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

3
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25 **ABSTRACT**

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27 The energy needs of dairy cows during the lactation period increase significantly.,
28 and the essential mineral zinc is crucial for their metabolic, production, and reproductive
29 processes, despite common deficiencies. This study aimed to evaluate the effects of
30 energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on
31 fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The four
32 treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil,
33 PO), T2 (basal diet + 5% partially ZPOS: 75% ZPOS + 25% PO), and T3 (basal diet +
34 5% ZPOS). The inoculum source was rumen liquid from fistulated female dairy goats.
35 The feed consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%,
36 NDF 35%). Our findings showed that both partial and full ZPOS supplementation (T2
37 and T3) resulted in higher VFA and microbial protein production, and lower NH₃ levels
38 compared to the control (P<0.05). While DM, OM, and CP digestibility were unaffected
39 by the treatments, digestibility of EE, CF, NDF, and ADF was significantly higher
40 (P<0.05). ZPOS supplementation increased acetate and propionate levels but did not
41 affect butyrate, reducing the A/P ratio and methane production. The PO treatment was
42 dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels of
43 trans 11 C18:1 and EPA compared to the control. In conclusion, adding protected palm
44 oil in the form of zinc soap enhances fermentability, digestibility, and MUFA content in
45 rumen liquid.

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

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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 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

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

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

84 Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300
85 enzymes, and is involved in DNA synthesis, growth, CO₂ transport, essential fatty acid
86 metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro
87 observed that supplementing zinc in ruminal fluid increased cellulose digestibility
88 (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and
89 acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation
90 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

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

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

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

109

110 **MATERIALS AND METHODS**

111 **Zinc Soap and Feed Preparation**

112 The preparation of zinc soap was carried out according to (Cabatit, 1979). The
113 palm oil used to make Zinc soap is commercial palm oil which generally sold on the
114 market. The Zinc soap of palm oil was made based on saponification number. Palm oil is
115 measured for its saponification number and the KOH added is proportional to the
116 saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an
117 amount equal to the KOH required to soak the oil, which is determined by the outcomes
118 of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated
119 in a water bath at 90°C. KOH that has been dissolved in ethanol is added to hot oil and
120 stirred until the oil is completely hydrolyzed. Next, add the $ZnCl_2$ solution and mix
121 continuously until a paste forms. The final step is to remove the remaining KOH by

122 adding water and washing using a centrifuge. This process produces cream soap called
123 Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract,
124 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

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

130

131 **In Vitro Experiment**

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

142 In vitro experiments were carried out according to the method of Tilley and Terry
143 (1963). Into the fermenter tube, put 0.55 grams of feed samples from each treatment then
144 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 :

$$161 \text{ Nutrient Digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100$$

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

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

$$175 \quad \text{Partial VFA (mM)} = \frac{\text{sample area} \times \text{standard concentration} \times 1000}{(\text{standard area} \times \text{MW})}$$

176 The methane gas concentration and Energy conversion efficiency was determined
177 by calculating the VFA stockiometry, which was the estimation using the formula Orskov
178 and Ryle (1990). The formula used for methane concentration were : Methane (mM) =
179 0.5 (% Asetate) - 0.25 (% Propionate) + 0.5 (% Butirate). Energy conversion efficiency
180 was calculated based on the stockyometry of the carbohydrate fermentation reaction from
181 hexose to VFA (acetate, propionate and butyrate). The calculation formula used was:

$$182 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB pA} + \text{pP} + 2 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

183

184 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

185 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
186 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
187 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
188 was methil formalin saline which was made from a mixture of 100 ml 35% formaldehyde,
189 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated
190 by using a microscope at magnification 100 times. Measurement of rumen liquid protein
191 microbes using the method of Makkar et al. (1982) on the principle of gradual

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

202

203 **Statistical Analysis**

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

207

207 **RESULTS**

208

208 **Feed Fermentability**

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

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

219

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Feed Digestibility

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

234

235

Relative Proportion of VFA

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

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

245

246 **Fatty Acids in Rumen Liquid**

247 Table 5 outlines the fatty acid composition in rumen liquid. The control diet
248 primarily contained short-chain fatty acids (SA), with a notably higher concentration of
249 C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and
250 ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1)
251 relative to the control ($P<0.05$). However, the proportion of stearate was significantly
252 lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9
253 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), and EPA (20:5), showed a
254 significant increase in the 75% and 100% ZSPO treatments ($P<0.01$), while the increase
255 in DHA was not statistically significant.

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

265

DISCUSSION

266 **Feed Fermentability**

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

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

284 Yanza et al. (2021) found that ruminants given protected medium chain fatty acids
285 (MCFA) experienced a decrease in total VFA production compared to those given
286 unprotected oil. Similarly, T2 and T3 treatments resulted in high VFA levels due to
287 increased propionate production. Propionate is formed from the breakdown of glycerol
288 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 used 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

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

316 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
317 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et
318 al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and
319 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
320 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
321 one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al.
322 (2021) elucidated that Zn supplementation precipitated increased acetate production and
323 a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion
324 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
325 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
326 protein compared to the control, specifically 13.37 (T2) and 11.41 (T3) versus 8.01 in the
327 control feed (Table 3).

328

329 **Relative Proportion of VFA**

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

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

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

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354 **Fatty Acids in Rumen Liquid**

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

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

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

377 Consistent with our study's findings, Mosley et al. (2002) observed that
378 supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis
379 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil
380 supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis
381 involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected
382 fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA
383 and PUFA. These findings are consistent with the research by Satir et al. (2023), which
384 demonstrated that 3% protected palm oil supplementation can increase the content of

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

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

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CONCLUSION

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

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CONFLICT OF INTEREST

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

423

424

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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
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

628 ¹Total 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).

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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

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642 **Table 3.** Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm
 643 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}

644 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

645 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75%
 646 POZS+25%PO); T3= basal diet+5% ZPOS

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648 **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

649 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

650 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal diet+partially ZPOS (75%
 651 POZS+25%PO); T3= basal diet+5% ZPOS

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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

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^{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

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



Anis Muktiani <anis.muktiani@gmail.com>

[TASJ] Revision Required of Your Manuscript

2 messages

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id>
To: Anis Muktiani <anismuktiani@gmail.com>

Tue, Jun 25, 2024 at 8:21 AM

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

Tropical Animal Science
Journal <http://journal.ipb.ac.id/index.php/tasj>

3 attachments

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Anis Muktiani <anis.muktiani@gmail.com>
To: "Prof. Dr. Komang G Wiryawan" <jurnal@apps.ipb.ac.id>

Thu, Jun 27, 2024 at 8:45 AM

Thank you, I will do that.

[Quoted text hidden]



Tropical Animal Science Journal



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Website: <https://journal.ipb.ac.id/index.php/tasj>

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.	

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

3
 4 **ABSTRACT**

5 The energy needs of dairy cows during the lactation period increase significantly,
 6 and the essential mineral zinc is crucial for their metabolic, production, and reproductive
 7 processes, despite common deficiencies. This study aimed to evaluate the effects of
 8 energy and organic zinc supplements, specifically zinc palm oil soap (ZPOS), on
 9 fermentability, digestibility, and unsaturated fatty acid profiles in vitro. The four
 10 treatments were: T0 (basal diet without supplementation), T1 (basal diet + 5% palm oil,
 11 PO), T2 (basal diet + 5% partially ZPOS: 75% ZPOS + 25% PO), and T3 (basal diet +
 12 5% ZPOS). The inoculum source was rumen liquid from fistulated female dairy goats.
 13 The feed consisted of corn straw, soybean hulls, and concentrate (TDN 63%, CP 14%,
 14 NDF 35%). Our findings/Results showed that both partial and full ZPOS supplementation
 15 (T2 and T3) resulted in higher VFA and microbial protein production, and lower NH₃
 16 levels compared to the control (P<0.05). While DM, OM, and CP digestibility were
 17 unaffected by similar among the treatments, digestibility of EE, CF, NDF, and ADF was
 18 significantly higher (P<0.05). ZPOS supplementation increased acetate and propionate
 19 levels but did not affect butyrate, reducing the A/P ratio and methane production. The PO
 20 treatment was dominated by stearate (C18:0), whereas the ZPOS treatments showed
 21 higher levels of trans 11 C18:1 and EPA compared to the control. In conclusion, adding
 22 protected palm oil in the form of zinc soap enhances fermentability, digestibility, and
 23 MUFA content in rumen liquid.

24 **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?

Commented [A5]: Please describe.

Commented [A6]: Please describe.

25

26

INTRODUCTION

27 Adequate energy intake often becomes a challenge for high-producing dairy
28 livestock, especially in early lactation. Energy requirements can be two to three times
29 higher than the basic maintenance needs because it is used for tissue maintenance after
30 giving birth and milk production (Khotijah et al., 2017). Energy deficiency has
31 detrimental effects, including low production and weight loss in livestock (Tribout et al.,
32 2023).

33 Palm oil is one of the most promising vegetable oils to be used as an energy source
34 with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is
35 also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil
36 is \$574 per metric ton (mt), which is cheaper compared to soybean oil at \$684/mt, coconut
37 oil at \$830/mt, and peanut oil at \$1,584/mt (World Bank, 2020). Palm oil contains high
38 levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et
39 al., 2015). Unsaturated fatty acids contribute to energy efficiency by reducing protozoa
40 (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane (CH₄) production
41 and the acetate/propionate (A/P) ratio by increasing propionate production (Gao et al.,
42 2016).

43 However, oil supplementation also has negative effects. Fat particles tend to coat
44 feed particles, which hinders the adhesion of rumen microbes, especially fibrolytic
45 microbes, thus reducing fiber digestibility (Sears et al., 2024). Rumen microbes defend
46 against lipid toxicity by performing biohydrogenation, which converts unsaturated fatty
47 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.

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

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

60 Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300
61 enzymes, and is involved in DNA synthesis, growth, CO₂ transport, essential fatty acid
62 metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro
63 observed that supplementing zinc in ruminal fluid increased cellulose digestibility
64 (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and
65 acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation
66 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

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

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

77 The purpose of this study ~~was~~ to ~~determine~~ ~~investigate~~ the effects of
78 supplementing protected palm oil Zinc soap on feed fermentability, digestibility, and the
79 profile of unsaturated fatty acids in the rumen in vitro. The benefit of this research is to
80 identify the most efficient form of palm oil supplementation for enhancing energy supply
81 and improving the profile of unsaturated fatty acids in the rumen without disrupting feed
82 fermentability in vitro. The discovery of the most effective palm oil management method
83 provides a practical alternative solution to energy and zinc deficiencies in dairy cattle,
84 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.

