc]	1,6[ab]	1,86[a]	0,084	0,000
C18:3	<0,1	<0,1	<0,1	<0,1	-	-
C18:3, omega 6	0,22[b]	0,26[b]	0,45[a]	0,53[a]	0,032	0,000
C18:3,gamma linolenat	0,79[a]	<0,1[c]	0,38[b]	0,41[b]	0,064	0,000
C20	1,31	0,83	0,64	0,67	0,050	0,058
cis 11 C20:1	0,16[b]	0,59[a]	0,31[b]	0,23[b]	0,041	0,000
cis 11-14 C20:2	<0,1	<0,1	<0,1	<0,1	-	-
C20:3	<0,1	<0,1	<0,1	<0,1	-	-
C20:4, arakidonat	0,48[a]	<0,1[b]	<0,1[b]	<0,1[b]	0,048	0,000
C20:5, EPA	0,07[c]	0,55[b]	0,59[b]	0,76[ab]	0,069	0,000
C21	1,38	0,31	0,2	0,26	0,017	0,000
C22, behenate	0,94[a]	0,43[ab]	0,23[b]	0,25[b]	0,042	0,050
C22:1	<0,1	<0,1	<0,1	<0,1	-	-
C22:6, DHA	3,19[b]	4,81[a]	4,57[a]	4,42[a]	0,159	0,000
C24	<0,1	<0,1	<0,1	<0,1	-	-
C24:1, nervonat omega 9	<0,1	<0,1	<0,1	<0,1	-	-

656 [a,b] different superscripts at the same column indicate significant differences (P<0.05).
657 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
658 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
659

Table 6. Proportion of short chain, long chain, saturated and unsaturated fatty acids in rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Amount of fatty acids :						
SCFA	7,34[a]	5,34[b]	5,27[b]	2,36[c]	0,453	0,000
MCFA	1,38[a]	1,15[b]	1,22[b]	1,2[b]	0,028	0,009
LCFA	91,23[c]	93,41[b]	93,50[b]	96,35[a]	0,443	0,000
SFA	77,9[a]	71,14[b]	61,85[c]	59,67[c]	1,766	0,000
USFA	22,05[c]	28,76[b]	38,14[a]	40,24[a]	1,722	0,000
MUFA	16,26[c]	21,63[b]	30,55[a]	32,26[a]	1,531	0,000
PUFA	5,79[c]	7,13[b]	7,59[a]	7,98[a]	0,201	0,000

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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[TASJ] Editor Decision

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id> Mon, Jul 29, 2024 at 8:59 AM
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Bogor, July 29, 2024

Dear A. Muktiani, W. Widiyanto, N. S. Pandupuspitasari:

I am pleased to inform you that your article submitted to Tropical Animal Science Journal, entitled "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid" has been accepted for publication in this journal. We will send you the COPYEDITING of your manuscript as we will ask you for some corrections of the typesetting.

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Bogor, July 29, 2024

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I am pleased to inform you that your article submitted to Tropical Animal Science Journal, entitled "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid" has been accepted for publication in this journal. We will send you the COPYEDITING of your manuscript as we will ask you for some corrections of the typesetting.

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7. Pengiriman draft perbaikan format penulisan dari penulis

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[TASJ] Editor Decision

Anis Muktiani <anis.muktiani@gmail.com> Tue, Jul 30, 2024 at 11:11 AM
To: "Prof. Dr. Komang G Wiryawan" <jurnal@apps.ipb.ac.id>

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Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid profiles in vitro. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated female dairy goats, and were homogenized. The goats were feed on consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH3 levels compared to the control ($P<0.05$). Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher than another treatment ($P<0.05$). ZPOS supplementation resulted higher acetate and propionate levels compared to the control and supplementation 5% palm oil ($P<0.05$) but did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment

was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content in rumen liquid.

Keywords: Palm oil soap, Zinc, fermentability, unsaturated fatty acid ruminal.

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to decreased dry matter intake (DMI). Energy requirements can be two to three times higher than the basic maintenance needs because it is used for tissue maintenance after giving birth and milk production (Khotijah et al., 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout et al., 2023). To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessement of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera et al., 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (Bos taurus x Bos indicus) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (Bos taurus). Therefore, understanding these

differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils to be used as an energy source with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears et al. (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al., 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural

components of biomembranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira et al., 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir et al., 2023; Vargas-bello-p et al., 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari et al., 2020). However, the use of Zn minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, acts as a structural component in gene expression and signal transduction (Franco et al., 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase which play a role in protein synthesis (Sloup et al., 2017). Studies in vitro observed that supplementing zinc in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zinc is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy et al., 2023). The most important of Zinc are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup

et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zinc soap supplementation research was conducted by Faizah et al., (2019), that compared between supplementation of 10% zinc soap from palm oil and 10% corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short chain fatty acids. However, there has been no research that explores the contribution of long chain fatty acids, MUFA and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. The results of this study was provide information regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due to zinc soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic zinc supplement, specifically zinc palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The benefit of this research is to identify the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability in vitro. Discovery of the most effective use of palm oil in providing an alternative solution to energy and zinc deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of zinc soap was carried out according to (Cabatit, 1979). The palm oil used to make Zinc soap was a commercial palm oil which generally sold on the market. The Zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, add the ZnCl2 solution and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

The feed consists of forage and concentrate that has been formulated for feeding lactating dairy goats with a content of crude protein (CP) 14% and total degestible nutrients (TDN) 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal and molasses. The composition of ingredients and nutrient content of the ration as shown in Table 1.

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0 = basal diet without supplementation, T1 = basal diet + 5% palm oil (PO), T2 = basal diet + 5% partially

144 ZPOS (3.75% ZPOS+1.25% PO), and T3 = basal diet + 5% ZPOS. Rumen liquid as a
145 source of inoculum was taken from three female goats with fistulas that belongs to the
146 Faculty of Animal and Agricultural Sciences Diponegoro University, and were
147 homogenized. The goats were given a dietary trial following the ration for in vitro
148 substrate for one week before rumen liquid was taken. Rumen liquid was collected before
149 morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese
150 cloth and placed into a 39 °C flasks under anaerobic conditions.

151 In vitro experiments were carried out according to the method of Tilley and Terry
152 (1963). In the fermenter tube, 0.55 grams of feed samples from each treatment were
153 added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The
154 fermentor cylinder was flowed by CO2 gas and closed to anaerobic conditions. The
155 fermentor tube was incubated in a 39°C water temperature.

156 **Nutrient Digestibility**

157 The digestibility of nutrients including dry matter (DMD), organic matter (OMD),
158 crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through
159 two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor
160 tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped
161 by immersing the fermenter tube in ice water for 20 minutes. Then the tube was
162 centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded.
163 Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for
164 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered
165 with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed
166 by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient

digestibility values. Fermentation is also carried out without feed samples called blanks. Nutrien digestibility samples are calculated by the formula :

$$\text{Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{nutrient sample, g}} x100$$

pH value, VFA, NH3 and Methane production

The process of measuring pH, VFA, and NH3 followed the same procedure as that for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A mililiterl of 95–97% H_2SO_4 was combined with a 10 ml incubation sample. A mililiter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7^{o}C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency was determined by calculating the VFA stoichiometry, which was the estimation using the formula Orskov and Ryle (1990). The formula used for methane concentration were : Methane (mM) = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion

efficiency was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$\text{E}\ (\%)\ = \frac{(0{,}622\ \text{pA}\ +\ 1{,}091\ \text{pP}\ +\ 1{,}558\ \text{pB})}{\text{pA} + \text{pP} + 2\text{pB}} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto and Imai (1981). The solution used was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde, 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein microbes using the method of Makkar et al. (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).

215 **Statistical Analysis**

216 The data were analyzed using a completely randomized design in SPSS 16.
217 Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments
218 when a significant effect was observed.

219

220 **RESULTS**

221 **Feed Fermentability**

222 The fermentability of feed is determined by various factors such as pH, total
223 protozoa count, microbial protein content, NH3 concentration, and total VFA production
224 in rumen liquid. These parameters are detailed in Table 2. The pH levels across all
225 treatments fell within the normal range of 6.75-6.82. While the treatment did not
226 significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total
227 protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil
228 supplementation had the most pronounced impact, notably reducing protozoa count and
229 microbial protein. When zinc soap palm oil protection was partially supplemented (T2),
230 it led to higher microbial protein and VFA production compared to feed supplemented
231 with total zinc palm oil soap, although the difference was not significant.

232 **Feed Digestibility**

233 Table 3 presents the feed digestibility results from the palm oil zinc soap
234 supplementation treatment. Statistical analysis revealed no significant differences in the
235 digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due
236 to the palm oil zinc soap treatment. However, there were significant differences ($P<0.05$)
237 in the digestibility of ether extract (EED), crude fiber (CFD), neutral detergent fiber
238 (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil

increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial zinc soap supplementation (T2), though it was not significantly different from the total protection zinc soap supplementation (T3).

Relative Proportion of VFA

Palm oil (PO and ZPOS) supplementation significantly influenced the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane ($P<0.05$), but did not impact the efficiency of hexose energy conversion into VFA. The relative persentation of VFAs in this study were 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. According to Duncan's Mulitiple Range Test results, both partial (T2) and total (T3) ZSPO supplementation resulted in higher levels of acetate, propionate, and butyrate compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZSPO supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SCFA), with a notably higher concentration of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($P<0.05$). However, the proportion of stearate was significantly lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6)

showed a significant increase in the 3.75% ZPOS (T2) and 5% ZSPO (T3) treatments ($P<0.05$).

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZSPO ($P<0.05$), but produced a lower amount of LCFA. SFAs showed higher in control and PO suplmentation, whereas ZSPO supplementation demonstrated an ability to elevate USFAs. Notably, the 3.75% ZPOS and 5% ZSPO supplementation resulted in higher levels of both MUFAs and PUFAs compared to the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil zinc soap did not disrupt the rumen fermentation process. Similar results have been reported in other studies, such as those conducted by Adeyemi et al. (2015), Amanullah et al. (2022), and Roy et al. (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman et al., (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos et al., 2022).

Ibrahim et al. (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears et al. (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased protein synthesis. The use of zinc soap palm oil protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The

experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be use d as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total zinc soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and zinc content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki et al., 2015). Research by Sears et al. (2024) indicate an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, zinc is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al. (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein

synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah et al. (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($P<0.05$). These findings are consistent with the increased microbial protein levels observed with ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, including DNA and RNA polymerase which play a role in protein synthesis (Franco et al., 2024; Sloup et al., 2017).

The increase in propionate in the ZSPO supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold et al., 2015). These findings are consistent with studies showing that ZSPO supplementation leads to an increase in protozoan populations (Table 4). Zinc supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal et al., 2016). Moreover, the lack of an increase in methane

production and a lower A/P ratio are advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short chain fatty acids in rumen fluid, while increasing the presence of long chain fatty acids. Mancini et al. (2015) argue that this transition is caused by the large number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated fatty acids (PUFA) . The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85% and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids including oleic acid (18:1) undergo a biohydrogenation process, it being converted into saturated fatty acids stearic acid (18:0) in the rumen by bacteria (Buccioni et al., 2012). Harvatine et al. (2009) who tracked the biohydrogenation process of oleic acid found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomesase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate

polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with zinc, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zinc's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic

acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZSPO supplementation holds potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed as much as 5% has beneficial effects, namely increasing fermentability, MUFA, EPA and DHA in the rumen . It was indicated that partial suplementation of ZPOS (3.75% ZPOS+1.25%PO) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS on dairy cow in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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612

Table 1. Ingredients and chemical composition of experimental diet (dry matter basis).

Item	Composition
Ingredient :	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molases	3.00
Vitamin and mineral mixture	0.80
Nutrient composition :	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calsium, %DM	0.33
Phospor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

[1]Total digestible nutrients (TDN) were calculated using TDN (%DM) = TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according to (Wardeh, 1981).

Table 2. The pH, total protozoa, microbial protein, NH3, and total VFA production of rumen liquid due to supplementation of the zinc soap palm oil (ZPOS)

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/ml)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/ml)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS(3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 3. Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm oil (ZPOS).

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Digestibility :						
Dry matter (%)	64.06	63.82	67.20	69.24	0.850	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 4. Fermentability of feed due to palm oil supplementation in vitro

Parameter	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 5. Proportion of fatty Acids in rumen liquid due supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Short chain Fatty Acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain Fatty Acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, kaprilat	<0.1	<0.1	<0.1	<0.1	-	-
C10, kaprat	<0.1	<0.1	<0.1	<0.1	-	-
C12, laurat	1.38	1.15	1.22	1.2	0.044	0.299
Long chain Fatty Acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, miristat	2.19	1.85	2.3	1.68	0.063	0.054
C14:1	1.00	0.5	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitat	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearate	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleat	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3,gamma linolenat	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arakidonat	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.2	0.26	0.017	0.000
C22, behenate	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonat omega 9	<0.1	<0.1	<0.1	<0.1	-	-

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

Table 6. Proportion of short chain, long chain, saturated and unsaturated fatty acids in rumen liquid due with supplemented of zinc soap palm Oil (ZPOS) In Vitro

Fatty Acids	Treatment				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
Amount of fatty acids :						
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.2[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

[a,b] different superscripts at the same column indicate significant differences (P<0.05).
SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,, UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

8. Permintaan copyediting dari editor jurnal

Gmail **Anis Muktiani <anis.muktiani@gmail.com>**

[TASJ] New notification from Tropical Animal Science Journal

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Supplementation of Zinc Palm Oil Soap Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on fermentability and unsaturated fatty acid profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated female dairy goats and was homogenized. The goats were fed ration consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and microbial protein production and lower NH3 levels compared to the control ($p<0.05$). Partial ZPOS supplementation (T2) resulted in DM, OM, and CP digestibility that was similar among the treatments, but the digestibility of EE, CF, NDF, and ADF was significantly higher than another treatment ($p<0.05$). ZPOS supplementation resulted in higher acetate and propionate levels than the control and supplementation of 5% palm oil ($p<0.05$) but did not affect butyrate, reducing the A/P ratio and methane production. The

Commented [mp1]: What do you mean with 5% parrtially ZPOS? Why used this treatment?

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PO treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to the control. In conclusion, adding protected palm oil in the form of zinc soap enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content in rumen liquid.

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Keywords: *fermentability; palm oil soap; unsaturated fatty acid ruminal; zinc*

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to the decreased dry matter intake (DMI). Energy requirements can be two to three times higher than basic maintenance needs because they are used for tissue maintenance after birth and milk production (Khotijah *et al.*, 2017). Energy deficiency has detrimental effects, including low production and weight loss in livestock (Tribout *et al.*, 2023). To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Commented [mp5]: Please check again, Do Khotijah et al. state their research about dairy?

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessment of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera *et al.*, 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (*Bos taurus* x *Bos indicus*) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (*Bos taurus*). Therefore, understanding these differences is very

important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils for energy sources, with a gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains high levels of unsaturated fatty acids, specifically oleic acid at 39.2% (Mancini *et al.*, 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao *et al.*, 2016).

Commented [mp6]: Palmitic acid is saturated fatty acid, not unsaturated fatty acid?

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibro lytic microbes, thus reducing fiber digestibility (Sears *et al.*, 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard polyunsaturated fatty acids to preserve their biological roles, such as being structural

Commented [mp7]: Palm oil rich in saturated fatty acid, so no need to prevent from biohydrogenation??

components of bio membranes that uphold membrane integrity. Polyunsaturated fatty acids in bio membranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira *et al.,* 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir *et al.,* 2023; Vargas-bello-p *et al.,* 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari *et al.,* 2020). However, the use of zinc (Zn) minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and acts as a structural component in gene expression and signal transduction (Franco *et al.,* 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase, which play a role in protein synthesis (Sloup *et al.,* 2017). Studies *in vitro* observed that supplementing Zn in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970) and concentrations of total volatile fatty acids (VFA) and acetate (Chen *et al.,* 2019). Research by Wang *et al.* (2021) found that supplementation of 20-30mg/kg DM Zn sulfate led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zn is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy *et al.,* 2023). The most important of Zn are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup

et al., 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zn soap supplementation research was conducted by Faizah *et al.* (2019), which compared the supplementation of 10% Zn soap from palm oil with 10% corn. Supplementation with 10% palm oil Zn soap resulted in no different digestibility of dry matter, organic matter, and crude fiber, but the total production of VFA, acetate, and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short-chain fatty acids. However, no research has explored the contribution of long-chain fatty acids, MUFA, and PUFA due to Zn soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. This study's results provided information regarding the concentration of long-chain fatty acids, MUFA and PUFA in the rumen due to Zn soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic Zn supplements, specifically Zn palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles *in vitro*. The benefit of this research is identifying the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability *in vitro*. Discovery of the most effective use of palm oil in providing an alternative solution to energy and Zn deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of Zn soap was carried out according to Cabatit (1979). The palm oil used to make Zn soap was a commercial palm oil that was generally sold on the market. The zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90 ^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, the ZnCl2 solution was added and mix continuously until a paste forms. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called zinc palm oil soap (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn, and gross energy 5257 kcal/kg.

The feed consists of forage and concentrates that have been formulated for feeding lactating dairy goats with a content of crude protein (CP) of 14% and total digestible nutrients (TDN) of 63%. The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal, and molasses. The composition of ingredients and nutrient content of the ration are shown in Table 1.

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In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partially ZPOS

(3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belong to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for *in vitro* substrate for one week before rumen liquid was collected. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flask under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley & Terry (1963). In the fermenter tube, 0.55 g of feed samples from each treatment were added, followed by 40 mL of McDaugall's solution and 10 mL of rumen liquid. The fermenter cylinder was flowed by CO2 gas and closed to anaerobic conditions. The fermenter tube was incubated at a 39 °C water temperature.

Nutrient Digestibility

The digestibility of nutrients, including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED), and crude fiber (CFD) were measured through two stages of incubation, namely fermentative and enzymatic. In the first stage, the fermenter tube was incubated at 39 °C water temperature for 2x24 hours, and the process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then, the tube was centrifuged for 15 minutes at a speed of 3,000 rpm, and the supernatant was discarded. In the second stage, the HCL pepsin solution was added 50 mL into the tube and incubated again for 2x24 hours in a 39 °C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed for DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed

Commented [mp9]: What is the consideration of this proportion?

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168 samples, which are called blanks. Nutrient digestibility samples are calculated by the
169 formula:

170 Nutrient digestibility (%)

171 $$= \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{Nutrient sample, g}} x100$$

172

173 **pH Value, VFA, NH3, and Methane Production**

Commented [mp11]: NH_3,??

174 The process of measuring pH, VFA, and NH3 followed the same procedure as that
175 for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After
176 centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to
177 measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH
178 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
179 was tested three times. NH3 levels were determined using a spectrophotometer,
180 employing a spectrophotometric method based on the catalyzed endophenol reaction to
181 form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968)
182 reported that gas chromatography was used to quantify the generation of partial VFAs
183 (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H_2SO_4 was combined
184 with a 10 mL incubation sample. A milliliter of the sample combination was mixed with
185 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10
186 minutes at 7 ^oC at 12,000 rpm. The samples were ready for gas chromatography
187 identification.

188 The methane gas concentration and energy conversion efficiency were determined
189 by calculating the VFA stoichiometry, which was the estimation using the formula of
190 Orskov & Ryle (1990). The formula used for methane concentration was: Methane (mM)
191 = 0.5 (% Acetate) - 0.25 (% Propionate) + 0.5 (% Butyrate). Energy conversion efficiency

Commented [mp12]: Please check again the formula using Moss et al (2002)??

was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate, and butyrate). The calculation formula used was:

$$E\ (\%) = \frac{(0.622\ pA + 1.091\ pP + 1.558\ pB)}{pA + pP + 2pB} x100$$

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto & Imai (1981). The solution used was methyl formalin saline, made from a mixture of 100 mL 35% formaldehyde, 2 g trypan blue, 9 g NaCl, and 900 mL distilled water. The number of protozoa was calculated by using a microscope at magnification 100 times. Measurement of rumen liquid protein microbes using the method of Makkar *et al.* (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of Cocks & Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang *et al.*, 2020).

Commented [mp13]: what is the reference?

Commented [mp14]: How is the formula?

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability

~~The fermentability of feed is determined by various factors such as pH, total protozoa count, microbial protein content, NH3 concentration, and total VFA production in rumen liquid~~. These parameters are detailed in Table 2. The pH levels across all treatments fell within the normal range of 6.75-6.82. While the treatment did not significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total protozoa count, microbial protein content, total VFA, and NH3 concentrations. Palm oil supplementation had the most pronounced impact, notably reducing protozoa count and microbial protein. When ZPOS protection was partially supplemented (T2), it led to higher microbial protein and VFA production compared to feed supplemented with total ZPOS, although the difference was not significant.

Feed Digestibility

Table 3 presents the feed digestibility results from the ZPOS supplementation treatment. Statistical analysis revealed no significant differences in the digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD) due to the palm oil Zn soap treatment. However, there were significant differences ($p<0.05$) in the digestibility

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Commented [mp18]: Nutrient Digestibility ??

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of ether extract (EED), crude fiber (CFD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD). Both non-protected and protected palm oil increased EED. Improvements in fiber digestibility (CFD, NDFD, and ADFD) were only observed with zinc soap supplementation (T2 and T3), whereas non-protected palm oil showed no difference from the control. The highest fiber digestibility was achieved with partial Zn soap supplementation (T2), though it was not significantly different from the total protection Zn soap supplementation (T3).

Relative Proportion of VFA

Palm oil (PO and ZPOS) supplementation significantly influenced ($p<0.05$) the partial VFA production of acetate, propionate, butyrate, the A/P ratio, and methane, but did not impact the efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. ~~According to Duncan's multiple range test results~~, both partial (T2) and total (T3) ZPOS supplementation ~~resulted in higher levels of~~ acetate, propionate, and butyrate compared to PO supplementation (T1) and the control (T0). Conversely, PO and ZSPO supplementation produced a lower A/P ratio. The estimated methane production also decreased with PO and ZPOS supplementation.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. The control diet primarily contained short-chain fatty acids (SCFA), with a notably higher concentration of C4 compared to the other treatments ($p<0.05$). Supplementation with palm oil (PO and ZPOS) led to the increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1) relative to the control ($p<0.05$). However, the proportion of stearate was significantly lower with ZPOS supplementation ($p<0.05$). Unsaturated fatty acids,

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particularly cis-9 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6), showed a significant increase in the 3.75% ZPOS (T2) and 5% ZPOS (T3) treatments ($p<0.05$).

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the control diet exhibited a higher total content of SCFAs compared to the diets supplemented with PO and ZPOS ($p<0.05$) but produced a lower amount of LCFA. SFAs showed higher in control and PO supplementation, whereas ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 3.75% ZPOS and 5% ZPOS supplementation resulted in higher levels of MUFAs and PUFAs than the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen fermentation process. Similar results have been reported in the other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.* (2022), and Roy *et al.* (2017). The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly lauric acid. According to Rahman *et al.* (2022), palm oil typically contains about 44% lauric acid (C12:0). Lauric acid is known to have antiprotozoal properties, which can potentially disrupt protozoan cell membranes and inhibit their growth (Santos *et al.*, 2022).

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Ibrahim *et al.* (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears *et al.* (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in the decreased protein synthesis. The use of ZPOS protection, both partially (T2) and fully (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza et al. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to the increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Feed Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% non-protected ZPOS supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p *et al.*, 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The

Commented [mp26]: Does palm oil rich in Unsaturated fatty acid or oleic acid? Please use the relevant references.

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312 experimental feed in this study, characterized by high fiber content and low ADF
313 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
314 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
315 (Benchaar *et al.,* 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
316 increased EED, supporting the conclusion that palm oil can be used as an energy
317 supplement without compromising rumen feed fermentability.

318 A notable finding from this research is that both partial and total Zn soap
319 supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and
320 ADFD. This effect is likely due to the fat and Zn content in palm oil. Palm oil contains
321 high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki *et al.,* 2015).
322 Research by Sears *et al.* (2024) indicates an increase in Prevotella and Fibrobacter
323 populations in response to palmitic acid supplementation. This suggests that cellulolytic
324 bacteria can incorporate palmitic acid into their cell membranes, thereby increasing
325 energy availability for metabolic processes. Furthermore, Zn is crucial for various
326 metabolic functions in rumen microbes.

327 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
328 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et*
329 *al.,* 2016; Uniyal *et al.,* 2017). It is indispensable for preserving the structural and
330 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
331 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
332 one Zn-dependent protein (Ranasinghe *et al.,* 2015). Recent research by Fellner *et al.*
333 (2021) elucidated that Zn supplementation precipitated increased acetate production and
334 a heightened acetate/propionate ratio. This delineates an augmented fiber digestion
335 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein

synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany *et al.* (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah *et al.* (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both partial and total ZPOS supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($p<0.05$). These findings are consistent with the increased microbial protein levels observed with ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, including DNA and RNA polymerase, which play a role in protein synthesis (Franco *et al.*, 2024; Sloup *et al.*, 2017).

Commented [mp30]: Please describe first why it is happening???

The increase in propionate in the ZPOS supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold *et al.*, 2015). These findings are consistent with studies showing that ZPOS supplementation leads to an increase in protozoan populations (Table 4). Zn supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal *et al.*, 2016). Moreover, the lack of an increase in methane production and a

lower A/P ratio is advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85%, and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids, including oleic acid (18:1), undergo a biohydrogenation process, being converted into saturated fatty acids, stearic acid (18:0), in the rumen by bacteria (Buccioni *et al.,* 2012). Harvatine *et al.* (2009), who tracked the biohydrogenation process of oleic acid, found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomerase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria

hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZPOS (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.* (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZPOS treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zn's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock

products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZPOS supplementation holds the potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Its supplementation in feed by as much as 5% has beneficial effects, namely increasing fermentability, MUFA, EPA, and DHA in the rumen. It was indicated that partial supplementation of ZPOS (3.75% ZPOS+1.25%PO) resulted in higher protozoa populations and fiber digestibility. Further investigation is required to test the ZPOS on dairy cows *in vivo*.

Commented [mp31]: In conclusion, Which level suggested the best treatment: 5% ZPOS or Partial ZPOS??

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENT

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606 medium-chain fatty acids on ruminal methanogenesis and fermentation *in vitro* and
607 *in vivo*: A meta-analysis. J. Anim. Physiol. Anim. Nutr. 105:874–889.
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609

610 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter basis).

Items	Composition
Ingredient:	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molasses	3.00
Vitamin and mineral mixture	0.80
Nutrient composition:	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcium, %DM	0.33
Phosphor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

611 Note: [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) =
612 TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK),
613 according to Wardeh (1981).
614
615
616 **Table 2**. The pH, total protozoa, microbial protein, NH3, and total VFA production of
617 rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/mL)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/mL)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

618 Note: [a,b] means in the same row with different superscripts differ significantly ($p<0.05$).
619 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
620 diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
621
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626 **Table 3**. Feed nutrient in vitro digestibility with supplementation of zinc palm oil soap
627 (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Dry matter (%)	64.06	63.82	67.20	69.24	0.850	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

628 Note: ^{a,b} means in the same row with different superscripts differ significantly ($p<0.05$).
629 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
630 diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
631
632
633 **Table 4**. Fermentability of feed with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

634 Note: ^{a,b} means in the same row with different superscripts differ significantly ($p<0.05$).
635 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
636 diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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653 **Table 5**. Proportion of fatty acids in rumen liquid with supplementation of zinc palm oil
654 soap (ZPOS)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
	----------% fat ----------					
Short-chain fatty acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain fatty acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, caprylic	<0.1	<0.1	<0.1	<0.1	-	-
C10, capric	<0.1	<0.1	<0.1	<0.1	-	-
C12, lauric	1.38	1.15	1.22	1.2	0.044	0.299
Long-chain fatty acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, myristic	2.19	1.85	2.3	1.68	0.063	0.054
C14:1	1.00	0.5	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitic	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearic	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleic	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3, gamma linolenic	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arachidonic	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.2	0.26	0.017	0.000
C22, behenic	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonic omega 9	<0.1	<0.1	<0.1	<0.1	-	-

655 Note: [a,b] means in the same row with different superscripts differ significantly ($p<0.05$).
656 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
657 diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
658

Table 6. Proportion of short-chain, long-chain, saturated, and unsaturated fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Fatty acids	Treatments				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.2[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

Note: [a,b] means in the same row with different superscripts differ significantly ($p<0.05$).
SA= short-chain fatty acids, LA= long-chain fatty acids, SFA= saturated fatty acids, UFA= unsaturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.
T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Commented [mp33]: There are not SA, LA, and UFA in the table

Please use the same abbreviations between the table and the notes

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Due date reminder

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I hereby submit the revised copyediting results of the manuscript entitled: Supplementation of Zinc Soap Palm Oil Improves Feed Digestibility, Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid" Thank you.