86 MATERIALS AND METHODS

87 Zinc Soap and Feed Preparation

88 The preparation of zinc soap was carried out according to (Cabatit, 1979). The
89 palm oil used to make Zinc soap ~~is~~ ~~was a~~ commercial palm oil which generally sold on
90 the market. The Zinc soap of palm oil was made based on saponification number. Palm
91 oil is measured for its saponification number and the KOH added is proportional to the
92 saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an
93 amount equal to the KOH required to soak the oil, which is determined by the outcomes
94 of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated
95 in a water bath at 90°C. KOH that has been dissolved in ethanol is added to hot oil and

96 stirred until the oil is completely hydrolyzed. Next, add the ZnCl₂ solution and mix
 97 continuously until a paste forms. The final step is to remove the remaining KOH by
 98 adding water and washing using a centrifuge. This process produces cream soap called
 99 Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract,
 100 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

Commented [A10]: Is there any proof that the saponification process is really working to produce ZPOS?

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

106 **In Vitro Experiment**

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

117 In vitro experiments were carried out according to the method of Tilley and Terry
 118 (1963). Into the fermenter tube, put 0.55 grams of feed samples from each treatment then
 119 added 40 ml of McDaugall's solution and 10 ml of rumen liquid. The fermentor cylinder

Commented [A11]: Please paraphrase the sentence into passive voice.

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

122 **Nutrient Digestibility**

123 The digestibility of nutrients including dry matter (DMD), organic matter (OMD),
124 crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through
125 ~~two~~ stages of incubation, namely fermentative and enzymatic. First stage, the fermentor
126 tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped
127 by immersing the fermenter tube in ice water for 20 minutes. Then the tube was
128 centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded.
129 Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for
130 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered
131 with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed
132 by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient
133 digestibility values. Fermentation is also carried out without feed samples called blanks.
134 Nutrien digestibility samples are calculated by the formula :

$$135 \text{ Nutrient Digestibility (\%)} \\ 136 = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100$$

137

138 **pH value, VFA, NH₃ and Methane production**

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

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

$$150 \quad \text{Partial VFA (mM)} = \frac{\text{sample area} \times \text{standard concentration} \times 1000}{(\text{standard area} \times \text{MW})}$$

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

$$158 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB pA} + \text{pP} + 2 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

159

160 Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid

161 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
 162 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
 163 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
 164 was methyl formalin saline which was made from a mixture of 100 ml 35% formaldehyde,
 165 2 g trypan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated
 166 by using a microscope at magnification 100 times. Measurement of rumen liquid protein

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

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

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

178 **Statistical Analysis**

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

182 **RESULTS**

183 **Feed Fermentability**

184 The fermentability of feed is determined by various factors such as pH, total
185 protozoa count, microbial protein content, NH₃ concentration, and total VFA production
186 in rumen liquid. These parameters are detailed in Table 2. The pH levels across all
187 treatments fell within the normal range of 6.75-6.82. While the treatment did not
188 significantly affect pH ($p > 0.05$), notable differences ($p < 0.05$) were observed in the total
189 protozoa count, microbial protein content, total VFA, and NH₃ concentrations. Palm oil
190

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

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

195 **Feed Digestibility**

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

207 **Relative Proportion of VFA**

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

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

217 **Fatty Acids in Rumen Liquid**

218 Table 5 outlines the fatty acid composition in rumen liquid. The control diet
219 primarily contained short-chain fatty acids (SA), with a notably higher concentration of
220 C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and
221 ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1)
222 relative to the control ($P<0.05$). However, the proportion of stearate was significantly
223 lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9
224 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), and EPA (20:5), showed a
225 significant increase in the 75% and 100% ZSPO treatments ($P<0.01$), while the increase
226 in DHA was not statistically significant.

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

236

237 **DISCUSSION**

238 **Feed Fermentability**

Commented [A15]: The discussion part has been nicely elaborated.

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

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

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

263 **Feed Digestibility**

264 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
265 unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this
266 level of supplementation is safe for rumen microbial growth. These results are consistent
267 with previous research, which found that 6% supplementation with vegetable oils (olive
268 oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable
269 to the control, although linseed oil resulted in higher digestibility than sunflower oil
270 (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly
271 dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The
272 experimental feed in this study, characterized by high fiber content and low ADF
273 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
274 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
275 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
276 increased EED, supporting the conclusion that palm oil can be use d as an energy
277 supplement without compromising rumen feed fermentability.

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

287 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
288 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et
289 al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and
290 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
291 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
292 one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al.
293 (2021) elucidated that Zn supplementation precipitated increased acetate production and
294 a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion
295 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
296 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
297 protein compared to the control, specifically 13.37 (T2) and 11.41 (T3) versus 8.01 in the
298 control feed (Table 3).

299 **Relative Proportion of VFA**

300 The relative proportion of acetate observed is slightly lower than that reported by
301 Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of
302 propionate and butyrate are comparatively higher. According to Amanullah et al. (2022),
303 palm oil supplementation modifies the rumen environment, thereby altering the ratio of
304 cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted
305 in significantly higher acetate production compared to non-protected palm oil ($P < 0.05$).
306 These findings are consistent with the increased microbial protein levels observed with
307 ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential
308 trace mineral that functions as a cofactor for over 300 enzymes, and is involved in DNA
309 synthesis, growth, CO₂ transport, essential fatty acid metabolism, and protein and nucleic
310 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).

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

346 Consistent with our study's findings, Mosley et al. (2002) observed that
347 supplementation with ZSPO (T2 and T3) resulted in elevated levels of trans18:1 and cis
348 18:1, alongside reduced stearate (18:0) concentrations compared to non-protected oil
349 supplementation (T1). Amanullah et al. (2022) conducted a comparative analysis
350 involving corn oil, linseed oil, and Ca salt (protected C18:2n-6), revealing that protected
351 fatty acids (Ca Salt) led to diminished stearate levels but higher proportions of MUFA
352 and PUFA. These findings are consistent with the research by Satir et al. (2023), which
353 demonstrated that 3% protected palm oil supplementation can increase the content of
354 palmitic acid (SFA) and oleic acid (MUFA). Stearate is the final product of the
355 biohydrogenation of oleic, linoleic, and linolenic acids. This indicates a complete
356 inhibition of the biohydrogenation process in the ZSPO treatment. The protection of
357 polyunsaturated fatty acids through saponification involves bonding the carboxyl group

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

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

371

372 CONCLUSION

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

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CONFLICT OF INTEREST

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

387

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571

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
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

573 ¹Total 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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588 **Table 3.** Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm
589 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}

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

590 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
591 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
592 diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS
593

594 **Table 4.** Fermentability of feed due to palm oil supplementation in vitro
595

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

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

596 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
597 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
598 diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS
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616**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

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)617 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).618 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
619 diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS

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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

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

Commented [A22]: SCFA

Commented [A23]: LCFA

625 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).
626 SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,,
627 UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA=
628 polyunsaturated fatty acids.
629 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
630 diet+partially ZPOS (75% POZS+25%PO); T3= basal diet+5% ZPOS
631



Anis Muktiani <anis.muktiani@gmail.com>

[TASJ] New notification from Tropical Animal Science Journal

Anis Muktiani <anis.muktiani@gmail.com>




Tue, Jul 9, 2024 at 3:51 PM

To: "Prof. Dr. Ir. Komang G. Wiryawan" <kgwiryawan@yahoo.com>

I hereby send a revised manuscript entitled : "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid".
Thank you for reminding me.

[Quoted text hidden]

3 attachments

-  **OUTPUT TABEL 2 - SPSS (File 3).spv**
165K
-  **C-Form C1_TASJ-56357- Already did (File 2).doc**
1123K
-  **C-Manuscript TASJ-56357-revised July 9 (File 1).docx**
117K



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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	<p>Introduction Please state the novelty clearly. The introduction should contain problems, previous research, novelty statements, and objectives.</p>	<p>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.</p> <p>Previous research : Supplementation palm oil. Supplementation protected oil with Calcium. Supplementation 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.</p> <p>Novelty :</p> <ul style="list-style-type: none"> - 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. <p>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.</p>



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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
	<p>d) Line 21 : - Please describe.</p> <p>e) Line 23 : - Please describe.</p>	<p>d) eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)</p> <p>e) monounsaturated fatty acid (MUFA)</p>
	<p>- Introduction</p> <p>a) Line 27 - 32 : - Is it similar between tropical and temperate regions? Please address such difference.</p> <p>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.</p> <p>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.</p>	<p>a) Already did (line 34-43)</p> <p>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 <i>Prevotella</i> and <i>Fibrobacter</i> populations in response to palmitic acid supplementation.</p> <p>c) Already did (line 91-103). Novelty :</p> <ul style="list-style-type: none"> - 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.
	<p>- Material and Methods</p> <p>a) Line 98 – 99 : - Is there any proof that the saponification process is really working to produce ZPOS?</p>	<p>a) The saponification reaction that occurs between palm oil (triglyceride, TG), KOH and ZnCl₂ is as follows:</p>



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No	Comments	Author's response
	<p>b) Line 118 – 119 : - Please paraphrase the sentence into passive voice.</p> <p>c) Line 146 – 148 : - I don't think that GC measurement of rumen VFA is according to GLP, please check again.</p> <p>d) Line 176 – 177 : - Should be categorized into SCFA, MCFA, and LCFA.</p>	<p style="text-align: center;">O </p> $2 \text{ TG} + 6 \text{ KOH} \rightarrow 2 \text{ Gly} + 6 \text{ R-C-OK}$ <p style="text-align: center;">O </p> $6 \text{ R-C-OK} + 3 \text{ ZnCl}_2 \rightarrow 3 (\text{R-COO})_2\text{Zn} + 6 \text{ KCl}$ $2 \text{ TG} + 6 \text{ KOH} + 3 \text{ ZnCl}_2 \rightarrow 2 \text{ Gly} + 3 (\text{R-COO})_2\text{Zn} + \text{KCl}$ <p>A soap precipitate $(\text{R-COO})_2\text{Zn}$ is formed.</p> <ul style="list-style-type: none"> - 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 biohydrogenation. <p>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.</p> <p>c) Already did. (line 172-177) Gas chromatography, as reported by Cottyn and Boucque (1968) was used to quantify the generation of partial VFAs</p> <p>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).</p>
	<p>- Result</p> <p>a) Line 186 : - Data on protozoa should be converted first into log</p>	<p>a) Already did (file 3)</p>



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No	Comments	Author's response
	scale, and then analyzed by using ANOVA.	
	<p>- Table</p> <p>a) Table 2 : - Please provide SEM and P-values for all parameters.</p> <p>b) Table 3 : - Please provide SEM and P-values for all parameters.</p> <p>c) Table 4 : - Please provide SEM and P-values for all parameters.</p> <p>d) Table 5 :</p> <ul style="list-style-type: none"> - Please provide SEM and P-values for all parameters. - Please categorize the fatty acids into three: <ol style="list-style-type: none"> 1. Short chain FA (SCFA) 2. Medium chain FA (MCFA) 3. Long chain FA (LCFA) <p>e) Table 6 : - Please provide SEM and P-values for all parameters.</p> <ul style="list-style-type: none"> - SCFA - LCFA 	<p>a) Already did</p> <p>b) Already did</p> <p>c) Already did</p> <p>d) Already did</p> <p>- Already did :</p> <ol style="list-style-type: none"> 1. SCFA : C<6 2. MCFA : C6-C12 3. LCFA : C>12 <p>e) Already did</p>

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

3
4 **ABSTRACT**

5 This study aimed to evaluate the effects of energy and organic zinc supplement,
6 specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid
7 profiles in vitro. The study used a completely randomized design with 4 treatments and 5
8 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet
9 + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and
10 T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated
11 female dairy goats, and were homogenized. The goats were feed on consisted of corn
12 straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed
13 that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and
14 microbial protein production, and lower NH₃ levels compared to the control (P<0.05).
15 Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar
16 among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher
17 than another treatment (P<0.05). ZPOS supplementation resulted higher acetate and
18 propionate levels compared to the control and supplementation 5% palm oil (P<0.05) but
19 did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment
20 was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels
21 of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)
22 compared to the control. In conclusion, adding protected palm oil in the form of zinc soap
23 enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content
24 in rumen liquid.

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

26 INTRODUCTION

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

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

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

49 levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et
50 al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria.
51 Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby
52 increasing energy availability for metabolic processes. Recent findings by Sears et al.
53 (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to
54 palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by
55 reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane
56 (CH₄) production and the acetate/propionate (A/P) ratio by increasing propionate
57 production (Gao et al., 2016).

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

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

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

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

75 Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300
76 enzymes, and is involved in DNA synthesis, growth, CO₂ transport, essential fatty acid
77 metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro
78 observed that supplementing zinc in ruminal fluid increased cellulose digestibility
79 (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and
80 acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation
81 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

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

92 *In vitro* Zinc soap supplementation research was conducted by Faizah et al.,
93 (2019), that compared between supplementation of 10% zinc soap from palm oil and 10%
94 corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility
95 of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and
96 A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support

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

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

111 MATERIALS AND METHODS

112 Zinc Soap and Feed Preparation

113 The preparation of zinc soap was carried out according to (Cabatit, 1979). The
114 palm oil used to make Zinc soap was a commercial palm oil which generally sold on the
115 market. The Zinc soap of palm oil was made based on saponification number. Palm oil is
116 measured for its saponification number and the KOH added is proportional to the
117 saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an
118 amount equal to the KOH required to soak the oil, which is determined by the outcomes
119 of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated
120 in a water bath at 90°C. KOH that has been dissolved in ethanol is added to hot oil and

121 stirred until the oil is completely hydrolyzed. Next, add the ZnCl₂ solution and mix
122 continuously until a paste forms. The final step is to remove the remaining KOH by
123 adding water and washing using a centrifuge. This process produces cream soap called
124 Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract,
125 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

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

131 In Vitro Experiment

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

142 In vitro experiments were carried out according to the method of Tilley and Terry
143 (1963). **In the fermenter tube, 0.55 grams of feed samples from each treatment were**
144 **added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The**

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

147

148 **Nutrient 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
 151 two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor
 152 tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped
 153 by immersing the fermenter tube in ice water for 20 minutes. Then the tube was
 154 centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded.
 155 Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for
 156 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered
 157 with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed
 158 by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient
 159 digestibility values. Fermentation is also carried out without feed samples called blanks.
 160 Nutrien digestibility samples are calculated by the formula :

$$\begin{aligned}
 & \text{Nutrient Digestibility (\%)} \\
 & = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100
 \end{aligned}$$

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164 **pH value, VFA, NH₃ and Methane production**

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

169 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
 170 was tested three times. NH₃ levels were determined using a spectrophotometer,
 171 employing a spectrophotometric method based on the catalyzed endophenol reaction to
 172 form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as
 173 reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial
 174 VFAs (acetic acid, propionate, and butyrate). A milliliter of 95–97% H₂SO₄ was
 175 combined with a 10 ml incubation sample. A milliliter of the sample combination was
 176 mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged
 177 for 10 minutes at 7°C at 12,000 rpm. The samples were ready for gas chromatography
 178 identification.

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

$$186 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

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188 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

189 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
 190 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
 191 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
 192 was methyl 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.

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

217 treatments fell within the normal range of 6.75-6.82. While the treatment did not
218 significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total
219 protozoa count, microbial protein content, total VFA, and NH_3 concentrations. Palm oil
220 supplementation had the most pronounced impact, notably reducing protozoa count and
221 microbial protein. When zinc soap palm oil protection was partially supplemented (T2),
222 it led to higher microbial protein and VFA production compared to feed supplemented
223 with total zinc palm oil soap, although the difference was not significant.

224 **Feed Digestibility**

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

236 **Relative Proportion of VFA**

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

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

246 **Fatty Acids in Rumens Liquid**

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

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

DISCUSSION

Feed Fermentability

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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).

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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 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.

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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 **Feed Digestibility**

292 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
293 unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this
294 level of supplementation is safe for rumen microbial growth. These results are consistent
295 with previous research, which found that 6% supplementation with vegetable oils (olive
296 oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable
297 to the control, although linseed oil resulted in higher digestibility than sunflower oil
298 (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly
299 dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The
300 experimental feed in this study, characterized by high fiber content and low ADF
301 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
302 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
303 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
304 increased EED, supporting the conclusion that palm oil can be used as an energy
305 supplement without compromising rumen feed fermentability.

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

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

315 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
316 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et
317 al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and
318 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
319 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
320 one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al.
321 (2021) elucidated that Zn supplementation precipitated increased acetate production and
322 a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion
323 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
324 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
325 protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3)
326 versus 9.37 mg/ml in the P1 treatment (Table 3).

327 **Relative Proportion of VFA**

328 The relative proportion of acetate observed is slightly lower than that reported by
329 Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of
330 propionate and butyrate are comparatively higher. According to Amanullah et al. (2022),
331 palm oil supplementation modifies the rumen environment, thereby altering the ratio of
332 cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted
333 in significantly higher acetate production compared to non-protected palm oil ($P < 0.05$).
334 These findings are consistent with the increased microbial protein levels observed with
335 ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential
336 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

361 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
362 61.85% and T3 59.67% (Table 6).

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

374 Consistent with our study's findings, supplementation with ZSPO (T2 and T3)
375 resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)
376 concentrations compared to non-protected oil supplementation (T1). Amanullah et al.
377 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
378 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished
379 stearate levels but higher proportions of MUFA and PUFA. These findings are consistent
380 with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil
381 supplementation can increase the content of palmitic acid (SFA) and oleic acid
382 (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and
383 linolenic acids. This indicates a complete inhibition of the biohydrogenation process in
384 the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification

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

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

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CONCLUSION

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

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409 **CONFLICT OF INTEREST**

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

413

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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
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 ¹Total 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).
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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	<i>p</i> -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
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626 **Table 3.** Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm
627 oil (ZPOS).

Parameter	Treatment				SEM	<i>p</i> -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

628 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

629 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
630 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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

Parameter	Treatment				SEM	<i>p</i> -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 ^{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
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 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

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

Fatty Acids	Treatment				SEM	<i>p-Value</i>
	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

662 ^{a,b} different superscripts at the same column indicate significant differences ($P < 0.05$).

663 SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,,

664 UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA=

665 polyunsaturated fatty acids.

666 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal

667 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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[TASJ] Revision Required of Your Manuscript

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id>
To: Anis Muktiani <anismuktiani@gmail.com>

Fri, Jul 19, 2024 at 1:55 PM

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:

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 Editor. 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
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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	<p>References</p> <p>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. <i>International Journal of Pure and Applied Biological Research and Sciences</i>,. 1(1):1–8. Koushki, M., M. Nahidi, & F. Cheraghali. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. <i>Journal of Paramedical Sciences (JPS)</i>. 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 <i>Bailey's Industrial Oil and Fat Products</i>. Wulandari, B.P. Widyobroto, & A. Agus. 2020. In Vitro Digestibility and Ruminant Fermentation Profile of Pangola Grass (<i>Digitaria Decumbens</i>) Supplemented with Crude Palm Oil Protected by Sodium Hydroxide. <i>Livestock Research for Rural Development</i>. 32.</p> <p>b) <i>Please check the writing of author's names.</i> They must be written following on the journal's guidelines</p> <p>c) Cottyn (1968): write all authors</p>	

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

3
4 **ABSTRACT**

5 This study aimed to evaluate the effects of energy and organic zinc supplement,
6 specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid
7 profiles in vitro. The study used a completely randomized design with 4 treatments and 5
8 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet
9 + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and
10 T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated
11 female dairy goats, and were homogenized. The goats were feed on consisted of corn
12 straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed
13 that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and
14 microbial protein production, and lower NH₃ levels compared to the control (P<0.05).
15 Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar
16 among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher
17 than another treatment (P<0.05). ZPOS supplementation resulted higher acetate and
18 propionate levels compared to the control and supplementation 5% palm oil (P<0.05) but
19 did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment
20 was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels
21 of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)
22 compared to the control. In conclusion, adding protected palm oil in the form of zinc soap
23 enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content
24 in rumen liquid.

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

26 INTRODUCTION

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

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

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

49 levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et
50 al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria.
51 Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby
52 increasing energy availability for metabolic processes. Recent findings by Sears et al.
53 (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to
54 palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by
55 reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane
56 (CH₄) production and the acetate/propionate (A/P) ratio by increasing propionate
57 production (Gao et al., 2016).

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

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

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

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

75 Zinc (Zn) is an essential trace mineral that functions as a cofactor for over 300
76 enzymes, and is involved in DNA synthesis, growth, CO₂ transport, essential fatty acid
77 metabolism, and protein and nucleic acid synthesis (Elamin et al., 2013). Studies in vitro
78 observed that supplementing zinc in ruminal fluid increased cellulose digestibility
79 (Martínez & Church, 1970), and concentrations of total volatile fatty acids (VFA) and
80 acetate (Chen et al., 2019). Research of Wang et al. (2021) found that supplementation
81 20-30mg/kg DM Zn sulfat led to increase nutrient digestibility and ruminal fermentation.