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Supplementation of Zinc Palm Oil Soap Improves Feed Digestibility, Fermentability, and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on digestibility and unsaturated fatty acid profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. The inoculum source was rumen liquid from three fistulated female dairy goats and was homogenized. The goats were fed ration consisted of corn straw, soybean hulls, and concentrate containing total digestible nutrients (TDN) 63% crude protein (CP) 14%, and neutral detergent fiber (NDF) 35%. Results showed that both 5% partial ZPOS and 5% ZPOS supplementation (T2 and T3) resulted increased of total volatile fatty acids (VFA), acetat, propionate, butirat, unsaturated fatty acids (USFA) and decreased in ratio of acetat/propionate (A/P) compared to the control and supplementation of 5% PO ($p<0.05$). Supplementation of 5% partial ZPOS (T2) is better than 5% ZPOS because increased the digestibility of ether ekstract (EE), crude fiber (CF), NDF, and acids detergent fiber (ADF) ($p<0.05$) and decreased of methane compared to the control

Commented [mp1]: What do you mean with 5% parrtial ZPOS? Why used this treatment?
- 5% partial ZPOS means that only part of the ZPOS is used (75%), while the remaining 25% uses palm oil without protection.
- The aim of supplementation 5% partial ZPOS is so that some of the polyunsaturated fatty acids are not saponified, so that they can have an effect in the form of barriers to methanogenic microbes in the formation of methane in order to cause hydrogen accumulation to encourage increased production of propionic acid.

Commented [mp2]: All of abbreviations, ie TDN, CP, NDF, VFA, please define first
Already did

Commented [mp3]: Please use a clear statement. Does the treatment increase or decrease the variable observed?
Already did

(p<0.05). In conclusion, adding of 5% partial ZPOS (3.5% ZPOS and 1.5% PO) increase fiber digestibility, concentration of VFA, LCFA and USFA, as well as decrease methane production in the rumen liquid.

Keywords: *fermentability; zinc palm oil soap; unsaturated fatty acids; rumen*

INTRODUCTION

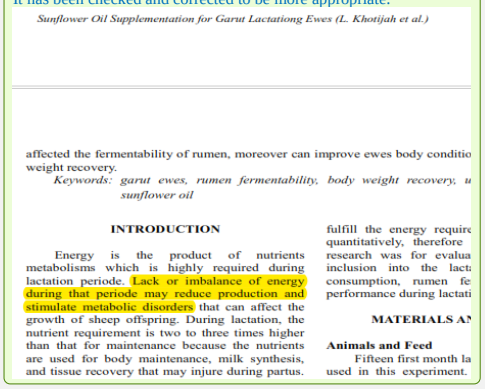
Early lactation dairy cows experience negative energy balance due to the decreased dry matter intake (DMI). Lack or imbalance of energy during that periode may reduce production and stimulate metabolic disorders (Khotijah *et al.*, 2017). This opinion was supported by (Tribout *et al.*, 2023) who stated that energy deficiency has detrimental effects, including low production and weight loss in livestock. To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessment of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera *et al.*, 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (*Bos taurus* x *Bos indicus*) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (*Bos taurus*). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Commented [A4]: which level?? Please state clearly.
Already did

Commented [mp5]: Please check again, Do Khotijah et al. state their research about dairy?

It has been checked and corrected to be more appropriate.


Sunflower Oil Supplementation for Garut Lactationg Ewes (L. Khotijah et al.)

affected the fermentability of rumen, moreover can improve ewes body conditio
weight recovery.
Keywords: garut ewes, rumen fermentability, body weight recovery, u
sunflower oil

INTRODUCTION

Energy is the product of nutrients metabolisms which is highly required during lactation periode. Lack or imbalance of energy during that periode may reduce production and stimulate metabolic disorders that can affect the growth of sheep offspring. During lactation, the nutrient requirement is two to three times higher than that for maintenance because the nutrients are used for body maintenance, milk synthesis, and tissue recovery that may injure during partus.

fulfill the energy require quantitatively, therefore research was for evalua inclusion into the lact consumption, rumen fe performance during lactati

MATERIALS AN

Animals and Feed

Fifteen first month la used in this experiment.

Palm oil is one of the most promising vegetable oils for energy sources, with a gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains saturated fatty acids in the form of palmitic acid 44%, as well as unsaturated fatty acids specifically oleic acid 39.2% and linoleic acid 10.1% (Mancini *et al.*, 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids in palm oil (oleic acids and linoleic acids) contribute to energy efficiency by reducing protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao *et al.*, 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibro lytic microbes, thus reducing fiber digestibility (Sears *et al.*, 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil contains 49.3% unsaturated fatty acids, when used in animal feed must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard unsaturated fatty acids to preserve their biological roles, such as being structural components of bio membranes that uphold

Commented [A6]: The fatty acid content of palm oil is clarified to support the sentence in the line 59

Commented [mp7]: Palmitic acid is saturated fatty acid, not unsaturated fatty acid?
Unsaturated fatty acids in this sentence are oleic acid and linoleic acids which is contained in palm oil as much as 39.2% and 10,1% (please read the line 54)

Corrections already done

Commented [mp8]: Palm oil rich in saturated fatty acid, so no need to prevent from biohydrogenation??

Please see line 54, palm oil contain 49,3% unsaturated from oleic and linoleic acids, more than unsaturated fatty acids from palmitic acids 44%.

membrane integrity. Polyunsaturated fatty acids in bio membranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism, and enhances nutrient utilization efficiency in livestock (Pereira *et al.*, 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir *et al.*, 2023; Vargas-bello-p *et al.*, 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari *et al.*, 2020). However, the use of zinc (Zn) minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, and acts as a structural component in gene expression and signal transduction (Franco *et al.*, 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase, which play a role in protein synthesis (Sloup *et al.*, 2017). Studies *in vitro* observed that supplementing Zn in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970) and concentrations of total volatile fatty acids (VFA) and acetate (Chen *et al.*, 2019). Research by Wang *et al.* (2021) found that supplementation of 20-30mg/kg DM Zn sulfate led to increase nutrient digestibility and ruminal fermentation.

In livestock, Zn is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy *et al.*, 2023). The most important of Zn are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup *et al.*, 2017). The use of Zn as an oil protector has a double benefit, namely protecting

unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zn soap supplementation research was conducted by Faizah *et al.* (2019), which compared the supplementation of 10% Zn soap from palm oil with 10% corn. Supplementation with 10% palm oil Zn soap resulted in no different digestibility of dry matter, organic matter, and crude fiber, but the total production of VFA, acetate, and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short-chain fatty acids. However, no research has explored the contribution of long-chain fatty acids, MUFA, and PUFA due to Zn soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. This study's results provided information regarding the concentration of long-chain fatty acids, MUFA and PUFA in the rumen due to Zn soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic Zn supplements, specifically Zn palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles *in vitro*. The benefit of this research is identifying the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability *in vitro*. Discovery of the most effective use of palm oil in providing an alternative solution to energy and Zn deficiencies in dairy cattle.

MATERIALS AND METHODS

120 **Zinc Soap and Feed Preparation**

121 The preparation of Zn soap was carried out according to Cabatit (1979). The palm
122 oil used to make Zn soap was a commercial palm oil that was generally sold on the market.
123 The zinc soap of palm oil was made based on saponification number. Palm oil is measured
124 for its saponification number and the KOH added is proportional to the saponification
125 number. In order to protect palm oil, zinc chloride (ZnCl2) is added in an amount equal
126 to the KOH required to soak the oil, which is determined by the outcomes of reaction
127 stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water
128 bath at 90 ^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until
129 the oil is completely hydrolyzed. Next, the ZnCl2 solution was added and mix
130 continuously until a paste forms. The final step is to remove the remaining KOH by
131 adding water and washing using a centrifuge. This process produces cream soap called
132 zinc palm oil soap (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2%
133 crude fiber, 4.94% Zn, and gross energy 5257 kcal/kg.

134 The basal feed consists of forage and concentrates contain CP 14% and TDN 63%,
135 that have been formulated for feeding lactating dairy cow with body weight 400 kg, milk
136 yield 15 kg and fat content 3.5% (National Research Council, 1988). The feed was
137 composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut
138 meal, soybean meal, and molasses. The composition of ingredients and nutrient content
139 of the basal feed are shown in Table 1.

Commented [mp9]: What the standard used for this nutrient?
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140 ***In Vitro* Experiment**

141 The experiment was designed using a completely randomized design with four
142 treatments and five replications. The treatments tested were T0= basal diet without
143 supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partially ZPOS

(3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belong to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for *in vitro* substrate for one week before rumen liquid was collected. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheese cloth and placed into a 39 °C flask under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley & Terry (1963). In the fermenter tube, 0.55 g of feed samples from each treatment were added, followed by 40 mL of McDaugall's solution and 10 mL of rumen liquid. The fermenter cylinder was flowed by CO2 gas and closed to anaerobic conditions. The fermenter tube was incubated at a 39 °C water temperature.

Nutrient Digestibility

The digestibility of nutrients, including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED), and crude fiber (CFD) were measured through two stages of incubation, namely fermentative and enzymatic. In the first stage, the fermenter tube was incubated at 39 °C water temperature for 2x24 hours, and the process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then, the tube was centrifuged for 15 minutes at a speed of 3,000 rpm, and the supernatant was discarded. In the second stage, the HCl pepsin solution was added 50 mL into the tube and incubated again for 2x24 hours in a 39 °C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed for DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed

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samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

$$\text{Nutrient digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})}{\text{Nutrient sample, g}} x100$$

pH Value, VFA, NH_3, and Methane Production

The process of measuring pH, VFA, and NH_3 followed the same procedure as that for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968) reported that gas chromatography was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H_2SO_4 was combined with a 10 mL incubation sample. A milliliter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7 ^oC at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency were determined by calculating the VFA stoichiometry, which was the estimation using the formula of Orskov & Ryle (1990). The formula used for methane concentration was: Methane (mM) = 0.5a-0.25p+0.5b, where a, p and b are moles of acetic, propionic and butyric acids respectively. The efficiency of conversion of hexose energy to VFA was calculated based

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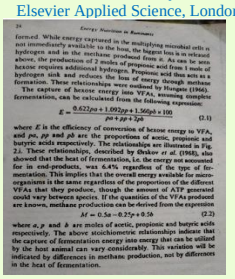
Orskov, E. R. & M. Ryle. 1990. Energy Nutrition in Ruminants. Elsevier Applied Science, London (UK).



on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate, and butyrate). The calculation formula used was:

$$E\ (\%) = \frac{(0.622\ pa + 1.091\ pp + 1.558\ pb)}{pa + pp + 2pb} x\ 100\%$$

Where pa, pp and pb are the proportion of acetic, propionic and butiric acids.

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto & Imai (1981). The solution used was trypan blue formalin saline (TBFS), made from a mixture of 100 mL 35% formaldehyde, 2 g trypan blue, 8 g NaCl, and 900 mL distilled water. The protozoa population was carried out on each treatment rumen fluid mixed with Trypan Blue Formalin Saline (TBFS) solution with a ratio of 5:1. The counting chamber with a thickness of 0.1 mm was dripped with 2 drops of the mixture. The area of the smallest box was 0.0625 mm, totaling 16 boxes with a box area of 5 boxes read. The number of protozoa was calculated by using a microscope at magnification 100 times. The formula used for total population of protozoa was : $\frac{1}{0.1x0.0625x16x5} x\ 1000xDFxC$, where C is protozoa population in the counting chamber and DF is diluent factor the specimen.

Measurement of rumen liquid protein microbes using the method of Makkar *et al.* (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of Cocks & Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50

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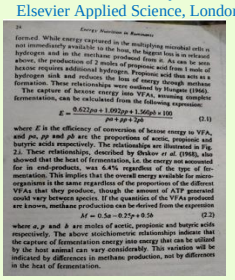
Orskov, E. R. & M. Ryle. 1990. Energy Nutrition in Ruminants. Elsevier Applied Science, London (UK).



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µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang *et al.*, 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability in The Rumen

Detailed rumen feed fermentation parameters can be seen in Table 2. The pH levels in all treatments were not significantly different ($P>0.05$) and were in the normal range of 6.75-6.82. Supplementation of 5% PO (T1) had an effect on reducing the number of protozoa and microbial protein compared to the control and other treatments ($P<0.05$). On the other hand, 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation caused a decreased in NH3 concentration and an increased in VFA production compared to the control and 5% PO supplementation ($P<0.05$).

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Nutrient Digestibility

Table 3 presents the nutrient digestibility due to 5% PO, 5% partial ZPOS and 5% ZPOS supplementation. All treatments did not show significant differences in dry matter (DMD), organic matter (OMD), and crude protein (CPD) digestibility, but there were significant differences ($p<0.05$) in ether extract digestibility (EED), crude fiber digestibility (CFD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber digestibility (ADFD). Both supplementation of 5% PO (T1), 5% partial ZPOS (T2) and 5% ZPOS (T3) increased EED, but fiber digestibility (CFD, NDFD, and ADFD) was only increased by 5% partial ZPOS supplementation (T2), while 5% PO and 5% ZPOS supplementation did not differ from the control.