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

92 *In vitro* Zinc soap supplementation research was conducted by Faizah et al.,
93 (2019), that compared between supplementation of 10% zinc soap from palm oil and 10%
94 corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility
95 of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and
96 A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support

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

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

111 MATERIALS AND METHODS

112 Zinc Soap and Feed Preparation

113 The preparation of zinc soap was carried out according to (Cabatit, 1979). The
114 palm oil used to make Zinc soap was a commercial palm oil which generally sold on the
115 market. The Zinc soap of palm oil was made based on saponification number. Palm oil is
116 measured for its saponification number and the KOH added is proportional to the
117 saponification number. In order to protect palm oil, zinc chloride ($ZnCl_2$) is added in an
118 amount equal to the KOH required to soak the oil, which is determined by the outcomes
119 of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated
120 in a water bath at 90°C. KOH that has been dissolved in ethanol is added to hot oil and

121 stirred until the oil is completely hydrolyzed. Next, add the ZnCl₂ solution and mix
122 continuously until a paste forms. The final step is to remove the remaining KOH by
123 adding water and washing using a centrifuge. This process produces cream soap called
124 Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract,
125 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

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

131 In Vitro Experiment

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

142 In vitro experiments were carried out according to the method of Tilley and Terry
143 (1963). **In the fermenter tube, 0.55 grams of feed samples from each treatment were**
144 **added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The**

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

147

148 **Nutrient 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
 151 two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor
 152 tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped
 153 by immersing the fermenter tube in ice water for 20 minutes. Then the tube was
 154 centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded.
 155 Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for
 156 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered
 157 with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed
 158 by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient
 159 digestibility values. Fermentation is also carried out without feed samples called blanks.
 160 Nutrien digestibility samples are calculated by the formula :

$$\begin{aligned}
 & \text{Nutrient Digestibility (\%)} \\
 & = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100
 \end{aligned}$$

163

164 **pH value, VFA, NH₃ and Methane production**

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

169 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
 170 was tested three times. NH₃ levels were determined using a spectrophotometer,
 171 employing a spectrophotometric method based on the catalyzed endophenol reaction to
 172 form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as
 173 reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial
 174 VFAs (acetic acid, propionate, and butyrate). A milliliter of 95–97% H₂SO₄ was
 175 combined with a 10 ml incubation sample. A milliliter of the sample combination was
 176 mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged
 177 for 10 minutes at 7°C at 12,000 rpm. The samples were ready for gas chromatography
 178 identification.

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

$$186 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

187

188 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

189 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
 190 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
 191 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
 192 was methyl 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 μ L of n-hexane, 50 mg of oil, and 50 μ L 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

217 treatments fell within the normal range of 6.75-6.82. While the treatment did not
218 significantly affect pH ($p>0.05$), notable differences ($p<0.05$) were observed in the total
219 protozoa count, microbial protein content, total VFA, and NH_3 concentrations. Palm oil
220 supplementation had the most pronounced impact, notably reducing protozoa count and
221 microbial protein. When zinc soap palm oil protection was partially supplemented (T2),
222 it led to higher microbial protein and VFA production compared to feed supplemented
223 with total zinc palm oil soap, although the difference was not significant.

224 **Feed Digestibility**

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

236 **Relative Proportion of VFA**

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

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

246 **Fatty Acids in Rumens Liquid**

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

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

DISCUSSION

Feed Fermentability

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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).

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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 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.

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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 **Feed Digestibility**

292 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
293 unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this
294 level of supplementation is safe for rumen microbial growth. These results are consistent
295 with previous research, which found that 6% supplementation with vegetable oils (olive
296 oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable
297 to the control, although linseed oil resulted in higher digestibility than sunflower oil
298 (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly
299 dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The
300 experimental feed in this study, characterized by high fiber content and low ADF
301 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
302 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
303 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
304 increased EED, supporting the conclusion that palm oil can be used as an energy
305 supplement without compromising rumen feed fermentability.

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

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

315 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
316 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et
317 al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and
318 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
319 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
320 one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al.
321 (2021) elucidated that Zn supplementation precipitated increased acetate production and
322 a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion
323 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
324 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
325 protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3)
326 versus 9.37 mg/ml in the P1 treatment (Table 3).

327 **Relative Proportion of VFA**

328 The relative proportion of acetate observed is slightly lower than that reported by
329 Anzhany et al. (2024), which ranged from 69% to 72%, while the proportions of
330 propionate and butyrate are comparatively higher. According to Amanullah et al. (2022),
331 palm oil supplementation modifies the rumen environment, thereby altering the ratio of
332 cellulolytic to amylolytic microbes. Both partial and total ZSPO supplementation resulted
333 in significantly higher acetate production compared to non-protected palm oil ($P < 0.05$).
334 These findings are consistent with the increased microbial protein levels observed with
335 ZSPO supplementation, suggesting enhanced bacterial growth. Zinc (Zn) is an essential
336 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

361 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
362 61.85% and T3 59.67% (Table 6).

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

374 Consistent with our study's findings, supplementation with ZSPO (T2 and T3)
375 resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)
376 concentrations compared to non-protected oil supplementation (T1). Amanullah et al.
377 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
378 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished
379 stearate levels but higher proportions of MUFA and PUFA. These findings are consistent
380 with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil
381 supplementation can increase the content of palmitic acid (SFA) and oleic acid
382 (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and
383 linolenic acids. This indicates a complete inhibition of the biohydrogenation process in
384 the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification

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

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

399

400

CONCLUSION

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

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CONFLICT OF INTEREST

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ACKNOWLEDGEMENT

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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
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 ¹Total 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).
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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	<i>p</i> -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
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626 **Table 3.** Feed nutrient in vitro digestibility due to supplementation of the zinc soap palm
627 oil (ZPOS).

Parameter	Treatment				SEM	<i>p-Value</i>
	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

628 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

629 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
630 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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

Parameter	Treatment				SEM	<i>p-Value</i>
	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 ^{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
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 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

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

Fatty Acids	Treatment				SEM	<i>p-Value</i>
	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

662 ^{a,b} different superscripts at the same column indicate significant differences ($P < 0.05$).

663 SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,,

664 UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA=

665 polyunsaturated fatty acids.

666 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal

667 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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[TASJ] Revision Required of Your Manuscript

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Fri, Jul 26, 2024 at 2:43 PM


To: "Prof. Dr. Komang G Wiryawan" <jurnal@apps.ipb.ac.id>

I hereby send a revised manuscript entitled : "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability, Digestibility and Unsaturated Fatty Acid Profile in Rumen Liquid".
Thank you for your attention.

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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

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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.	Thank you very much. I will revise it.
2	<p>References</p> <p>a) Please provide link for the references below:</p> <p>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. <i>International Journal of Pure and Applied Biological Research and Sciences</i>,. 1(1):1–8.</p> <p>Koushki, M., M. Nahidi, & F. Cheraghali. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. <i>Journal of Paramedical Sciences (JPS)</i>. 6(3):117–34.</p> <p>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 <i>Bailey's Industrial Oil and Fat Products</i>.</p>	<p>a) Link not found, replaced with another reference. Lines 75-79 Lines 341-343 (written in red ink)</p> <p>Koushki, M., M. Nahidi, & F. Cheraghali. 2015. Physico-Chemical Properties, Fatty Acid Profile and Nutrition in Palm Oil. <i>Journal of Paramedical Sciences (JPS)</i>.6:117–34. https://doi.org/10.22037/jps.v6i3.9772</p> <p>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). <i>Bailey's Industrial Oil and Fat Products: 7th ed.</i> John Wiley & Sons, Ltd. https://doi.org/10.1002/047167849X.bio100.</p>



Tropical Animal Science Journal



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	<p>Wulandari, B.P. Widyobroto, & A. Agus. 2020. In Vitro Digestibility and Ruminal Fermentation Profile of Pangola Grass (<i>Digitaria Decumbens</i>) Supplemented with Crude Palm Oil Protected by Sodium Hydroxide. <i>Livestock Research for Rural Development</i>. 32.</p> <p>b) <i>Please check the writing of author's names.</i> They must be written following on the journal's guidelines</p> <p>c) Cottyn (1968): write all authors</p>	<p>Wulandari, B. P. Widyobroto, & A. Agus. 2020. In Vitro Digestibility and Ruminal Fermentation Profile of Pangola Grass (<i>Digitaria Decumbens</i>) Supplemented with Crude Palm Oil Protected by Sodium Hydroxide. <i>Livestock Research for Rural Development</i>. 32. https://www.lrrd.cipav.org.co/lrrd32/7/wulan32102.html.</p> <p>b) Already did. Line 568 Lines 587-588 Line 592</p> <p>c) Already did. Cottyn, B. G., & C. V. Boucque. 1968. Rapid Method for the Gas-Chromatographic Determination of Volatile Fatty Acids in Rumen Fluid. <i>J.Agric.Food Chem.</i> 16:105-7. https://doi.org/10.1021/jf60155a002.</p>

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

3
4 **ABSTRACT**

5 This study aimed to evaluate the effects of energy and organic zinc supplement,
6 specifically zinc palm oil soap (ZPOS), on fermentability, and unsaturated fatty acid
7 profiles in vitro. The study used a completely randomized design with 4 treatments and 5
8 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet
9 + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and
10 T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated
11 female dairy goats, and were homogenized. The goats were feed on consisted of corn
12 straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed
13 that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and
14 microbial protein production, and lower NH₃ levels compared to the control (P<0.05).
15 Partial ZPOS supplementation (T2) resulted DM, OM, and CP digestibility were similar
16 among the treatments, but digestibility of EE, CF, NDF, and ADF was significantly higher
17 than another treatment (P<0.05). ZPOS supplementation resulted higher acetate and
18 propionate levels compared to the control and supplementation 5% palm oil (P<0.05) but
19 did not affect butyrate, reducing the A/P ratio and methane production. The PO treatment
20 was dominated by stearate (C18:0), whereas the ZPOS treatments showed higher levels
21 of trans 11 C18:1, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)
22 compared to the control. In conclusion, adding protected palm oil in the form of zinc soap
23 enhances fermentability, digestibility, and monounsaturated fatty acid (MUFA) content
24 in rumen liquid.

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

26 INTRODUCTION

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

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

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

49 levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et
50 al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria.
51 Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby
52 increasing energy availability for metabolic processes. Recent findings by Sears et al.
53 (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to
54 palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by
55 reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane
56 (CH₄) production and the acetate/propionate (A/P) ratio by increasing propionate
57 production (Gao et al., 2016).

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

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

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

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

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

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

94 In vitro Zinc soap supplementation research was conducted by Faizah et al.,
95 (2019), that compared between supplementation of 10% zinc soap from palm oil and 10%
96 corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility

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

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

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MATERIALS AND METHODS

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Zinc Soap and Feed Preparation

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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

121 amount equal to the KOH required to soak the oil, which is determined by the outcomes
122 of reaction stoichiometric calculations. Palm oil was put into a glass beaker and incubated
123 in a water bath at 90°C. KOH that has been dissolved in ethanol is added to hot oil and
124 stirred until the oil is completely hydrolyzed. Next, add the ZnCl₂ solution and mix
125 continuously until a paste forms. The final step is to remove the remaining KOH by
126 adding water and washing using a centrifuge. This process produces cream soap called
127 Zinc soap of palm oil (ZPOS). The nutrient content of ZPOS is 24.36% ether extract,
128 4.2% crude fiber, 4.94% Zn and gross energy 5257 kcal/kg.

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

134 **In Vitro Experiment**

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

145 In vitro experiments were carried out according to the method of Tilley and Terry
 146 (1963). In the fermenter tube, 0.55 grams of feed samples from each treatment were
 147 added, followed by 40 ml of McDaugall's solution and 10 ml of rumen liquid. The
 148 fermentor cylinder was flowed by CO₂ gas and closed to anaerobic conditions. The
 149 fermentor tube was incubated in a 39°C water temperature.

150 **Nutrient Digestibility**

151 The digestibility of nutrients including dry matter (DMD), organic matter (OMD),
 152 crude protein (CPD), ether extract (EED) and crude fiber (CFD) was measured through
 153 two stages of incubation, namely fermentative and enzymatic. First stage, the fermentor
 154 tube was incubated in a 39°C water temperature for 2x24 hours, and process was stopped
 155 by immersing the fermenter tube in ice water for 20 minutes. Then the tube was
 156 centrifuged for 15 minutes at a speed of 3,000 rpm and the supernatant was discarded.
 157 Second stage, the HCL pensin solution was added 50 ml into the tube and reincubated for
 158 2x24 hours in a 39°C water temperature under aerobic conditions. The sample was filtered
 159 with Whatman No.41 filter paper using a vacuum pump. The filter results were analyzed
 160 by DM, Ash, CP, EE and C, NDF, and ADF (AOAC, 2012) to calculate nutrient
 161 digestibility values. Fermentation is also carried out without feed samples called blanks.
 162 Nutrien digestibility samples are calculated by the formula :

$$\begin{aligned}
 & \text{Nutrient Digestibility (\%)} \\
 & = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100
 \end{aligned}$$

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166 **pH value, VFA, NH₃ and Methane production**

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

169 centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to
 170 measure the rumen pH, NH₃, and total and partial VFA concentrations. A digital pH
 171 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
 172 was tested three times. NH₃ levels were determined using a spectrophotometer,
 173 employing a spectrophotometric method based on the catalyzed endophenol reaction to
 174 form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as
 175 reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial
 176 VFAs (acetic acid, propionate, and butyrate). A milliliter of 95–97% H₂SO₄ was
 177 combined with a 10 ml incubation sample. A milliliter of the sample combination was
 178 mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged
 179 for 10 minutes at 7°C at 12,000 rpm. The samples were ready for gas chromatography
 180 identification.

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

$$188 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

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190 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

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

193 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
194 was methyl formalin saline which was made from a mixture of 100 ml 35% formaldehyde,
195 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated
196 by using a microscope at magnification 100 times. Measurement of rumen liquid protein
197 microbes using the method of Makkar et al. (1982) on the principle of gradual
198 centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas
199 chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were
200 stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty
201 acid composition was determined by converting oil to fatty acid methyl esters. This
202 involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The
203 peaks of the fatty acid methyl esters were identified by comparing their retention times
204 with those of authentic standards. The relative percentage of each fatty acid was
205 calculated based on its peak area relative to the total peak area of all fatty acids in the
206 sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer
207 than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and
208 long-chain fatty acids (LCFA), which are greater than C12 (Wang et al., 2020).

209 **Statistical Analysis**

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

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RESULTS

Feed Fermentability

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

Feed Digestibility

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

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

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DISCUSSION

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Feed Fermentability

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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).

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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 decreased protein synthesis. The use of zinc soap palm oil protection, both partially

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

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

297 **Feed Digestibility**

298 The digestibility values for DMD, OMD, CPD, NDFD, and ADFD were
299 unaffected by 5% non-protected palm oil zinc soap supplementation, indicating that this
300 level of supplementation is safe for rumen microbial growth. These results are consistent
301 with previous research, which found that 6% supplementation with vegetable oils (olive
302 oil, sunflower oil, and linseed oil) yielded OMD, NDFD, and ADFD values comparable
303 to the control, although linseed oil resulted in higher digestibility than sunflower oil
304 (Vargas-bello-p et al., 2020). The effect of oil supplementation on digestibility is highly
305 dependent on the nutrient composition of the feed and the fatty acid profile of the oil. The
306 experimental feed in this study, characterized by high fiber content and low ADF
307 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
308 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
309 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
310 increased EED, supporting the conclusion that palm oil can be used as an energy
311 supplement without compromising rumen feed fermentability.

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

321 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
322 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain et
323 al., 2016; Uniyal et al., 2017). It is indispensable for preserving the structural and
324 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
325 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
326 one Zn-dependent protein (Ranasinghe et al., 2015). Recent research by Fellner et al.
327 (2021) elucidated that Zn supplementation precipitated increased acetate production and
328 a heightened acetate/prothionate ratio. This delineates an augmented fiber digestion
329 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
330 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
331 protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3)
332 versus 9.37 mg/ml in the P1 treatment (Table 3).

333 **Relative Proportion of VFA**

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

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

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

356 **Fatty Acids in Rumen Liquid**

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

360 number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
361 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which
362 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
363 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated
364 fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes
365 to the range of rumen saturated fatty acid levels, where PO supplementation treatment
366 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
367 61.85% and T3 59.67% (Table 6).

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

379 Consistent with our study's findings, supplementation with ZSPO (T2 and T3)
380 resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)
381 concentrations compared to non-protected oil supplementation (T1). Amanullah et al.
382 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
383 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished

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

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

404

405

CONCLUSION

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

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

412

413

414

CONFLICT OF INTEREST

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

418

419

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610

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
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

612 ¹Total 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	<i>p</i> -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
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626

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

Parameter	Treatment				SEM	<i>p-Value</i>
	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

629 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

630 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
631 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

632

633

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

Parameter	Treatment				SEM	<i>p-Value</i>
	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

635 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

636 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
637 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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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

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660

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

Fatty Acids	Treatment				SEM	<i>p-Value</i>
	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

663 ^{a,b} different superscripts at the same column indicate significant differences ($P < 0.05$).

664 SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,,

665 UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA=

666 polyunsaturated fatty acids.

667 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal

668 diet+partially ZPOS (3.75% ZPOS+1.25% PO); T3= basal diet+5% ZPOS

669



Anis Muktiani <anis.muktiani@gmail.com>

[TASJ] Editor Decision

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id>

Mon, Jul 29, 2024 at 8:59 AM

To: "A. Muktiani" <anismuktiani@gmail.com>, "W. Widiyanto" <widiyanto@lecturer.undip.ac.id>, "N. S. Pandupuspitasari" <shin_tse@yahoo.com>

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.

Please find the attached invoice of the publication charge of your manuscript.

Thank you for your article submission, and we are looking forward to receiving your incoming articles.

Prof. Dr. Komang G Wiryawan
Chief Editor
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Please find the attached invoice of the publication charge of your manuscript.

Thank you for your article submission, and we are looking forward to receiving your incoming articles.

Prof. Dr. Komang G Wiryawan
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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>

I hereby send the manuscript, entitled "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid" which has been corrected for typos. I also sent proof of payment of the invoice. Thank you.

Anis Muktiani

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113K

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

3
4 **A. Muktiani*, W. Widiyanto, & N. S. Pandupuspitasari**

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8
9 **ABSTRACT**

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

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

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

31

32

INTRODUCTION

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

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

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

50 Palm oil is one of the most promising vegetable oils to be used as an energy source
51 with gross energy (GE) content was 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is
52 also easily obtainable, resistant to rancidity, and affordable. The global price of palm oil
53 is \$574 per metric ton (mt), which is cheaper compared to soybean oil at \$684/mt, coconut
54 oil at \$830/mt, and peanut oil at \$1,584/mt (World Bank, 2020). Palm oil contains high
55 levels of unsaturated fatty acids, specifically oleic acid at oleic acid at 39.2% (Mancini et
56 al., 2015). High levels of palmitate are very beneficial for cellulolytic bacteria.
57 Cellulolytic bacteria can incorporate palmitic acid into their cell membranes, thereby
58 increasing energy availability for metabolic processes. Recent findings by Sears et al.
59 (2024) showed an increase in *Prevotella* and *Fibrobacter* populations in response to
60 palmitic acid supplementation. Unsaturated fatty acids contribute to energy efficiency by
61 reducing protozoa (Muktiani et al., 2020; Vargas-bello-p et al., 2020), reducing methane
62 (CH₄) production and the acetate/propionate (A/P) ratio by increasing propionate
63 production (Gao et al., 2016).

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

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

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

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

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

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

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

100 In vitro Zinc soap supplementation research was conducted by Faizah et al.,
101 (2019), that compared between supplementation of 10% zinc soap from palm oil and 10%
102 corn. Supplementation with 10% of palm oil zinc soap resulted no different digestibility
103 of dry matter, organic matter and crude fiber, but the total production of VFA, acetate and
104 A/P ratio was higher compared to corn oil Zn soap. High acetate levels strongly support
105 the synthesis of milk fatty acids in dairy cows, especially short chain fatty acids. However,
106 there has been no research that explores the contribution of long chain fatty acids, MUFA
107 and PUFA due to zinc soap supplementation as a precursor for the synthesis of long fatty
108 acids and unsaturated fatty acids in milk. The results of this study was provide information
109 regarding the concentration of long chain fatty acids, MUFA and PUFA in the rumen due
110 to zinc soap supplementation, which is expected to be used as a consideration in providing
111 unsaturated fatty acids to increase production and health of dairy livestock.

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

119

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 ($ZnCl_2$) 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°C. KOH that has been dissolved in ethanol is added to hot oil and stirred until the oil is completely hydrolyzed. Next, add the $ZnCl_2$ 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 degradable 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

167 digestibility values. Fermentation is also carried out without feed samples called blanks.

168 Nutrien digestibility samples are calculated by the formula :

169 Nutrient Digestibility (%)

$$170 = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{nutrient sample, g}} \times 100$$

171

172 **pH value, VFA, NH₃ and Methane production**

173 The process of measuring pH, VFA, and NH₃ followed the same procedure as that
 174 for nutrient digestibility, but with the fermentation stopped at the 4-hour mark. After
 175 centrifuging the fermentor tube at 3,000 rpm for 15 minutes, the supernatant was taken to
 176 measure the rumen pH, NH₃, and total and partial VFA concentrations. A digital pH
 177 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
 178 was tested three times. NH₃ levels were determined using a spectrophotometer,
 179 employing a spectrophotometric method based on the catalyzed endophenol reaction to
 180 form a stable blue compound (Chaney & Marbach, 1962). Gas chromatography, as
 181 reported by (Cottyn & Boucque, 1968), was used to quantify the generation of partial
 182 VFAs (acetic acid, propionate, and butyrate). A milliliterl of 95–97% H₂SO₄ was
 183 combined with a 10 ml incubation sample. A milliliter of the sample combination was
 184 mixed with 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged
 185 for 10 minutes at 7°C at 12,000 rpm. The samples were ready for gas chromatography
 186 identification.