Relative Proportion of VFA

Table 4 shows the partial VFA and methane production in rumen liquid. Supplementation of 5% PO, 5% partial ZPOS and 5% ZPOS) significantly influenced ($p<0.05$) acetate, propionate, butyrate, the A/P ratio, and methane, but did not impact the efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. Both 5% partial ZPOS (T2) and 5% ZPOS supplementation increased acetate, propionate, and butyrate compared to 5% PO supplementation (T1) and the control (T0). Conversely, A/P ratio and methane production decreased with 5% PO and 5% partial ZPOS supplementation. The efficiency of hexose energy conversion into VFA between treatments was not significantly different.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. Suplementation of 5% PO (T1), 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation decreased

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significantly (P<0.05) short-chain fatty acids (SCFA). The long chain fatty acidc (LCFA) specially palmitate (C16:0) was increased, but stearate (C18:0) was decreased by all treatments (P<0.05). Unsaturated fatty acids (USFA), particularly trans 11 C18:1, cis 9 C18:1, C18:2, C20:5 (EPA) and C22:6 (DHA) were increased by 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation.

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These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the diets supplemented with 5% PO, 5% partial ZPOS and 5% ZPOS decreased of SCFAs compared to the control diet (p<0.05) but increased amount of LCFA. SFAs showed increased in control and 5% PO supplementation, whereas 5% partial ZPOS and 5% ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 5% partial 5% ZPOS and 5% ZPOS supplementation increased of MUFAs and PUFAs than the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen fermentation process. Palm oil contains high palmitic acid, which actually supports the growth of rumen bacteria (Sears *et al*., 2024). Apart from being high in palmitate content, palm oil is also high in unsaturated fatty acids (oleic acids), palm oil protection causes unsaturated fatty acids to escape rumen microbial biohydrogenation so that negative

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effects on the rumen environment can be eliminated. Similar results have been reported in the other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.* (2022), and Roy *et al.* (2017).

The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly palmitic acids (C16:0). According to Rahman *et al.* (2022), palm oil contains about 44% palmitic acid. It has been reported that supplementation of LCFA in the form of palmitate (C16:0) and stearate (C18:0) resulted in a decrease in the protozoa population, although the effect of the decrease did not as strong as MCFA, especially capric acids (C10:0) and lauric acid (C12:0). (Matsumoto *et al*., 1991). The decrease is caused by the inability of protozoa to digest fat because they do not have lipolytic enzymes, as a result the addition of oil can interfere with the metabolic activity of protozoa and subsequently cause the number of protozoa to decrease (Hartanto *et al*., 2017).

Ibrahim *et al.* (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears *et al.* (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in the decreased protein synthesis. The use of ZPOS protection, both 5% partial ZPOS (T2) and 5% ZPOS (T3), can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza *et al*. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to the

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This sentence discusses the decline in the protozoa population. To avoid confusion, I separated it into another paragraph.

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That right, palm oil rich in unsaturated fatty acids (oleic acids 41%), also saturated fatty acids (palmitic acids 44%).
➔See Rahman et al., 2022 (line 292)
➔ Mancini *et al.*, 2015 (line 54)

311 increased propionate production. Propionate is formed from the breakdown of glycerol
312 during fat hydrolysis. The NH3 concentration in the T2 treatment was the lowest among
313 all treatments, while microbial protein production was the highest. This indicates that
314 NH3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

315 **Nutrient Digestibility**

316 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
317 unaffected by 5% PO supplementation, indicating that this level of supplementation is
318 safe for rumen microbial growth. These results are consistent with previous research,
319 which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and
320 linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although
321 linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p *et al.*, 2020).
322 The effect of oil supplementation on digestibility is highly dependent on the nutrient
323 composition of the feed and the fatty acid profile of the oil. The experimental feed in this
324 study, characterized by high fiber content and low ADF (13.77%), supports the
325 proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential
326 negative impact of oil on fiber digestion by rumen microbes (Benchaar *et al.*, 2015). Both
327 non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the
328 conclusion that palm oil can be used as an energy supplement without compromising
329 rumen feed fermentability.

330 A notable finding from this research is that both partial and total Zn soap
331 supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and
332 ADFD. This effect is likely due to the fat and Zn content in palm oil. Palm oil contains
333 high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki *et al.*, 2015).
334 Research by Sears *et al.* (2024) indicates an increase in Prevotella and Fibrobacter

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Commented [mp29]: What do you mean by 5% Non-protected ZPOS? Does ZPOS mean protected zinc palm oil?
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Commented [mp30]: If palm oil at level 5% is safe for nutrient digestibility, does it mean there is no need to be protected??
The purpose of palm oil protection is to protect unsaturated fatty acids from biohydrogenation, namely preventing oleic acid from being converted into stearic acid. This will increase the supply of unsaturated fatty acids to the post-rumen, which is beneficial for livestock and livestock products

populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, Zn is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et al.*, 2016; Uniyal *et al.*, 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe *et al.*, 2015). Recent research by Fellner *et al.* (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/propionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/ml in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany *et al.* (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah *et al.* (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both 5% partial ZPOS and 5% ZPOS supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($p<0.05$). Acetate is the yield of digestion of fibrous carbohydrates

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by cellulolytic bacteria, which means that the increase in acetate is in line with the increase in the number of cellulolytic bacteria in the rumen. This is likely related to the decreasing protozoa population resulting from oil supplementation. Protozoa are predators of bacteria (Dayyani *et al*., 2013), so a decrease in the number of protozoa will increase the number of bacteria because it reduces competition for nutrients.. These findings are consistent with the increased microbial protein levels observed with 5% partial ZPOS and 5% ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes, including DNA and RNA polymerase, which play a role in protein synthesis (Franco *et al.*, 2024; Sloup *et al.*, 2017).

The increase in propionate in the ZPOS supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold *et al.*, 2015). These findings are consistent with studies showing that ZPOS supplementation leads to an increase in protozoan populations (Table 4). Zn supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal *et al.*, 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio is advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10 .5% polyunsaturated fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85%, and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids, including oleic acid (18:1), undergo a biohydrogenation process, being converted into saturated fatty acids, stearic acid (18:0), in the rumen by bacteria (Buccioni *et al.,* 2012). Harvatine *et al.* (2009), who tracked the biohydrogenation process of oleic acid, found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomerase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZPOS (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)

concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.* (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZPOS treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zn's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZPOS supplementation holds the potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Supplementation of 5% partial ZPOS (3.75% ZPOS+1.25% PO) is better than 5% ZPOS. Beneficial effects this supplementation are increase fiber digestibility, VFA, LCFA and USFA, as well as decrease methane production in the rumen. Further investigation is required to test the ZPOS on dairy cows *in vivo*.

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CONFLICT OF INTEREST

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

ACKNOWLEDGEMENT

The research was funded by the Faculty of Animal and Agricultural Science, Diponegoro University at 2023 with the contract number 4/UN7.F5/HK/III/2023.

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634 *in vivo*: A meta-analysis. J. Anim. Physiol. Anim. Nutr. 105:874–889.
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636 **Table 1.** Ingredients and chemical composition of experimental diet (dry matter
637 basis).

Items	Composition
Ingredient:	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9,93
Coconut meal	17.01
Soybean meal	6.98
Molasses	3.00
Vitamin and mineral mixture	0.80
Nutrient composition:	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcium, %DM	0.33
Phosphor, %DM	0.28
Zinc, mg/kg DM	16,93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

638 Note: [1]Total digestible nutrients (TDN) were calculated using TDN (%DM) =
639 TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK),
640 according to Wardeh (1981).
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643 **Table 2**. In vitro feed fermentability in rumen liquid with supplementation of zinc palm
644 oil soap (ZPOS)

Variables	Treatments				SEM	*p-Value*
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/mL)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/mL)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

645 Note: [a,b] means in the same row with different superscripts differ significantly ($p<0.05$).
646 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
647 diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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653 **Table 3**. In vitro nutrient digestibility in rumen liquid with supplementation of zinc palm
654 oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Dry matter (%)	64.06	63.82	67.20	69.24	0.850	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

655 Note: ^{a,b} means in the same row with different superscripts differ significantly ($p<0.05$).
656 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
657 diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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660 **Table 4**. In vitro VFA and methane production in rumen liquid with supplementation of
661 zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

662 Note: ^{a,b} means in the same row with different superscripts differ significantly ($p<0.05$).
663 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
664 diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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681 **Table 5**. In vitro proportion of fatty acids in rumen liquid with supplementation of zinc
682 palm oil soap (ZPOS)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
	----------% fat ----------					
Short-chain fatty acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain fatty acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, caprylic	<0.1	<0.1	<0.1	<0.1	-	-
C10, capric	<0.1	<0.1	<0.1	<0.1	-	-
C12, lauric	1.38	1.15	1.22	1.2	0.044	0.299
Long-chain fatty acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, myristic	2.19	1.85	2.3	1.68	0.063	0.054
C14:1	1.00	0.5	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitic	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearic	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleic	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3, gamma linolenic	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arachidonic	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.2	0.26	0.017	0.000
C22, behenic	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonic omega 9	<0.1	<0.1	<0.1	<0.1	-	-

683 Note: [a,b] means in the same row with different superscripts differ significantly (p<0.05).
684 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
685 diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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688 **Table 6**. In vitro proportion of short-chain, long-chain, saturated, and unsaturated fatty
689 acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Fatty acids	Treatments				SEM	*p-Value*
	T0	T1	T2	T3		
	----------% fat ----------					
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.2[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

690 Note: [a,b] means in the same row with different superscripts differ significantly (p<0.05).
691 SCFA= short-chain fatty acids, MCFA= medium-chain fatty acids, LCFA= long-chain
692 fatty acids, SFA= saturated fatty acids, USFA= unsaturated fatty acids, MUFA=
693 monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.
694 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
695 diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
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Commented [mp34]: There are not SA, LA, and UFA in the table

Please use the same abbreviations between the table and the notes

Corected already done

Gmail **Anis Muktiani <anis.muktiani@gmail.com>**

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Messages

Note	From
Dear A. Muktiani, W. Widiyanto, & N. S. Pandupuspitasari: I am pleased to inform you that your article submitted to Tropical Animal Science Journal, entitled: "Supplementation of Zinc Palm Oil Soap Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid" has been approved to be published in Tropical Animal Science Journal in the upcoming edition (Vol. 47 No. 3, September 2024). Submission No. TASJ-56357 Please find the PROOF of your manuscript in the attached file. If there are still any corrections in the manuscript, please respond and return the document within 5 days. Thank you for your article submission, and we are looking forward to receiving your incoming articles. Prof. Dr. Komang G Wiryawan Chief Editor Tropical Animal Science Journal kgwiryawan@yahoo.com komang, TASJSept2024_371.pdf	komang 2024-08-20 09:14 AM

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Supplementation of Zinc Palm Oil Soap Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on digestibility and unsaturated fatty acid profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. The inoculum source was rumen liquid from three fistulated female dairy goats and was homogenized. The goats were fed ration consisting of corn straw, soybean hulls, and concentrate containing total digestible nutrients (TDN) 63%, crude protein (CP) 14%, and neutral detergent fiber (NDF) 35%. Results showed that both 5% partial ZPOS and 5% ZPOS supplementation (T2 and T3) resulted in the increase of total volatile fatty acids (VFA), acetate, propionate, butyrate, unsaturated fatty acids (USFA) and a decrease in the ratio of acetate/propionate (A/P) compared to the control and supplementation of 5% PO ($p<0.05$). Supplementation of 5% partial ZPOS (T2) is better than 5% ZPOS because increased the digestibility of ether extract (EE), crude fiber (CF), NDF, and acids detergent fiber (ADF) ($p<0.05$) and decreased of methane compared to the control ($p<0.05$). In conclusion, adding 5% partial ZPOS (3.5% ZPOS and 1.5% PO) increases fiber digestibility, VFA, LCFA, and USFA concentration, and decreases methane production in the rumen liquid.

Keywords: fermentability; rumen; zinc palm oil soap; unsaturated fatty acids

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to the decreased dry matter intake (DMI). Lack or energy imbalance during that period may reduce production and stimulate metabolic disorders (Khotijah *et al.,* 2017). This opinion was supported by (Tribout *et al.,* 2023), who stated that energy deficiency has detrimental effects, including low production and weight loss in livestock. To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessment of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera *et al.,* 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (*Bos taurus* x *Bos indicus*) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (*Bos taurus*). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils for energy sources, with a gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains saturated fatty acids in the form of palmitic acid 44% and unsaturated fatty acids, specifically oleic acid 39.2% and linoleic acid 10.1% (Mancini *et al.,* 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids in palm oil (oleic acids and linoleic acids) contribute to energy efficiency by reducing protozoa (Muktiani *et al.,* 2020; Vargas-bello-p *et al.,* 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao *et al.,* 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears *et al.*, 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil contains 49.3% unsaturated fatty acids, and when used in animal feed, it must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard unsaturated fatty acids to preserve their biological roles, such as being structural components of bio membranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism and enhances nutrient utilization efficiency in livestock (Pereira *et al.*, 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir *et al.*, 2023; Vargas-bello-p *et al.*, 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari *et al.*, 2020). However, the use of zinc (Zn) minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes and acts as a structural component in gene expression and signal transduction (Franco *et al.*, 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase, which play a role in protein synthesis (Sloup *et al.*, 2017). Studies *in vitro* observed that supplementing Zn in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970) and concentrations of total volatile fatty acids (VFA) and acetate (Chen *et al.*, 2019). Research by Wang *et al.* (2021) found that supplementation of 20-30 mg/kg DM Zn sulfate led to increasing nutrient digestibility and ruminal fermentation.

In livestock, Zn is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy *et al.*, 2023). The most important of Zn are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup *et al.*, 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zn soap supplementation research was conducted by Faizah *et al.* (2019), which compared the supplementation of 10% Zn soap from palm oil with 10% corn. Supplementation with 10% palm oil Zn soap resulted in no different digestibility of dry matter, organic matter, and crude fiber, but the total production of VFA, acetate, and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short-chain fatty acids. However, no research has explored the contribution of long-chain fatty acids, MUFA, and PUFA due to Zn soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. This study's results provided information regarding the concentration of long-chain fatty acids, MUFA and PUFA in the rumen due to Zn soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase the production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic Zn supplements, specifically Zn palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles *in vitro*. The benefit of this research is identifying the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability *in vitro*. Discover the most effective use of palm oil in providing an alternative solution to energy and Zn deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of Zn soap was carried out according to Cabatit (1979). The palm oil used to make Zn soap was a commercial palm oil that was generally sold on the market. The zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90 ^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, the $ZnCl_2$ solution was added and mixed continuously until a paste formed. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called zinc palm oil soap (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn, and gross energy 5257 kcal/kg.