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

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

$$194 \quad E (\%) = \frac{(0,622 \text{ pA} + 1,091 \text{ pP} + 1,558 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

195

196 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

197 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
 198 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
 199 were calculated following the procedures of Ogimoto and Imai (1981). The solution used
 200 was methyl formalin saline which was made from a mixture of 100 ml 35% formaldehyde,
 201 2 g tryhan blue, 9 g NaCl and 900 ml aquades. The number of protozoa was calculated
 202 by using a microscope at magnification 100 times. Measurement of rumen liquid protein
 203 microbes using the method of Makkar et al. (1982) on the principle of gradual
 204 centrifugation. The fatty acid profile of rumen liquid was analyzed using a gas
 205 chromatograph (Model GC-14A, Shimadzu Corporation, Kyoto, Japan). Samples were
 206 stored at -20°C until analysis. Following the method of Cocks and Rede (1966), the fatty
 207 acid composition was determined by converting oil to fatty acid methyl esters. This
 208 involved adding 950 µL of n-hexane, 50 mg of oil, and 50 µL of sodium methoxide. The
 209 peaks of the fatty acid methyl esters were identified by comparing their retention times
 210 with those of authentic standards. The relative percentage of each fatty acid was
 211 calculated based on its peak area relative to the total peak area of all fatty acids in the
 212 sample. Fatty acids were categorized into short-chain fatty acids (SCFA) as having fewer
 213 than six carbon atoms, medium chain fatty acids (MCFA) ranging from C6 to C12, and
 214 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

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

244 **Relative Proportion of VFA**

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

254 **Fatty Acids in Rumen Liquid**

255 Table 5 outlines the fatty acid composition in rumen liquid. The control diet
256 primarily contained short-chain fatty acids (SCFA), with a notably higher concentration
257 of C4 compared to other treatments ($P<0.05$). Supplementation with palm oil (PO and
258 ZSPO) led to increased levels of palmitate (C16:0), stearate (C18:0), and oleate (C18:1)
259 relative to the control ($P<0.05$). However, the proportion of stearate was significantly
260 lower with ZSPO supplementation ($P<0.05$). Unsaturated fatty acids, particularly cis-9
261 18:1, trans-11 18:1, linoleate (18:2), linolenic acid (18:3), EPA (20:5), and DHA (C22:6)

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

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

273

274

DISCUSSION

275

Feed Fermentability

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

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

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

300 **Feed Digestibility**

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

309 experimental feed in this study, characterized by high fiber content and low ADF
310 (13.77%), supports the proliferation of cellulolytic microbes. A high-fiber diet may
311 mitigate the potential negative impact of oil on fiber digestion by rumen microbes
312 (Benchaar et al., 2015). Both non-protected (T1) and protected (T2 and T3) palm oil
313 increased EED, supporting the conclusion that palm oil can be used as an energy
314 supplement without compromising rumen feed fermentability.

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

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

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

336 **Relative Proportion of VFA**

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

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

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

359 **Fatty Acids in Rumen Liquid**

360 Previous research showed that oil supplementation caused a decrease in the
361 proportion of short chain fatty acids in rumen fluid, while increasing the presence of long
362 chain fatty acids. Mancini et al. (2015) argue that this transition is caused by the large
363 number of long chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
364 1.7% consists of short chain fatty acids (SCFA). Among long-chain fatty acids, which
365 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
366 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated
367 fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes
368 to the range of rumen saturated fatty acid levels, where PO supplementation treatment
369 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
370 61.85% and T3 59.67% (Table 6).

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

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

382 Consistent with our study's findings, supplementation with ZSPO (T2 and T3)
383 resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)
384 concentrations compared to non-protected oil supplementation (T1). Amanullah et al.
385 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
386 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished
387 stearate levels but higher proportions of MUFA and PUFA. These findings are consistent
388 with the research by Satir et al. (2023), which demonstrated that 3% protected palm oil
389 supplementation can increase the content of palmitic acid (SFA) and oleic acid
390 (MUFA). Stearate is the final product of the biohydrogenation of oleic, linoleic, and
391 linolenic acids. This indicates a complete inhibition of the biohydrogenation process in
392 the ZSPO treatment. The protection of polyunsaturated fatty acids through saponification
393 involves bonding the carboxyl group with zinc, resulting in the inhibition of
394 isomerization, which is a prerequisite for the hydrogenation of unsaturated fatty acids into
395 saturated fatty acids. This is reflected in the higher proportion of MUFA in the ZSPO
396 treatment compared to PO and the control (Table 6). The substantial presence of PUFA,
397 notably EPA and DHA, underscores Zinc's role in the elongation and desaturation
398 process, facilitated by the formation of arachidonic acid (20:4) through delta-6-desaturase
399 (D6D) and delta-5-desaturase (D5D) enzymes (Ridgway, 2016). Zinc's indispensability
400 is further highlighted as a pivotal mineral cofactor for D6D and D5D enzymes (Noland
401 & Drisko, 2020).

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

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

407

408

CONCLUSION

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

415

416

CONFLICT OF INTEREST

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

420

421

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- 612

613 **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
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

614 ¹Total digestible nutrients (TDN) were calculated using $TDN (\%DM) = TDN =$
615 $-17.2649 + 1.2120(PK) + 0.8352(BETN) + 2.4637(LK) + 0.4475(SK)$, according
616 to (Wardeh, 1981).
617
618

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

Parameter	Treatment				SEM	<i>p</i> -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

621 ^{a,b} different superscripts at the same column indicate significant differences ($P < 0.05$).

622 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
623 diet+partially ZPOS(3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS
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628

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

Parameter	Treatment				SEM	<i>p</i> -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

631 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

632 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
633 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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

Parameter	Treatment				SEM	<i>p</i> -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

637 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

638 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
639 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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

Fatty Acids	Treatment				SEM	<i>p</i> -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	-	-

658 ^{a,b} different superscripts at the same column indicate significant differences (P<0.05).

659 T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal
 660 diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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

Fatty Acids	Treatment				SEM	<i>p-Value</i>
	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

665

^{a,b} different superscripts at the same column indicate significant differences ($P < 0.05$).

666

SA= short chain fatty acids, LA= long chain fatty acids, SFA= saturated fatty acids,,

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UFA= unsaturated fatty acids (UFA), MUFA= monounsaturated fatty acids, dan PUFA=

668

polyunsaturated fatty acids.

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T0= basal diet without supplementation; T1= basal diet+5% palm oil; T2= basal

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diet+partially ZPOS (3.75% ZPOS+1.25%PO); T3= basal diet+5% ZPOS

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[TASJ] New notification from Tropical Animal Science Journal

Prof. Dr. Komang G Wiryawan <jurnal@apps.ipb.ac.id>
Reply-To: "Prof. Dr. Ir. Komang G. Wiryawan" <kgwiryawan@yahoo.com>
To: Dr Anis Muktiani <anismuktiani@gmail.com>

Fri, Aug 9, 2024 at 8:53 AM

You have a new notification from Tropical Animal Science Journal:

You have been added to a discussion titled "Copyediting review request" regarding the submission "Supplementation of Zinc Soap Palm Oil Improves Feed Fermentability and Unsaturated Fatty Acid Profile in Rumen Liquid".

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Prof. Dr. Ir. Komang G. Wiryawan

Tropical Animal Science

Journal <http://journal.ipb.ac.id/index.php/tasj>

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

3
4 **A. Mukhtiani*, W. Widiyanto, & N. S. Pandupuspitasari**

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8
9 **ABSTRACT**

10 This study aimed to evaluate the effects of energy and organic zinc supplements,
11 specifically zinc palm oil soap (ZPOS), on fermentability and unsaturated fatty acid
12 profiles *in vitro*. The study used a completely randomized design with 4 treatments and 5
13 replications. The treatments were: T0 (basal diet without supplementation), T1 (basal diet
14 + 5% palm oil, PO), T2 (basal diet + 5% partially ZPOS: 3.75% ZPOS + 1.25% PO), and
15 T3 (basal diet + 5% ZPOS). The inoculum source was rumen liquid from three fistulated
16 female dairy goats and was homogenized. The goats were fed ration consisted of corn
17 straw, soybean hulls, and concentrate (TDN 63%, CP 14%, NDF 35%). Results showed
18 that both partial and full ZPOS supplementation (T2 and T3) resulted in higher VFA and
19 microbial protein production and lower NH₃ levels compared to the control (p<0.05).
20 Partial ZPOS supplementation (T2) resulted in DM, OM, and CP digestibility that was
21 similar among the treatments, but the digestibility of EE, CF, NDF, and ADF was
22 significantly higher than another treatment (p<0.05). ZPOS supplementation resulted in
23 higher acetate and propionate levels than the control and supplementation of 5% palm oil
24 (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% partially ZPOS? Why used this treatment?

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

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

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

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

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INTRODUCTION

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

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

Commented [mp4]: which level?? Please state clearly.

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

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

50 Palm oil is one of the most promising vegetable oils for energy sources, with a
51 gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also
52 easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is
53 \$574 per metric ton (mt), which is cheaper compared to soybean oil at \$684/mt, coconut
54 oil at \$830/mt, and peanut oil at \$1,584/mt (World Bank, 2020). Palm oil contains high
55 levels of unsaturated fatty acids, specifically oleic acid at 39.2% (Mancini *et al.*, 2015).
56 High levels of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria
57 can incorporate palmitic acid into their cell membranes, thereby increasing energy
58 availability for metabolic processes. Recent findings by Sears *et al.* (2024) showed an
59 increase in *Prevotella* and *Fibrobacter* populations in response to palmitic acid
60 supplementation. Unsaturated fatty acids contribute to energy efficiency by reducing
61 protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020), reducing methane (CH₄)
62 production and the acetate/propionate (A/P) ratio by increasing propionate production
63 (Gao *et al.*, 2016).

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

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

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

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

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

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

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

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

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

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

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

119

MATERIALS AND METHODS

Zinc Soap and Feed Preparation

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

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

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

Commented [mp8]: What the standard used for this nutrient?

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

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

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

156 Nutrient Digestibility

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

Commented [mp10]: HCl??

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

$$170 \quad \text{Nutrient digestibility (\%)} \\ 171 \quad = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{Nutrient sample, g}} \times 100$$

172

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

Commented [mp11]: NH₃,??

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% H₂SO₄ 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 °C 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)??

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

$$194 \quad E (\%) = \frac{(0.622 \text{ pA} + 1.091 \text{ pP} + 1.558 \text{ pB})}{\text{pA} + \text{pP} + 2\text{pB}} \times 100$$

195

196 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

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

215

Commented [mp13]: what is the reference?

Commented [mp14]: How is the formula?

216 **Statistical Analysis**

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

220

221 **RESULTS**222 **Feed Fermentability**

223

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

234 **Feed Digestibility**

235

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

Commented [mp15]: In Rumen ??

Commented [mp16]: Please describe the results to the point and how they affect the treatment of the observed variables.

Commented [mp17]: what treatment? Please specify the treatment

Commented [mp18]: Nutrient Digestibility ??

Commented [mp19]: Please describe the results to the point and how they affect the treatment of the observed variables.

Commented [mp20]: Please state directly which treatment that has significant effect on the variable, and is it increase or decrease??

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

247 **Relative Proportion of VFA**

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

257 **Fatty Acids in Rumen Liquid**

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

Commented [mp21]: at what level....??

Commented [mp22]: increased

Commented [mp23]: Please describe the results to the point and how they affect the treatment of the observed variables.

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

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

276

277 DISCUSSION

278 Feed Fermentability

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

Commented [mp24]: Why palm oil did not disrupt the rumen pH?? Please describe the reason.

Commented [mp25]: What the correlation with pH value??

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

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

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

303 **Feed Digestibility**

Commented [mp27]: Nutrient Digestibility ??

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

Commented [mp28]: What do you mean by 5% Non-protected ZPOS? Does ZPOS mean protected zinc palm oil?

Commented [mp29]: If palm oil at level 5% is safe for nutrient digestibility, does it mean there is no need to be protected??

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 al.*,
329 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

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

339 **Relative Proportion of VFA**

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

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

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

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

362 **Fatty Acids in Rumen Liquid**

363 Previous research showed that oil supplementation caused a decrease in the
364 proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-
365 chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large
366 number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
367 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which
368 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
369 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated
370 fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes
371 to the range of rumen saturated fatty acid levels, where PO supplementation treatment
372 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
373 61.85%, and T3 59.67% (Table 6).

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

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

385 Consistent with our study's findings, supplementation with ZPOS (T2 and T3)
386 resulted in elevated levels of trans18:1 and cis 18:1, alongside reduced stearate (18:0)
387 concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.*
388 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
389 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished
390 stearate levels but higher proportions of MUFA and PUFA. These findings are consistent
391 with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil
392 supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).
393 Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids.
394 This indicates a complete inhibition of the biohydrogenation process in the ZPOS
395 treatment. The protection of polyunsaturated fatty acids through saponification involves
396 bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is
397 a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids.
398 This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to
399 PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA,
400 underscores Zn's role in the elongation and desaturation process, facilitated by the
401 formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-
402 desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted
403 as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

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

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

409

410

CONCLUSION

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

417

418

CONFLICT OF INTEREST

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

422

423

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426

427

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

Commented [mp32]: Please add the contract number and the year of the project.

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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: ¹Total 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).

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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.

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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.

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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.

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

Fatty acids	Treatments				SEM	<i>p</i> -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

662 Note: ^{a,b} means in the same row with different superscripts differ significantly ($p < 0.05$).
663 SA= short-chain fatty acids, LA= long-chain fatty acids, SFA= saturated fatty acids,
664 UFA= unsaturated fatty acids, MUFA= monounsaturated fatty acids, PUFA=
665 polyunsaturated fatty acids.
666 T0= basal diet without supplementation; T1= basal diet + 5% palm oil (PO); T2= basal
667 diet + partially ZPOS (3.75% ZPOS + 1.25% PO); T3= basal diet + 5% ZPOS.
668

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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Fri, Aug 16, 2024 at 12:21 PM

To: Tropical Animal Science Journal <mediapeternakan@apps.ipb.ac.id>

Dear Chief Editor
Tropical Animal Science Journal

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.

[Quoted text hidden]

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

3
 4 **A. Mukhtiani*, W. Widiyanto, & N. S. Pandupuspitasari**

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8
 9 **ABSTRACT**

10 This study aimed to evaluate the effects of energy and organic zinc supplements,
 11 specifically zinc palm oil soap (ZPOS), on digestibility and unsaturated fatty acid profiles
 12 *in vitro*. The study used a completely randomized design with 4 treatments and 5
 13 replications. The treatments were: T0= basal diet without supplementation, T1= basal diet
 14 + 5% palm oil (PO), T2= basal diet + 5% partial ZPOS (3.75% ZPOS + 1.25% PO), and
 15 T3= basal diet + 5% ZPOS. The inoculum source was rumen liquid from three fistulated
 16 female dairy goats and was homogenized. The goats were fed ration consisted of corn
 17 straw, soybean hulls, and concentrate containing total digestible nutrients (TDN) 63%
 18 crude protein (CP) 14%, and neutral detergent fiber (NDF) 35%. Results showed that both
 19 5% partial ZPOS and 5% ZPOS supplementation (T2 and T3) resulted increased of total
 20 volatile fatty acids (VFA), acetat, propionate, butirat, unsaturated fatty acids (USFA) and
 21 decreased in ratio of acetat/propionate (A/P) compared to the control and supplementation
 22 of 5% PO (p<0.05). Supplementation of 5% partial ZPOS (T2) is better than 5% ZPOS
 23 because increased the digestibility of ether ekstrakt (EE), crude fiber (CF), NDF, and
 24 acids detergent fiber (ADF) (p<0.05) and decreased of methane compared to the control

Commented [mp1]: What do you mean with 5% partial 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?
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25 (p<0.05). In conclusion, adding of 5% partial ZPOS (3.5% ZPOS and 1.5% PO) increase
 26 fiber digestibility, concentration of VFA, LCFA and USFA, as well as decrease methane
 27 production in the rumen liquid.

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

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

29

30 INTRODUCTION

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

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 Lactating Ewes (L. Khotijah et al.)

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

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 AND METHODS

Animals and Feed
 Fifteen first month la
 used in this experiment.

48 Palm oil is one of the most promising vegetable oils for energy sources, with a
 49 gross energy (GE) content of 9219 kcal/kg (Khaskheli & Chou, 2020). Palm oil is also
 50 easily obtainable, resistant to rancidity, and affordable. The global price of palm oil is
 51 \$574 per metric ton (mt), which is cheaper compared to soybean oil at \$684/mt, coconut
 52 oil at \$830/mt, and peanut oil at \$1,584/mt (World Bank, 2020). Palm oil contains
 53 saturated fatty acids in the form of palmitic acid 44%, as well as unsaturated fatty acids
 54 specifically oleic acid 39.2% and linoleic acid 10.1% (Mancini *et al.*, 2015). High levels
 55 of palmitate are very beneficial for cellulolytic bacteria. Cellulolytic bacteria can
 56 incorporate palmitic acid into their cell membranes, thereby increasing energy availability
 57 for metabolic processes. Recent findings by Sears *et al.* (2024) showed an increase in
 58 *Prevotella* and *Fibrobacter* populations in response to palmitic acid supplementation.
 59 Unsaturated fatty acids in palm oil (oleic acids and linoleic acids) contribute to energy
 60 efficiency by reducing protozoa (Muktiani *et al.*, 2020; Vargas-bello-p *et al.*, 2020),
 61 reducing methane (CH₄) production and the acetate/propionate (A/P) ratio by increasing
 62 propionate production (Gao *et al.*, 2016).

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

68 Palm oil contains 49.3% unsaturated fatty acids, when used in animal feed must
 69 be protected to prevent biohydrogenation and to avoid disrupting rumen bacterial
 70 fermentation. It is essential to safeguard unsaturated fatty acids to preserve their
 71 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%.

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

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

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

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

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

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

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

118

119

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 °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.

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

Commented [mp9]: What the standard used for this nutrient?
 Already did

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

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

156 **Nutrient Digestibility**

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

Commented [mp10]: What is the consideration of this proportion?

The consideration of this treatment is the result of previous research conducted by authors (Faizah et al., 2019), that partial supplementation of protected oil (75%) produces higher energy efficiency compared to total protected oil supplementation.

Commented [mp11]: HCl??

Already did

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

170
$$\text{Nutrient digestibility (\%)} = \frac{(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g}))}{\text{Nutrient sample, g}} \times 100$$

171

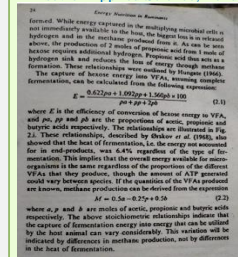
172 **pH Value, VFA, NH₃, and Methane Production**

173 The process of measuring pH, VFA, and NH₃ followed the same procedure as that
 174 for nutrient digestibility, but the fermentation stopped at the 4-hour mark. After
 175 centrifuging the fermenter tube at 3,000 rpm for 15 minutes, the supernatant was taken to
 176 measure the rumen pH, NH₃, and total and partial VFA concentrations. A digital pH
 177 meter from the brand "ACT" was used to measure the rumen liquid pH, and each sample
 178 was tested three times. NH₃ levels were determined using a spectrophotometer,
 179 employing a spectrophotometric method based on the catalyzed endophenol reaction to
 180 form a stable blue compound (Chaney & Marbach, 1962). Cottyn & Boucque (1968)
 181 reported that gas chromatography was used to quantify the generation of partial VFAs
 182 (acetic acid, propionate, and butyrate). A milliliter of 95%–97% H₂SO₄ was combined
 183 with a 10 mL incubation sample. A milliliter of the sample combination was mixed with
 184 0.0003 g of dehydrated sulfosalicylate. After that, the mixture was centrifuged for 10
 185 minutes at 7 °C at 12,000 rpm. The samples were ready for gas chromatography
 186 identification.

187 The methane gas concentration and energy conversion efficiency were determined
 188 by calculating the VFA stoichiometry, which was the estimation using the formula of
 189 Orskov & Ryle (1990). The formula used for methane concentration was: Methane (mM)
 190 $= 0.5a - 0.25p + 0.5b$, where a, p and b are moles of acetic, propionic and butyric acids
 191 respectively. The efficiency of conversion of hexose energy to VFA was calculated based

Commented [mp12]: NH₃,??
 Already did

Commented [mp13]: Please check again the formula using Moss et al (2002)??
 It has been checked. The formula is in this book :
 Orskov, E. R. & M. Ryle. 1990. Energy Nutrition in Ruminants.
 Elsevier Applied Science, London (UK).



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

$$194 E (\%) = \frac{(0.622 pa + 1.091 pp + 1.558 pb)}{pa + pp + 2pb} \times 100\%$$

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

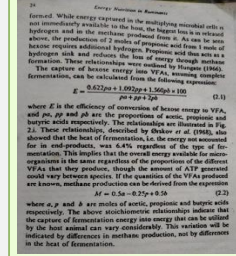
196 **Protozoa, Microbial Protein, and Fatty Acids Rumen Liquid**

197 To calculate protozoa, microbial protein and fatty acid concentrations in the rumen
 198 liquid, the fermentation process was carried out for 24 hours. The populations of protozoa
 199 were calculated following the procedures of Ogimoto & Imai (1981). The solution used

200 was trypan blue formalin saline (TBFS), made from a mixture of 100 mL 35%
 201 formaldehyde, 2 g trypan blue, 8 g NaCl, and 900 mL distilled water. The protozoa
 202 population was carried out on each treatment rumen fluid mixed with Trypan Blue
 203 Formalin Saline (TBFS) solution with a ratio of 5:1. The counting chamber with a
 204 thickness of 0.1 mm was dripped with 2 drops of the mixture. The area of the smallest
 205 box was 0.0625 mm, totaling 16 boxes with a box area of 5 boxes read. The number of
 206 protozoa was calculated by using a microscope at magnification 100 times. The formula
 207 used for total population of protozoa was : $\frac{1}{0.1 \times 0.0625 \times 16 \times 5} \times 1000 \times DF \times C$, where C is
 208 protozoa population in the counting chamber and DF is diluent factor the specimen.