The basal feed consists of forage and concentrates containing CP 14% and TDN 63%, formulated for feeding lactating dairy cows with a body weight 400 kg, milk yield 15 kg, and fat content 3.5% (National Research Council, 1988). The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal, and molasses. The composition of ingredients and nutrient content of the basal feed are shown in Table 1.

Table 1. Ingredients and chemical composition of experimental diet (dry matter basis)

Items	Composition
Ingredient:	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9.93
Coconut meal	17.01
Soybean meal	6.98
Molasses	3.00
Vitamin and mineral mixture	0.80
Nutrient composition:	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcium, %DM	0.33
Phosphor, %DM	0.28
Zinc, mg/kg DM	16.93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

Note: [1]Total digestible nutrients (TDN) were calculated using TDN (%DM). TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according to Wardeh (1981).

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belong to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for *in vitro* substrate for one week before rumen liquid was collected. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheesecloth and placed into a 39 °C flask under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley & Terry (1963). In the fermenter tube, 0.55 g of feed samples from each treatment were added, followed by 40 mL of McDaugall's solution and 10 mL of rumen liquid. The fermenter cylinder was flowed by CO_2 gas and closed to anaerobic conditions. The fermenter tube was incubated at a 39 °C water temperature.

Nutrient Digestibility

The digestibility of nutrients, including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED), and crude fiber (CFD) were measured through two stages of incubation, namely fermentative and enzymatic. In the first stage, the fermenter tube was incubated at 39 °C water temperature for 2x24 hours, and the process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then, the tube was centrifuged for 15 minutes at a speed of 3,000 rpm, and the supernatant was discarded. In the second stage, the HCl pepsin solution was added 50 mL into the tube and incubated again for 2x24 hours in a 39 °C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed for DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

Nutrient digestibility (%)=
{[Nutrient sample, g - (Nutrient residue, g - Nutrient blank, g)] / Nutrient sample, g} x 100

pH Value, VFA, NH_3, and Methane Production

The process of measuring pH, VFA, and NH_3 followed the same procedure as that for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH_3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968) reported that gas chromatography was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H_2SO_4 was combined with a 10 mL incubation sample. A milliliter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7 °C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency were determined by calculating the VFA stoichiometry, which was the estimation using the formula of Orskov & Ryle (1990). The formula used for methane concentration was Methane (mM)= 0.5a - 0.25p + 0.5b, where a, p, and b are moles of acetic, propionic, and butyric acids, respectively. The efficiency of conversion of hexose energy to VFA was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate, and butyrate). The calculation formula used was:

E (%)= [(0.622 pA + 1.091 pP + 1.558 pB) / (pA + pP + 2pB)] x 100

Where E is energy, and pA, pP, and pB are the proportions of acetic, propionic, and butyric acids.

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto & Imai (1981). The solution used was trypan blue formalin saline (TBFS), made from a mixture of 100 mL 35% formaldehyde, 2 g trypan blue, 8 g NaCl, and 900 mL distilled water. The protozoa population was carried out on each treatment rumen fluid mixed with Trypan Blue Formalin Saline (TBFS) solution with a ratio of 5:1. The counting chamber with a thickness of 0.1 mm was dripped with 2 drops of the mixture. The area of the smallest box was 0.0625 mm, totaling 16 boxes with a box area of 5 boxes read. The number of protozoa was calculated by using a microscope at magnification 100 times. The formula used for total population of protozoa was: [1 / (0.1 x 0.0625 x 16 x 5)] x 1000 x DF x C, where C is protozoa population in the counting chamber and DF is diluent factor the specimen.

Measurement of rumen liquid protein microbes using the method of Makkar *et al.* (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of Cocks & Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang *et al.*, 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability in The Rumen

Detailed rumen feed fermentation parameters can be seen in Table 2. The pH levels in all treatments were not significantly different and were in the normal range of 6.75-6.82. Supplementation of 5% PO (T1) had an effect on reducing the number of protozoa and microbial proteins compared to the control and other treatments ($p<0.05$). On the other hand, 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation caused a decrease in NH3 concentration and an increase in VFA production compared to the control and 5% PO supplementation ($p<0.05$).

Nutrient Digestibility

Table 3 presents the nutrient digestibility due to 5% PO, 5% partial ZPOS, and 5% ZPOS supplementation. All treatments did not show significant differences in dry matter (DMD), organic matter (OMD), and crude protein (CPD) digestibility, but there were significant differences ($p<0.05$) in ether extract digestibility (EED), crude fiber digestibility (CFD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber digestibility (ADFD). Both supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) increased EED, but fiber digestibility (CFD, NDFD, and ADFD) was only increased by 5% partial ZPOS supplementation (T2), while 5% PO and 5% ZPOS supplementation did not differ from the control.

Relative Proportion of VFA

Table 4 shows the partial VFA and methane production in rumen liquid. Supplementation of 5% PO, 5% partial ZPOS, and 5% ZPOS) significantly influenced ($p<0.05$) acetate, propionate, butyrate, the A/P ratio, and methane but did not impact the efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. Both 5% partial ZPOS (T2) and 5% ZPOS supplementation increased acetate, propionate, and butyrate compared to 5% PO supplementation (T1) and the control (T0). Conversely, A/P ratio and methane production decreased with 5% PO and 5% partial ZPOS supplementation. The efficiency of hexose energy conversion into VFA between treatments was not significantly different.

Table 2. *In vitro* feed fermentability in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/mL)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/mL)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 3. *In vitro* nutrient digestibility in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Dry matter (%)	64.06	63.82	67.20	69.24	0.85	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 4. *In vitro* VFA and methane production in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. Supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) supplementation decreased significantly ($p<0.05$) short-chain fatty acids (SCFA). The long chain fatty acids (LCFA), especially palmitate (C16:0), increased, but stearate (C18:0) decreased by all treatments ($p<0.05$). Unsaturated fatty acids (USFA), particularly trans 11 C18:1, cis 9 C18:1, C18:2, C20:5 (EPA), and C22:6 (DHA), increased by 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation.

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the diets supplemented with 5% PO, 5% partial ZPOS, and 5% ZPOS decreased SCFAs compared to the control diet ($p<0.05$) but increased LCFA. SFAs showed an increase in control and 5% PO supplementation, whereas 5% partial ZPOS and 5% ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 5% partial 5% ZPOS and 5% ZPOS supplementation increased MUFAs and PUFAs than the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen fermentation process. Palm oil contains high palmitic acid, which actually supports the growth of rumen bacteria (Sears *et al*., 2024). Apart from being high in palmitate content, palm oil is also high in unsaturated fatty acids (oleic acids), palm oil protection causes unsaturated fatty acids to escape rumen microbial biohydrogenation so that negative effects on the rumen environment can be eliminated. Similar results have been reported in other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.* (2022), and Roy *et al.* (2017).

The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly palmitic acids (C16:0). According to Rahman *et al.* (2022), palm oil contains about 44% palmitic acid. It has been reported that supplementation of LCFA in the form of palmitate (C16:0) and stearate (C18:0) resulted in a decrease in the protozoa population, although the effect of the decrease was not as strong as MCFA, especially capric acids (C10:0) and lauric acid (C12:0). (Matsumoto *et al*., 1991). The decrease is caused by the inability of protozoa to digest fat because they do not have lipolytic enzymes; as a result, the addition of oil can interfere with the metabolic activity of protozoa and subsequently cause the number of protozoa to decrease (Hartanto *et al*., 2017).

Ibrahim *et al.* (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears *et al.* (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased

Table 5. *In vitro* proportion of fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Short-chain fatty acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain fatty acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, caprylic	<0.1	<0.1	<0.1	<0.1	-	-
C10, capric	<0.1	<0.1	<0.1	<0.1	-	-
C12, lauric	1.38	1.15	1.22	1.20	0.044	0.299
Long-chain fatty acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, myristic	2.19	1.85	2.30	1.68	0.063	0.054
C14:1	1.00	0.50	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitic	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearic	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleic	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3, gamma linolenic	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arachidonic	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.20	0.26	0.017	0.000
C22, behenic	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonic omega 9	<0.1	<0.1	<0.1	<0.1	-	-

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 6. *In vitro* proportion of short-chain, long-chain, saturated, and unsaturated fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.20[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). SCFA= short-chain fatty acids, MCFA= medium-chain fatty acids, LCFA= long-chain fatty acids, SFA= saturated fatty acids, USFA= unsaturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids. T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

protein synthesis. The use of ZPOS protection, both 5% partial ZPOS (T2) and 5% ZPOS (T3) can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza *et al*. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to the increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH_3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH_3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Nutrient Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% PO supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p *et al.*, 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar *et al.*, 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be used as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total Zn soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and Zn content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki *et al.*, 2015). Research by Sears *et al.* (2024) indicates an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, Zn is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et al.*, 2016; Uniyal *et al.*, 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe *et al.*, 2015). Recent research by Fellner *et al.* (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/propionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/mL in the P1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany *et al.* (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah *et al.* (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both 5% partial ZPOS and 5% ZPOS supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($p<0.05$). Acetate is the yield of digestion of fibrous carbohydrates by cellulolytic bacteria, which means that the increase in acetate is in line with the increase in the number of cellulolytic bacteria in the rumen. This is likely related to the decreasing protozoa population resulting from oil supplementation. Protozoa are predators of bacteria (Dayyani *et al*., 2013), so a decrease in the number of protozoa will increase the number of bacteria because it reduces competition for nutrients. These findings are consistent with the increased microbial protein levels observed with 5% partial ZPOS and 5% ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral cofactor for over 300 enzymes, including DNA and RNA polymerase, which play a role in protein synthesis (Franco *et al.*, 2024; Sloup *et al.*, 2017).

The increase in propionate in the ZPOS supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold *et al.*, 2015). These findings are consistent with studies showing that ZPOS supplementation leads to an increase in protozoan populations (Table 4). Zn supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal *et al.*, 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio is advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85%, and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids, including oleic acid (18:1), undergo a biohydrogenation process, being converted into saturated fatty acids, stearic acid (18:0), in the rumen by bacteria (Buccioni *et al.*, 2012). Harvatine *et al.* (2009), who tracked the biohydrogenation process of oleic acid, found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomerase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZPOS (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.* (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZPOS treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zn's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZPOS supplementation holds the potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Supplementation of 5% partial ZPOS (3.75% ZPOS + 1.25% PO) is better than 5% ZPOS. The beneficial effects of this supplementation are increased fiber digestibility, VFA, LCFA, and USFA, as well as decreased methane production in the rumen. Further investigation is required to test the ZPOS on dairy cows *in vivo*.

CONFLICT OF INTEREST

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

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Supplementation of Zinc Palm Oil Soap Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on digestibility and unsaturated fatty acid profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. The inoculum source was rumen liquid from three fistulated female dairy goats and was homogenized. The goats were fed ration consisting of corn straw, soybean hulls, and concentrate containing total digestible nutrients (TDN) 63%, crude protein (CP) 14%, and neutral detergent fiber (NDF) 35%. Results showed that both 5% partial ZPOS and 5% ZPOS supplementation (T2 and T3) resulted in the increase of total volatile fatty acids (VFA), acetate, propionate, butyrate, unsaturated fatty acids (USFA) and a decrease in the ratio of acetate/propionate (A/P) compared to the control and supplementation of 5% PO ($p<0.05$). Supplementation of 5% partial ZPOS (T2) is better than 5% ZPOS because increased the digestibility of ether extract (EE), crude fiber (CF), NDF, and acids detergent fiber (ADF) ($p<0.05$) and decreased of methane compared to the control ($p<0.05$). In conclusion, adding 5% partial ZPOS (3.5% ZPOS and 1.5% PO) increases fiber digestibility, VFA, LCFA, and USFA concentration, and decreases methane production in the rumen liquid.

Keywords: fermentability; rumen; zinc palm oil soap; unsaturated fatty acids

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to the decreased dry matter intake (DMI). Lack or energy imbalance during that period may reduce production and stimulate metabolic disorders (Khotijah *et al.*, 2017). This opinion was supported by Tribout *et al.* (2023), who stated that energy deficiency has detrimental effects, including low production and weight loss in livestock. To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessment of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera *et al.*, 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (*Bos taurus* x *Bos indicus*) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (*Bos taurus*). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils for energy sources, with a gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains saturated fatty acids in the form of palmitic acid 44% and unsaturated fatty acids, specifically oleic acid 39.2% and linoleic acid 10.1% (Mancini *et al.*, 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids in palm oil (oleic acids and linoleic acids) contribute to energy efficiency by reducing protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao *et al.*, 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears *et al.*, 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil contains 49.3% unsaturated fatty acids, and when used in animal feed, it must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard unsaturated fatty acids to preserve their biological roles, such as being structural components of bio membranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism and enhances nutrient utilization efficiency in livestock (Pereira *et al.*, 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir *et al.*, 2023; Vargas-bello-p *et al.*, 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari *et al.*, 2020). However, the use of zinc (Zn) minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes and acts as a structural component in gene expression and signal transduction (Franco *et al.*, 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase, which play a role in protein synthesis (Sloup *et al.*, 2017). Studies *in vitro* observed that supplementing Zn in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970) and concentrations of total volatile fatty acids (VFA) and acetate (Chen *et al.*, 2019). Research by Wang *et al.* (2021) found that supplementation of 20-30 mg/kg DM Zn sulfate led to increasing nutrient digestibility and ruminal fermentation.

In livestock, Zn is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy *et al.*, 2023). The most important of Zn are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup *et al.*, 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zn soap supplementation research was conducted by Faizah *et al.* (2019), which compared the supplementation of 10% Zn soap from palm oil with 10% corn. Supplementation with 10% palm oil Zn soap resulted in no different digestibility of dry matter, organic matter, and crude fiber, but the total production of VFA, acetate, and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short-chain fatty acids. However, no research has explored the contribution of long-chain fatty acids, MUFA, and PUFA due to Zn soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. This study's results provided information regarding the concentration of long-chain fatty acids, MUFA and PUFA in the rumen due to Zn soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase the production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic Zn supplements, specifically Zn palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles *in vitro*. The benefit of this research is identifying the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability *in vitro*. Discover the most effective use of palm oil in providing an alternative solution to energy and Zn deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of Zn soap was carried out according to Cabatit (1979). The palm oil used to make Zn soap was a commercial palm oil that was generally sold on the market. The zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90 ^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, the $ZnCl_2$ solution was added and mixed continuously until a paste formed. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called zinc palm oil soap (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn, and gross energy 5257 kcal/kg.

The basal feed consists of forage and concentrates containing CP 14% and TDN 63%, formulated for feeding lactating dairy cows with a body weight 400 kg, milk yield 15 kg, and fat content 3.5% (National Research Council, 1988). The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal, and molasses. The composition of ingredients and nutrient content of the basal feed are shown in Table 1.

Table 1. Ingredients and chemical composition of experimental diet (dry matter basis)

Items	Composition
Ingredient:	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9.93
Coconut meal	17.01
Soybean meal	6.98
Molasses	3.00
Vitamin and mineral mixture	0.80
Nutrient composition:	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcium, %DM	0.33
Phosphor, %DM	0.28
Zinc, mg/kg DM	16.93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

Note: [1]Total digestible nutrients (TDN) were calculated using TDN (%DM). TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according to Wardeh (1981).

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belong to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for *in vitro* substrate for one week before rumen liquid was collected. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheesecloth and placed into a 39 °C flask under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley & Terry (1963). In the fermenter tube, 0.55 g of feed samples from each treatment were added, followed by 40 mL of McDaugall's solution and 10 mL of rumen liquid. The fermenter cylinder was flowed by CO_2 gas and closed to anaerobic conditions. The fermenter tube was incubated at a 39 °C water temperature.

Nutrient Digestibility

The digestibility of nutrients, including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED), and crude fiber (CFD) were measured through two stages of incubation, namely fermentative and enzymatic. In the first stage, the fermenter tube was incubated at 39 °C water temperature for 2x24 hours, and the process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then, the tube was centrifuged for 15 minutes at a speed of 3,000 rpm, and the supernatant was discarded. In the second stage, the HCl pepsin solution was added 50 mL into the tube and incubated again for 2x24 hours in a 39 °C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed for DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

Nutrient digestibility (%)=
{[Nutrient sample, g - (Nutrient residue, g - Nutrient blank, g)] / Nutrient sample, g} x 100

pH Value, VFA, NH_3, and Methane Production

The process of measuring pH, VFA, and NH_3 followed the same procedure as that for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH_3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968) reported that gas chromatography was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H_2SO_4 was combined with a 10 mL incubation sample. A milliliter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7 °C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency were determined by calculating the VFA stoichiometry, which was the estimation using the formula of Orskov & Ryle (1990). The formula used for methane concentration was Methane (mM)= 0.5a - 0.25p + 0.5b, where a, p, and b are moles of acetic, propionic, and butyric acids, respectively. The efficiency of conversion of hexose energy to VFA was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate, and butyrate). The calculation formula used was:

E (%)= [(0.622 pA + 1.091 pP + 1.558 pB) / (pA + pP + 2pB)] x 100

Where E is energy, and pA, pP, and pB are the proportions of acetic, propionic, and butyric acids.

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto & Imai (1981). The solution used was trypan blue formalin saline (TBFS), made from a mixture of 100 mL 35% formaldehyde, 2 g trypan blue, 8 g NaCl, and 900 mL distilled water. The protozoa population was carried out on each treatment rumen fluid mixed with Trypan Blue Formalin Saline (TBFS) solution with a ratio of 5:1. The counting chamber with a thickness of 0.1 mm was dripped with 2 drops of the mixture. The area of the smallest box was 0.0625 mm, totaling 16 boxes with a box area of 5 boxes read. The number of protozoa was calculated by using a microscope at magnification 100 times. The formula used for total population of protozoa was: [1 / (0.1 x 0.0625 x 16 x 5)] x 1000 x DF x C, where C is protozoa population in the counting chamber and DF is diluent factor the specimen.

Measurement of rumen liquid protein microbes using the method of Makkar *et al.* (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of Cocks & Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang *et al.*, 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability in The Rumen

Detailed rumen feed fermentation parameters can be seen in Table 2. The pH levels in all treatments were not significantly different and were in the normal range of 6.75-6.82. Supplementation of 5% PO (T1) had an effect on reducing the number of protozoa and microbial proteins compared to the control and other treatments ($p<0.05$). On the other hand, 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation caused a decrease in NH3 concentration and an increase in VFA production compared to the control and 5% PO supplementation ($p<0.05$).

Nutrient Digestibility

Table 3 presents the nutrient digestibility due to 5% PO, 5% partial ZPOS, and 5% ZPOS supplementation. All treatments did not show significant differences in dry matter (DMD), organic matter (OMD), and crude protein (CPD) digestibility, but there were significant differences ($p<0.05$) in ether extract digestibility (EED), crude fiber digestibility (CFD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber digestibility (ADFD). Both supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) increased EED, but fiber digestibility (CFD, NDFD, and ADFD) was only increased by 5% partial ZPOS supplementation (T2), while 5% PO and 5% ZPOS supplementation did not differ from the control.

Relative Proportion of VFA

Table 4 shows the partial VFA and methane production in rumen liquid. Supplementation of 5% PO, 5% partial ZPOS, and 5% ZPOS) significantly influenced ($p<0.05$) acetate, propionate, butyrate, the A/P ratio, and methane but did not impact the efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. Both 5% partial ZPOS (T2) and 5% ZPOS supplementation increased acetate, propionate, and butyrate compared to 5% PO supplementation (T1) and the control (T0). Conversely, A/P ratio and methane production decreased with 5% PO and 5% partial ZPOS supplementation. The efficiency of hexose energy conversion into VFA between treatments was not significantly different.

Table 2. *In vitro* feed fermentability in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/mL)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/mL)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 3. *In vitro* nutrient digestibility in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Dry matter (%)	64.06	63.82	67.20	69.24	0.85	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 4. *In vitro* VFA and methane production in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. Supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) supplementation decreased significantly ($p<0.05$) short-chain fatty acids (SCFA). The long chain fatty acids (LCFA), especially palmitate (C16:0), increased, but stearate (C18:0) decreased by all treatments ($p<0.05$). Unsaturated fatty acids (USFA), particularly trans 11 C18:1, cis 9 C18:1, C18:2, C20:5 (EPA), and C22:6 (DHA), increased by 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation.

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the diets supplemented with 5% PO, 5% partial ZPOS, and 5% ZPOS decreased SCFAs compared to the control diet ($p<0.05$) but increased LCFA. SFAs showed an increase in control and 5% PO supplementation, whereas 5% partial ZPOS and 5% ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 5% partial 5% ZPOS and 5% ZPOS supplementation increased MUFAs and PUFAs than the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen fermentation process. Palm oil contains high palmitic acid, which actually supports the growth of rumen bacteria (Sears *et al*., 2024). Apart from being high in palmitate content, palm oil is also high in unsaturated fatty acids (oleic acids), palm oil protection causes unsaturated fatty acids to escape rumen microbial biohydrogenation so that negative effects on the rumen environment can be eliminated. Similar results have been reported in other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.* (2022), and Roy *et al.* (2017).

The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly palmitic acids (C16:0). According to Rahman *et al.* (2022), palm oil contains about 44% palmitic acid. It has been reported that supplementation of LCFA in the form of palmitate (C16:0) and stearate (C18:0) resulted in a decrease in the protozoa population, although the effect of the decrease was not as strong as MCFA, especially capric acids (C10:0) and lauric acid (C12:0). (Matsumoto *et al*., 1991). The decrease is caused by the inability of protozoa to digest fat because they do not have lipolytic enzymes; as a result, the addition of oil can interfere with the metabolic activity of protozoa and subsequently cause the number of protozoa to decrease (Hartanto *et al*., 2017).

Ibrahim *et al.* (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears *et al.* (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased

Table 5. *In vitro* proportion of fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Short-chain fatty acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain fatty acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, caprylic	<0.1	<0.1	<0.1	<0.1	-	-
C10, capric	<0.1	<0.1	<0.1	<0.1	-	-
C12, lauric	1.38	1.15	1.22	1.20	0.044	0.299
Long-chain fatty acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, myristic	2.19	1.85	2.30	1.68	0.063	0.054
C14:1	1.00	0.50	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitic	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearic	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleic	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3, gamma linolenic	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arachidonic	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.20	0.26	0.017	0.000
C22, behenic	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonic omega 9	<0.1	<0.1	<0.1	<0.1	-	-

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 6. *In vitro* proportion of short-chain, long-chain, saturated, and unsaturated fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.20[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). SCFA= short-chain fatty acids, MCFA= medium-chain fatty acids, LCFA= long-chain fatty acids, SFA= saturated fatty acids, USFA= unsaturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids. T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

protein synthesis. The use of ZPOS protection, both 5% partial ZPOS (T2) and 5% ZPOS (T3) can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza *et al*. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to the increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH_3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH_3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Nutrient Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% PO supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p *et al.*, 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar *et al.*, 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be used as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total Zn soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and Zn content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki *et al.*, 2015). Research by Sears *et al.* (2024) indicates an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, Zn is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et al.*, 2016; Uniyal *et al.*, 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe *et al.*, 2015). Recent research by Fellner *et al.* (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/propionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (T1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/mL in the T1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany *et al.* (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah *et al.* (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both 5% partial ZPOS and 5% ZPOS supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($p<0.05$). Acetate is the yield of digestion of fibrous carbohydrates by cellulolytic bacteria, which means that the increase in acetate is in line with the increase in the number of cellulolytic bacteria in the rumen. This is likely related to the decreasing protozoa population resulting from oil supplementation. Protozoa are predators of bacteria (Dayyani *et al*., 2013), so a decrease in the number of protozoa will increase the number of bacteria because it reduces competition for nutrients. These findings are consistent with the increased microbial protein levels observed with 5% partial ZPOS and 5% ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral cofactor for over 300 enzymes, including DNA and RNA polymerase, which play a role in protein synthesis (Franco *et al.*, 2024; Sloup *et al.*, 2017).

The increase in propionate in the ZPOS supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold *et al.*, 2015). These findings are consistent with studies showing that ZPOS supplementation leads to an increase in protozoan populations (Table 4). Zn supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal *et al.*, 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio is advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85%, and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids, including oleic acid (18:1), undergo a biohydrogenation process, being converted into saturated fatty acids, stearic acid (18:0), in the rumen by bacteria (Buccioni *et al.*, 2012). Harvatine *et al.* (2009), who tracked the biohydrogenation process of oleic acid, found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomerase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZPOS (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.* (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZPOS treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zn's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZPOS supplementation holds the potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Supplementation of 5% partial ZPOS (3.75% ZPOS + 1.25% PO) is better than 5% ZPOS. The beneficial effects of this supplementation are increased fiber digestibility, VFA, LCFA, and USFA, as well as decreased methane production in the rumen. Further investigation is required to test the ZPOS on dairy cows *in vivo*.

CONFLICT OF INTEREST

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

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Supplementation of Zinc Palm Oil Soap Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid

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ABSTRACT

This study aimed to evaluate the effects of energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on digestibility and unsaturated fatty acid profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5 replications. The treatments were: T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. The inoculum source was rumen liquid from three fistulated female dairy goats and was homogenized. The goats were fed ration consisting of corn straw, soybean hulls, and concentrate containing total digestible nutrients (TDN) 63%, crude protein (CP) 14%, and neutral detergent fiber (NDF) 35%. Results showed that both 5% partial ZPOS and 5% ZPOS supplementation (T2 and T3) resulted in the increase of total volatile fatty acids (VFA), acetate, propionate, butyrate, unsaturated fatty acids (USFA) and a decrease in the ratio of acetate/propionate (A/P) compared to the control and supplementation of 5% PO ($p<0.05$). Supplementation of 5% partial ZPOS (T2) is better than 5% ZPOS because increased the digestibility of ether extract (EE), crude fiber (CF), NDF, and acids detergent fiber (ADF) ($p<0.05$) and decreased of methane compared to the control ($p<0.05$). In conclusion, adding 5% partial ZPOS (3.5% ZPOS and 1.5% PO) increases fiber digestibility, VFA, LCFA, and USFA concentration, and decreases methane production in the rumen liquid.

Keywords: fermentability; rumen; zinc palm oil soap; unsaturated fatty acids

INTRODUCTION

Early lactation dairy cows experience negative energy balance due to the decreased dry matter intake (DMI). Lack or energy imbalance during that period may reduce production and stimulate metabolic disorders (Khotijah *et al.*, 2017). This opinion was supported by Tribout *et al.* (2023), who stated that energy deficiency has detrimental effects, including low production and weight loss in livestock. To address this challenge, especially during early lactation, providing additional energy sources through supplements is crucial.

Energy requirements of dairy livestock in tropical regions differ from those in temperate areas due to varying environmental conditions and feed resources. Assessment of the energy balance of tropical and temperate crossbred dairy cows revealed significant differences in serum metabolic profiles, indicating variations in energy utilization and metabolism between the two regions (Ranaweera *et al.*, 2020). Meta-analysis research by Oliveira (2015) found that tropical dairy cows (*Bos taurus* x *Bos indicus*) have lower MEm requirements and net energy efficiency for milk production is also lower than temperate dairy cattle (*Bos taurus*). Therefore, understanding these differences is very important to optimize feeding strategies, especially energy source supplementation in dairy livestock.

Palm oil is one of the most promising vegetable oils for energy sources, with a gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is $574 per metric ton (mt), which is cheaper compared to soybean oil at $684/mt, coconut oil at $830/mt, and peanut oil at $1,584/mt (World Bank, 2020). Palm oil contains saturated fatty acids in the form of palmitic acid 44% and unsaturated fatty acids, specifically oleic acid 39.2% and linoleic acid 10.1% (Mancini *et al.*, 2015). High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation. Unsaturated fatty acids in palm oil (oleic acids and linoleic acids) contribute to energy efficiency by reducing protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020), reducing methane (CH4) production and the acetate/propionate (A/P) ratio by increasing propionate production (Gao *et al.*, 2016).

However, oil supplementation also has negative effects. Fat particles tend to coat feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic microbes, thus reducing fiber digestibility (Sears *et al.*, 2024). Rumen microbes defend against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty acids into saturated fatty acids in the rumen (Shingfield & Wallace, 2014).

Palm oil contains 49.3% unsaturated fatty acids, and when used in animal feed, it must be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial fermentation. It is essential to safeguard unsaturated fatty acids to preserve their biological roles, such as being structural components of bio membranes that uphold membrane integrity. Polyunsaturated fatty acids in biomembranes ensure membrane fluidity, which supports the activity of membrane-bound enzymes, facilitates intracellular metabolism and enhances nutrient utilization efficiency in livestock (Pereira *et al.*, 2022).

One effective method is saponification, which involves binding the free carboxyl groups of fatty acids with minerals to form fatty acid salts or soaps. Calcium (Ca) is commonly used for this purpose (Satir *et al.*, 2023; Vargas-bello-p *et al.*, 2020). In addition to Ca, NaOH can also be used to protect the oil (Wulandari *et al.*, 2020). However, the use of zinc (Zn) minerals for this purpose has not been widely explored.

Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300 enzymes and acts as a structural component in gene expression and signal transduction (Franco *et al.*, 2024). Zn is also a major component of metalloenzymes, lactate dehydrogenase, carboxypeptidase, and DNA and RNA polymerase, which play a role in protein synthesis (Sloup *et al.*, 2017). Studies *in vitro* observed that supplementing Zn in ruminal fluid increased cellulose digestibility (Martínez & Church, 1970) and concentrations of total volatile fatty acids (VFA) and acetate (Chen *et al.*, 2019). Research by Wang *et al.* (2021) found that supplementation of 20-30 mg/kg DM Zn sulfate led to increasing nutrient digestibility and ruminal fermentation.

In livestock, Zn is involved in multiple biochemical functions such as bone metabolism, the metabolism of carbohydrates, fats, and proteins, sexual development and spermatogenesis, immune function, and appetite regulation via its effects on the central nervous system (Duffy *et al.*, 2023). The most important of Zn are cellular respiration, cellular use of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, sequestration of free radicals, and protection against lipid peroxidation (Sloup *et al.*, 2017). The use of Zn as an oil protector has a double benefit, namely protecting unsaturated fatty acids from the biohydrogenation process of rumen microbes while providing Zn needed for rumen microbes and livestock, whose needs increase in the early stages of lactation.

In vitro Zn soap supplementation research was conducted by Faizah *et al.* (2019), which compared the supplementation of 10% Zn soap from palm oil with 10% corn. Supplementation with 10% palm oil Zn soap resulted in no different digestibility of dry matter, organic matter, and crude fiber, but the total production of VFA, acetate, and A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support the synthesis of milk fatty acids in dairy cows, especially short-chain fatty acids. However, no research has explored the contribution of long-chain fatty acids, MUFA, and PUFA due to Zn soap supplementation as a precursor for the synthesis of long fatty acids and unsaturated fatty acids in milk. This study's results provided information regarding the concentration of long-chain fatty acids, MUFA and PUFA in the rumen due to Zn soap supplementation, which is expected to be used as a consideration in providing unsaturated fatty acids to increase the production and health of dairy livestock.

This study aimed to evaluate the effects of energy and organic Zn supplements, specifically Zn palm oil soap (ZPOS), on fermentability, digestibility, and unsaturated fatty acid profiles *in vitro*. The benefit of this research is identifying the most efficient form of palm oil supplementation to increase energy supply and improve the profile of unsaturated fatty acids in the rumen without disrupting feed fermentability *in vitro*. Discover the most effective use of palm oil in providing an alternative solution to energy and Zn deficiencies in dairy cattle.

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

The preparation of Zn soap was carried out according to Cabatit (1979). The palm oil used to make Zn soap was a commercial palm oil that was generally sold on the market. The zinc soap of palm oil was made based on saponification number. Palm oil is measured for its saponification number and the KOH added is proportional to the saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an amount equal to the KOH required to soak the oil, which is determined by the outcomes of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated in a water bath at 90 ^{o}C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, the $ZnCl_2$ solution was added and mixed continuously until a paste formed. The final step is to remove the remaining KOH by adding water and washing using a centrifuge. This process produces cream soap called zinc palm oil soap (ZPOS). The nutrient content of ZPOS is 24.36% ether extract, 4.2% crude fiber, 4.94% Zn, and gross energy 5257 kcal/kg.

The basal feed consists of forage and concentrates containing CP 14% and TDN 63%, formulated for feeding lactating dairy cows with a body weight 400 kg, milk yield 15 kg, and fat content 3.5% (National Research Council, 1988). The feed was composed of corn straw, soybean hulls, rice bran, pollard, cassava waste meal, coconut meal, soybean meal, and molasses. The composition of ingredients and nutrient content of the basal feed are shown in Table 1.

Table 1. Ingredients and chemical composition of experimental diet (dry matter basis)

Items	Composition
Ingredient:	
Corn straw	33.38
Soybean hull	12.25
Rice bran	7.42
Pollard	9.32
Cassava waste meal	9.93
Coconut meal	17.01
Soybean meal	6.98
Molasses	3.00
Vitamin and mineral mixture	0.80
Nutrient composition:	
Dry matter (DM), %	84.68
Ash, %DM	8.63
Crude protein, %DM	14.32
Ether extract, %DM	4.43
Crude fiber, %DM	20.02
Calcium, %DM	0.33
Phosphor, %DM	0.28
Zinc, mg/kg DM	16.93
Neutral detergent fiber, %DM	35.51
Acid detergent fiber, %DM	14.77
Total digestible nutrient (TDN) [1], %	63.15

Note: [1]Total digestible nutrients (TDN) were calculated using TDN (%DM). TDN = -17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK), according to Wardeh (1981).

In Vitro Experiment

The experiment was designed using a completely randomized design with four treatments and five replications. The treatments tested were T0= basal diet without supplementation, T1= basal diet + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and T3= basal diet + 5% ZPOS. Rumen liquid as a source of inoculum was taken from three female goats with fistulas that belong to the Faculty of Animal and Agricultural Sciences Diponegoro University, and were homogenized. The goats were given a dietary trial following the ration for *in vitro* substrate for one week before rumen liquid was collected. Rumen liquid was collected before morning feeding from a fistulated rumen. The rumen liquid was filtered using cheesecloth and placed into a 39 °C flask under anaerobic conditions.

In vitro experiments were carried out according to the method of Tilley & Terry (1963). In the fermenter tube, 0.55 g of feed samples from each treatment were added, followed by 40 mL of McDaugall's solution and 10 mL of rumen liquid. The fermenter cylinder was flowed by CO_2 gas and closed to anaerobic conditions. The fermenter tube was incubated at a 39 °C water temperature.

Nutrient Digestibility

The digestibility of nutrients, including dry matter (DMD), organic matter (OMD), crude protein (CPD), ether extract (EED), and crude fiber (CFD) were measured through two stages of incubation, namely fermentative and enzymatic. In the first stage, the fermenter tube was incubated at 39 °C water temperature for 2x24 hours, and the process was stopped by immersing the fermenter tube in ice water for 20 minutes. Then, the tube was centrifuged for 15 minutes at a speed of 3,000 rpm, and the supernatant was discarded. In the second stage, the HCl pepsin solution was added 50 mL into the tube and incubated again for 2x24 hours in a 39 °C water temperature under aerobic conditions. The sample was filtered with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed for DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient digestibility values. Fermentation is also carried out without feed samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

Nutrient digestibility (%)=
{[Nutrient sample, g - (Nutrient residue, g - Nutrient blank, g)] / Nutrient sample, g} x 100

pH Value, VFA, NH_3, and Methane Production

The process of measuring pH, VFA, and NH_3 followed the same procedure as that for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to measure the rumen pH, NH_3, and total and partial VFA concentrations. A digital pH meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample was tested three times. NH3 levels were determined using a spectrophotometer, employing a spectrophotometric method based on the catalyzed endophenol reaction to form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968) reported that gas chromatography was used to quantify the generation of partial VFAs (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H_2SO_4 was combined with a 10 mL incubation sample. A milliliter of the sample combination was mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10 minutes at 7 °C at 12,000 rpm. The samples were ready for gas chromatography identification.

The methane gas concentration and energy conversion efficiency were determined by calculating the VFA stoichiometry, which was the estimation using the formula of Orskov & Ryle (1990). The formula used for methane concentration was Methane (mM)= 0.5a - 0.25p + 0.5b, where a, p, and b are moles of acetic, propionic, and butyric acids, respectively. The efficiency of conversion of hexose energy to VFA was calculated based on the stoichiometry of the carbohydrate fermentation reaction from hexose to VFA (acetate, propionate, and butyrate). The calculation formula used was:

E (%)= [(0.622 pA + 1.091 pP + 1.558 pB) / (pA + pP + 2pB)] x 100

Where E is energy, and pA, pP, and pB are the proportions of acetic, propionic, and butyric acids.

Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

To calculate protozoa, microbial protein and fatty acid concentrations in the rumen liquid, the fermentation process was carried out for 24 hours. The populations of protozoa were calculated following the procedures of Ogimoto & Imai (1981). The solution used was trypan blue formalin saline (TBFS), made from a mixture of 100 mL 35% formaldehyde, 2 g trypan blue, 8 g NaCl, and 900 mL distilled water. The protozoa population was carried out on each treatment rumen fluid mixed with Trypan Blue Formalin Saline (TBFS) solution with a ratio of 5:1. The counting chamber with a thickness of 0.1 mm was dripped with 2 drops of the mixture. The area of the smallest box was 0.0625 mm, totaling 16 boxes with a box area of 5 boxes read. The number of protozoa was calculated by using a microscope at magnification 100 times. The formula used for total population of protozoa was: [1 / (0.1 x 0.0625 x 16 x 5)] x 1000 x DF x C, where C is protozoa population in the counting chamber and DF is diluent factor the specimen.

Measurement of rumen liquid protein microbes using the method of Makkar *et al.* (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of Cocks & Rede (1966), the fatty acid composition was determined by converting oil to fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The peaks of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. The relative percentage of each fatty acid was calculated based on its peak area relative to the total peak area of all fatty acids in the sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and long-chain fatty acids (LCFA), which are greater than C12 (Wang *et al.*, 2020).

Statistical Analysis

The data were analyzed using a completely randomized design in SPSS 16. Duncan's Multiple Range Test with significance was set at $p<0.05$ to compare treatments when a significant effect was observed.

RESULTS

Feed Fermentability in The Rumen

Detailed rumen feed fermentation parameters can be seen in Table 2. The pH levels in all treatments were not significantly different and were in the normal range of 6.75-6.82. Supplementation of 5% PO (T1) had an effect on reducing the number of protozoa and microbial proteins compared to the control and other treatments ($p<0.05$). On the other hand, 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation caused a decrease in NH3 concentration and an increase in VFA production compared to the control and 5% PO supplementation ($p<0.05$).

Nutrient Digestibility

Table 3 presents the nutrient digestibility due to 5% PO, 5% partial ZPOS, and 5% ZPOS supplementation. All treatments did not show significant differences in dry matter (DMD), organic matter (OMD), and crude protein (CPD) digestibility, but there were significant differences ($p<0.05$) in ether extract digestibility (EED), crude fiber digestibility (CFD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber digestibility (ADFD). Both supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) increased EED, but fiber digestibility (CFD, NDFD, and ADFD) was only increased by 5% partial ZPOS supplementation (T2), while 5% PO and 5% ZPOS supplementation did not differ from the control.

Relative Proportion of VFA

Table 4 shows the partial VFA and methane production in rumen liquid. Supplementation of 5% PO, 5% partial ZPOS, and 5% ZPOS) significantly influenced ($p<0.05$) acetate, propionate, butyrate, the A/P ratio, and methane but did not impact the efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid. Both 5% partial ZPOS (T2) and 5% ZPOS supplementation increased acetate, propionate, and butyrate compared to 5% PO supplementation (T1) and the control (T0). Conversely, A/P ratio and methane production decreased with 5% PO and 5% partial ZPOS supplementation. The efficiency of hexose energy conversion into VFA between treatments was not significantly different.

Table 2. *In vitro* feed fermentability in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
pH	6.76	6.75	6.73	6.82	0.018	0.224
Protozoa (10^3 cell/mL)	98.14^{a}	32.57^{c}	82.83^{ab}	69.31^{b}	0.044	0.000
Microbial protein (mg/mL)	13.01^{a}	9.37^{b}	13.37^{a}	11.41^{ab}	0.469	0.002
NH^3 (mM)	14.06^{a}	14.45^{a}	9.64^{b}	10.36^{b}	0.543	0.000
VFA (mM)	87.52^{b}	101.78^{b}	164.38^{a}	157.89^{a}	8.134	0.000

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 3. *In vitro* nutrient digestibility in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Dry matter (%)	64.06	63.82	67.20	69.24	0.85	0.054
Organic matter (%)	69.16	65.89	69.65	69.98	0.632	0.068
Crude protein (%)	68.69	67.99	66.39	65.11	0.569	0.100
Ether extract (%)	88.45^{b}	94.34^{a}	93.22^{a}	92.35^{a}	0.587	0.000
Crude fiber (%)	54.46^{b}	51.46^{b}	73.63^{a}	66.11^{ab}	3.057	0.020
Neutral detergent fiber (%)	56.20^{b}	46.33^{b}	70.03^{a}	64.62^{ab}	2.830	0.006
Acid detergent fiber (%)	45.12^{b}	44.56^{b}	65.81^{a}	53.76^{ab}	2.704	0.006

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 4. *In vitro* VFA and methane production in rumen liquid with supplementation of zinc palm oil soap (ZPOS)

Variables	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Acetate (mM)	41.17^{b}	51.10^{a}	59.31^{a}	65.80^{a}	2.579	0.000
Propionate (mM)	15.91^{c}	25.41^{b}	28.73^{a}	29.89^{a}	1.348	0.000
Butyrate (mM)	8.73^{b}	10.28^{b}	12.55^{a}	14.20^{a}	0.546	0.000
A/P	2.59^{a}	2.01^{b}	2.06^{b}	2.20^{b}	0.061	0.000
Methan (mM)	31.87^{a}	28.04^{b}	28.58^{b}	29.57^{ab}	0.489	0.014
Efficiency of energy conversion (%)	73.05	73.19	74.74	74.47	0.357	0.226

Note: ^{a,b} Means in the same row with different superscripts differ significantly ($p<0.05$). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Fatty Acids in Rumen Liquid

Table 5 outlines the fatty acid composition in rumen liquid. Supplementation of 5% PO (T1), 5% partial ZPOS (T2), and 5% ZPOS (T3) supplementation decreased significantly ($p<0.05$) short-chain fatty acids (SCFA). The long chain fatty acids (LCFA), especially palmitate (C16:0), increased, but stearate (C18:0) decreased by all treatments ($p<0.05$). Unsaturated fatty acids (USFA), particularly trans 11 C18:1, cis 9 C18:1, C18:2, C20:5 (EPA), and C22:6 (DHA), increased by 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation.

These fatty acids are classified into various categories, including short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs), saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the diets supplemented with 5% PO, 5% partial ZPOS, and 5% ZPOS decreased SCFAs compared to the control diet ($p<0.05$) but increased LCFA. SFAs showed an increase in control and 5% PO supplementation, whereas 5% partial ZPOS and 5% ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 5% partial 5% ZPOS and 5% ZPOS supplementation increased MUFAs and PUFAs than the other treatments.

DISCUSSION

Feed Fermentability

The pH is one of the crucial factors in assessing rumen health. The findings indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen fermentation process. Palm oil contains high palmitic acid, which actually supports the growth of rumen bacteria (Sears *et al*., 2024). Apart from being high in palmitate content, palm oil is also high in unsaturated fatty acids (oleic acids), palm oil protection causes unsaturated fatty acids to escape rumen microbial biohydrogenation so that negative effects on the rumen environment can be eliminated. Similar results have been reported in other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.* (2022), and Roy *et al.* (2017).

The reduction in protozoa is attributed to the high content of saturated fatty acids in palm oil, particularly palmitic acids (C16:0). According to Rahman *et al.* (2022), palm oil contains about 44% palmitic acid. It has been reported that supplementation of LCFA in the form of palmitate (C16:0) and stearate (C18:0) resulted in a decrease in the protozoa population, although the effect of the decrease was not as strong as MCFA, especially capric acids (C10:0) and lauric acid (C12:0). (Matsumoto *et al*., 1991). The decrease is caused by the inability of protozoa to digest fat because they do not have lipolytic enzymes; as a result, the addition of oil can interfere with the metabolic activity of protozoa and subsequently cause the number of protozoa to decrease (Hartanto *et al*., 2017).

Ibrahim *et al.* (2021) discovered that supplementing with palm oil altered the rumen microbial population in ruminants, potentially affecting their overall protein synthesis capability. Sears *et al.* (2024) reported that oleic acid significantly reduces Fibrobacteraceae. The study further indicated that high levels of unsaturated fatty acids could adversely affect cellulolytic bacteria, which are crucial for fiber digestion, resulting in decreased

Table 5. *In vitro* proportion of fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
Short-chain fatty acids (SCFA)						
C4	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.435	0.000
Medium chain fatty acids (SCFA)						
C6	<0.1	<0.1	<0.1	<0.1	-	-
C8, caprylic	<0.1	<0.1	<0.1	<0.1	-	-
C10, capric	<0.1	<0.1	<0.1	<0.1	-	-
C12, lauric	1.38	1.15	1.22	1.20	0.044	0.299
Long-chain fatty acids (LCFA)						
C13	0.3[a]	0.17[a]	<0.1[b]	<0.1[b]	0.030	0.000
C14, myristic	2.19	1.85	2.30	1.68	0.063	0.054
C14:1	1.00	0.50	0.57	0.44	0.072	0.053
C15,	0.51	0.75	0.36	0.33	0.056	0.055
C15:1	0.34	0.23	0.16	0.21	0.025	0.051
C16, palmitic	22.03[b]	29.34[a]	29.23[a]	31.14[a]	0.854	0.000
C16:1, n7	1.12[b]	1.04[b]	1.36[ab]	1.5[a]	0.058	0.005
C17	0.39	0.23	0.23	0.28	0.025	0.052
C17:1	<0.1	<0.1	<0.1	<0.1	-	-
C18, stearic	40.13[a]	30.74[b]	23.17[b]	21.5[b]	2.198	0.002
trans 11 C18:1	12.55[c]	17.84[b]	26.16[a]	28.05[a]	1.476	0.834
cis 9 C18:1, oleic	1.09[b]	1.43[b]	1.99[a]	1.83[a]	0.099	0.000
C18:2	1.04[c]	1.31[bc]	1.6[ab]	1.86[a]	0.084	0.000
C18:3	<0.1	<0.1	<0.1	<0.1	-	-
C18:3, omega 6	0.22[b]	0.26[b]	0.45[a]	0.53[a]	0.032	0.000
C18:3, gamma linolenic	0.79[a]	<0.1[c]	0.38[b]	0.41[b]	0.064	0.000
C20	1.31	0.83	0.64	0.67	0.050	0.058
cis 11 C20:1	0.16[b]	0.59[a]	0.31[b]	0.23[b]	0.041	0.000
cis 11-14 C20:2	<0.1	<0.1	<0.1	<0.1	-	-
C20:3	<0.1	<0.1	<0.1	<0.1	-	-
C20:4, arachidonic	0.48[a]	<0.1[b]	<0.1[b]	<0.1[b]	0.048	0.000
C20:5, EPA	0.07[c]	0.55[b]	0.59[b]	0.76[ab]	0.069	0.000
C21	1.38	0.31	0.20	0.26	0.017	0.000
C22, behenic	0.94[a]	0.43[ab]	0.23[b]	0.25[b]	0.042	0.050
C22:1	<0.1	<0.1	<0.1	<0.1	-	-
C22:6, DHA	3.19[b]	4.81[a]	4.57[a]	4.42[a]	0.159	0.000
C24	<0.1	<0.1	<0.1	<0.1	-	-
C24:1, nervonic omega 9	<0.1	<0.1	<0.1	<0.1	-	-

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

Table 6. *In vitro* proportion of short-chain, long-chain, saturated, and unsaturated fatty acids in rumen liquid with supplementation of zinc palm oil soap (ZPOS) (% fat)

Fatty acids	Treatments				SEM	p-Value
	T0	T1	T2	T3		
SCFA	7.34[a]	5.34[b]	5.27[b]	2.36[c]	0.453	0.000
MCFA	1.38[a]	1.15[b]	1.22[b]	1.20[b]	0.028	0.009
LCFA	91.23[c]	93.41[b]	93.50[b]	96.35[a]	0.443	0.000
SFA	77.9[a]	71.14[b]	61.85[c]	59.67[c]	1.766	0.000
USFA	22.05[c]	28.76[b]	38.14[a]	40.24[a]	1.722	0.000
MUFA	16.26[c]	21.63[b]	30.55[a]	32.26[a]	1.531	0.000
PUFA	5.79[c]	7.13[b]	7.59[a]	7.98[a]	0.201	0.000

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05). SCFA= short-chain fatty acids, MCFA= medium-chain fatty acids, LCFA= long-chain fatty acids, SFA= saturated fatty acids, USFA= unsaturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids. T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal diet + partial ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.

protein synthesis. The use of ZPOS protection, both 5% partial ZPOS (T2) and 5% ZPOS (T3) can mitigate the harmful effects of unsaturated fatty acids, enabling microbes to develop as effectively as in the control group.

Yanza *et al*. (2021) found that ruminants given protected medium chain fatty acids (MCFA) experienced a decrease in total VFA production compared to those given unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to the increased propionate production. Propionate is formed from the breakdown of glycerol during fat hydrolysis. The NH_3 concentration in the T2 treatment was the lowest among all treatments, while microbial protein production was the highest. This indicates that NH_3 is utilized for microbial protein synthesis (Getabalew & Negash, 2020).

Nutrient Digestibility

The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were unaffected by 5% PO supplementation, indicating that this level of supplementation is safe for rumen microbial growth. These results are consistent with previous research, which found that 6% supplementation with vegetable oils (olive oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable to the control, although linseed oil resulted in higher digestibility than sunflower oil (Vargas-bello-p *et al.*, 2020). The effect of oil supplementation on digestibility is highly dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The experimental feed in this study, characterized by high fiber content and low ADF (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may mitigate the potential negative impact of oil on fiber digestion by rumen microbes (Benchaar *et al.*, 2015). Both non-protected (T1) and protected (T2 and T3) palm oil increased EED, supporting the conclusion that palm oil can be used as an energy supplement without compromising rumen feed fermentability.

A notable finding from this research is that both partial and total Zn soap supplementation with palm oil (T2 and T3) significantly enhances CFD, NDFD, and ADFD. This effect is likely due to the fat and Zn content in palm oil. Palm oil contains high levels of palmitic acid (39.8%) and oleic acid (46.14%) (Koushki *et al.*, 2015). Research by Sears *et al.* (2024) indicates an increase in Prevotella and Fibrobacter populations in response to palmitic acid supplementation. This suggests that cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby increasing energy availability for metabolic processes. Furthermore, Zn is crucial for various metabolic functions in rumen microbes.

Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et al.*, 2016; Uniyal *et al.*, 2017). It is indispensable for preserving the structural and functional integrity of over 2000 transcription factors and 300 enzymes. It can be contended that virtually all metabolic pathways are reliant, to varying degrees, on at least one Zn-dependent protein (Ranasinghe *et al.*, 2015). Recent research by Fellner *et al.* (2021) elucidated that Zn supplementation precipitated increased acetate production and a heightened acetate/propionate ratio. This delineates an augmented fiber digestion process facilitated by cellulolytic bacteria, consequent to heightened microbial protein synthesis. This conclusion aligns with findings indicating elevated levels of microbial protein compared to PO supplementation (T1), specifically 13.37 (T2) and 11.41 (T3) versus 9.37 mg/mL in the T1 treatment (Table 3).

Relative Proportion of VFA

The relative proportion of acetate observed is slightly lower than that reported by Anzhany *et al.* (2024), which ranged from 69% to 72%, while the proportions of propionate and butyrate are comparatively higher. According to Amanullah *et al.* (2022), palm oil supplementation modifies the rumen environment, thereby altering the ratio of cellulolytic to amylolytic microbes. Both 5% partial ZPOS and 5% ZPOS supplementation resulted in significantly higher acetate production compared to non-protected palm oil ($p<0.05$). Acetate is the yield of digestion of fibrous carbohydrates by cellulolytic bacteria, which means that the increase in acetate is in line with the increase in the number of cellulolytic bacteria in the rumen. This is likely related to the decreasing protozoa population resulting from oil supplementation. Protozoa are predators of bacteria (Dayyani *et al*., 2013), so a decrease in the number of protozoa will increase the number of bacteria because it reduces competition for nutrients. These findings are consistent with the increased microbial protein levels observed with 5% partial ZPOS and 5% ZPOS supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential trace mineral cofactor for over 300 enzymes, including DNA and RNA polymerase, which play a role in protein synthesis (Franco *et al.*, 2024; Sloup *et al.*, 2017).

The increase in propionate in the ZPOS supplementation treatment resulted from the hydrolysis of fat into fatty acids and glycerol, with glycerol subsequently converted into propionate. Protozoa in the rumen contribute significantly to the flow of unsaturated fatty acids leaving the rumen, potentially protecting chloroplast unsaturated fatty acids from rumen bio-hydrogenation and increasing the duodenal flows of mono and polyunsaturated fatty acids (Newbold *et al.*, 2015). These findings are consistent with studies showing that ZPOS supplementation leads to an increase in protozoan populations (Table 4). Zn supplementation has been associated with shifts in rumen volatile fatty acid profiles, notably an increase in propionate and a reduction in the acetate-to-propionate ratio (Hilal *et al.*, 2016). Moreover, the lack of an increase in methane production and a lower A/P ratio is advantageous, as higher propionate levels provide a carbon framework and synthetic energy for livestock.

Fatty Acids in Rumen Liquid

Previous research showed that oil supplementation caused a decrease in the proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA), consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes to the range of rumen saturated fatty acid levels, where PO supplementation treatment (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2 61.85%, and T3 59.67% (Table 6).

Palm oil contains MUFA in the form of oleic acid (18:0) in large quantities, namely 39.2%. In the rumen, feed fat will undergo lipolysis to produce fatty acids and glycerol. Unsaturated fatty acids, including oleic acid (18:1), undergo a biohydrogenation process, being converted into saturated fatty acids, stearic acid (18:0), in the rumen by bacteria (Buccioni *et al.,* 2012). Harvatine *et al.* (2009), who tracked the biohydrogenation process of oleic acid, found that oleate in the rumen can change into trans and cis forms because rumen microbes produce cis/trans isomerase enzymes, and there is an isomerase that changes the 18:2 and 18:3 cis bonds (linoleate and linolenate) to trans 18:1. The dominant biohydrogenation process that occurs in the rumen is bacteria hydrogenate polyunsaturated fatty acids (PUFA) into trans-11 18:1 and cis-9, trans-11 fatty acids, then trans 18:1 fatty acids into stearic acid (18:0).

Consistent with our study's findings, supplementation with ZPOS (T2 and T3) resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.* (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA and PUFA. These findings are consistent with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete inhibition of the biohydrogenation process in the ZPOS treatment. The protection of polyunsaturated fatty acids through saponification involves bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA, underscores Zn's role in the elongation and desaturation process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

Trans 18:1 acts as a precursor for saturated fatty acid formation in the rumen, particularly stearate, while on a tissue level, it serves as a precursor for conjugated linoleic acids (CLA), known for their antioxidant and anticarcinogenic properties in livestock products. Hence, the augmentation of trans 18:1 fatty acids attributed to ZPOS supplementation holds the potential for enhancing the quality of meat and milk fat.

CONCLUSION

Based on the results of this research, it can be concluded that zinc soap from palm oil (ZPOS) can be developed as a source of energy and organic Zn. Supplementation of 5% partial ZPOS (3.75% ZPOS + 1.25% PO) is better than 5% ZPOS. The beneficial effects of this supplementation are increased fiber digestibility, VFA, LCFA, and USFA, as well as decreased methane production in the rumen. Further investigation is required to test the ZPOS on dairy cows *in vivo*.

CONFLICT OF INTEREST

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

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