209 Measurement of rumen liquid protein microbes using the method of Makkar *et al.*
 210 (1982) on the principle of gradual centrifugation. The fatty acid profile of rumen liquid
 211 was analyzed using a gas chromatograph (Model GC-14A, Shimadzu Corporation,
 212 Kyoto, Japan). Samples were stored at -20 °C until analysis. Following the method of
 213 Cocks & Rede (1966), the fatty acid composition was determined by converting oil to
 214 fatty acid methyl esters. This involved adding 950 µL of n-hexane, 50 mg of oil, and 50

Commented [mp14]: what is the reference?
 The formula is in this book :
 Orskov, E. R. & M. Ryle. 1990. Energy Nutrition in Ruminants.
 Elsevier Applied Science, London (UK).



Commented [mp15]: How is the formula?
 Already did.

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

222

223 Statistical Analysis

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

227

228 RESULTS

229 Feed Fermentability in The Rumen

230 Detailed rumen feed fermentation parameters can be seen in Table 2. The pH
231 levels in all treatments were not significantly different ($P > 0.05$) and were in the normal
232 range of 6.75-6.82. Supplementation of 5% PO (T1) had an effect on reducing the number
233 of protozoa and microbial protein compared to the control and other treatments ($P < 0.05$).
234 On the other hand, 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation caused a
235 decreased in NH_3 concentration and an increased in VFA production compared to the
236 control and 5% PO supplementation ($P < 0.05$).

237

238

Commented [mp16]: In Rumen ??
Already did

Commented [mp17]: Please describe the results to the point
and how they affect the treatment of the observed variables.
Already did

Nutrient Digestibility

239
240 Table 3 presents the nutrient digestibility due to 5% PO, 5% partial ZPOS and
241 5% ZPOS supplementation. All treatments did not show significant differences in dry
242 matter (DMD), organic matter (OMD), and crude protein (CPD) digestibility, but there
243 were significant differences ($p < 0.05$) in ether extract digestibility (EED), crude fiber
244 digestibility (CFD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber
245 digestibility (ADFD). Both supplementation of 5% PO (T1), 5% partial ZPOS (T2) and
246 5% ZPOS (T3) increased EED, but fiber digestibility (CFD, NDFD, and ADFD) was only
247 increased by 5% partial ZPOS supplementation (T2), while 5% PO and 5% ZPOS
248 supplementation did not differ from the control.

Commented [mp18]: Nutrient Digestibility ??
Already did

Commented [mp19]: Please describe the results to the point
and how they affect the treatment of the observed variables.
Already did

Relative Proportion of VFA

249
250 Table 4 shows the partial VFA and methane production in rumen liquid.
251 Supplementation of 5% PO, 5% partial ZPOS and 5% ZPOS significantly influenced
252 ($p < 0.05$) acetate, propionate, butyrate, the A/P ratio, and methane, but did not impact the
253 efficiency of hexose energy conversion into VFA. The relative proportion of VFAs in this
254 study was 59%–62% acetic acid, 24%–29% propionic acid, and 12%–13% butyric acid.
255 Both 5% partial ZPOS (T2) and 5% ZPOS supplementation increased acetate,
256 propionate, and butyrate compared to 5% PO supplementation (T1) and the control (T0).
257 Conversely, A/P ratio and methane production decreased with 5% PO and 5% partial
258 ZPOS supplementation. The efficiency of hexose energy conversion into VFA between
259 treatments was not significantly different.

Commented [mp20]: at what level...??
Already did.

Commented [mp21]: Increased
Already did.

Fatty Acids in Rumen Liquid

260
261 Table 5 outlines the fatty acid composition in rumen liquid. Supplementation of 5%
262 PO (T1), 5% partial ZPOS (T2) and 5% ZPOS (T3) supplementation decreased

Commented [mp22]: Please describe the results to the point
and how they affect the treatment of the observed variables.
Already did.

Commented [mp23]: at what level...??
Already did.

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

Commented [mp24]: at what level...??
 Already did.

268 These fatty acids are classified into various categories, including short-chain fatty
 269 acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs),
 270 saturated fatty acids (SFAs), unsaturated fatty acids (USFAs), monounsaturated fatty
 271 acids (MUFAs), and polyunsaturated fatty acids (PUFAs). According to Table 6, the diets
 272 supplemented with 5% PO, 5% partial ZPOS and 5% ZPOS decreased of SCFAs
 273 compared to the control diet ($p < 0.05$) but increased amount of LCFA. SFAs showed
 274 increased in control and 5% PO supplementation, whereas 5% partial ZPOS and 5%
 275 ZPOS supplementation demonstrated an ability to elevate USFAs. Notably, the 5% partial
 276 5% ZPOS and 5% ZPOS supplementation increased of MUFAs and PUFAs than the other
 277 treatments.

278 DISCUSSION

279 Feed Fermentability

280 The pH is one of the crucial factors in assessing rumen health. The findings
 281 indicated that adding palm oil and protected palm oil Zn soap did not disrupt the rumen
 282 fermentation process. Palm oil contains high palmitic acid, which actually supports the
 283 growth of rumen bacteria (Sears *et al.*, 2024). Apart from being high in palmitate content,
 284 palm oil is also high in unsaturated fatty acids (oleic acids), palm oil protection causes
 285 unsaturated fatty acids to escape rumen microbial biohydrogenation so that negative
 286

Commented [mp25]: Why palm oil did not disrupt the rumen
 pH?? Please describe the reason.
 Already did

287 effects on the rumen environment can be eliminated. Similar results have been reported
 288 in the other studies, such as those conducted by Adeyemi *et al.* (2015), Amanullah *et al.*
 289 (2022), and Roy *et al.* (2017).

290 The reduction in protozoa is attributed to the high content of saturated fatty acids
 291 in palm oil, particularly palmitic acids (C16:0). According to Rahman *et al.* (2022), palm
 292 oil contains about 44% palmitic acid. It has been reported that supplementation of LCFA
 293 in the form of palmitate (C16:0) and stearate (C18:0) resulted in a decrease in the protozoa
 294 population, although the effect of the decrease did not as strong as MCFA, especially
 295 capric acids (C10:0) and lauric acid (C12:0). (Matsumoto *et al.*, 1991). The decrease is
 296 caused by the inability of protozoa to digest fat because they do not have lipolytic
 297 enzymes, as a result the addition of oil can interfere with the metabolic activity of
 298 protozoa and subsequently cause the number of protozoa to decrease (Hartanto *et al.*,
 299 2017).

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

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

Commented [mp26]: What the correlation with pH value??
 This sentence discusses the decline in the protozoa population. To avoid confusion, I separated it into another paragraph.

Commented [mp27]: Does palm oil rich in Unsaturated fatty acid or oleic acid? Please use the relevant references.
 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

Commented [mp28]: Nutrient Digestibility ??
 Already did

Commented [mp29]: What do you mean by 5% Non-protected ZPOS? Does ZPOS mean protected zinc palm oil?
 Correction already done.

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

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

339 Zinc (Zn) plays a pivotal role in numerous biological processes, encompassing
340 enzyme activity, cell membrane stability, gene expression, and cell signaling (Swain *et al.*,
341 2016; Uniyal *et al.*, 2017). It is indispensable for preserving the structural and
342 functional integrity of over 2000 transcription factors and 300 enzymes. It can be
343 contended that virtually all metabolic pathways are reliant, to varying degrees, on at least
344 one Zn-dependent protein (Ranasinghe *et al.*, 2015). Recent research by Fellner *et al.*
345 (2021) elucidated that Zn supplementation precipitated increased acetate production and
346 a heightened acetate/propionate ratio. This delineates an augmented fiber digestion
347 process facilitated by cellulolytic bacteria, consequent to heightened microbial protein
348 synthesis. This conclusion aligns with findings indicating elevated levels of microbial
349 protein compared to PO supplementation (P1), specifically 13.37 (T2) and 11.41 (T3)
350 versus 9.37 mg/ml in the P1 treatment (Table 3).

351 **Relative Proportion of VFA**

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

Commented [mp31]: Please describe first why it is happening???
Already did

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

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

381 **Fatty Acids in Rumen Liquid**

382 Previous research showed that oil supplementation caused a decrease in the
383 proportion of short-chain fatty acids in rumen fluid while increasing the presence of long-
384 chain fatty acids. Mancini *et al.* (2015) argue that this transition is caused by the large
385 number of long-chain fatty acids (LSFA) in palm oil, namely 98.3%, and the remaining
386 1.7% consists of short-chain fatty acids (SCFA). Among long-chain fatty acids, which
387 account for 98.3% of the total, almost half (49.9%) are unsaturated fatty acids (USFA),
388 consisting of 39.2% monounsaturated fatty acids (MUFA) and 10.5% polyunsaturated
389 fatty acids (PUFA). The prevalence of saturated fatty acids (SFA) in palm oil contributes
390 to the range of rumen saturated fatty acid levels, where PO supplementation treatment
391 (T1) has higher SFA levels compared to ZPOS supplementation, namely T1 71.14%, T2
392 61.85%, and T3 59.67% (Table 6).

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

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

406 concentrations compared to non-protected oil supplementation (T1). Amanullah *et al.*
407 (2022) conducted a comparative analysis involving corn oil, linseed oil, and Ca salt
408 (protected C18:2n-6), revealing that protected fatty acids (Ca Salt) led to diminished
409 stearate levels but higher proportions of MUFA and PUFA. These findings are consistent
410 with the research by Satir *et al.* (2023), which demonstrated that 3% protected palm oil
411 supplementation can increase the content of palmitic acid (SFA) and oleic acid (MUFA).
412 Stearate is the final product of the biohydrogenation of oleic, linoleic, and linolenic acids.
413 This indicates a complete inhibition of the biohydrogenation process in the ZPOS
414 treatment. The protection of polyunsaturated fatty acids through saponification involves
415 bonding the carboxyl group with Zn, resulting in the inhibition of isomerization, which is
416 a prerequisite for the hydrogenation of unsaturated fatty acids into saturated fatty acids.
417 This is reflected in the higher proportion of MUFA in the ZPOS treatment compared to
418 PO and the control (Table 6). The substantial presence of PUFA, notably EPA and DHA,
419 underscores Zn's role in the elongation and desaturation process, facilitated by the
420 formation of arachidonic acid (20:4) through delta-6-desaturase (D6D) and delta-5-
421 desaturase (D5D) enzymes (Ridgway, 2016). Zn's indispensability is further highlighted
422 as a pivotal mineral cofactor for D6D and D5D enzymes (Noland & Drisko, 2020).

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

428

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CONCLUSION

430 Based on the results of this research, it can be concluded that zinc soap from palm
 431 oil (ZPOS) can be developed as a source of energy and organic Zn. [Supplementation of](#)
 432 [5% partial ZPOS \(3.75% ZPOS+1.25% PO\) is better than 5% ZPOS](#). Beneficial effects
 433 this supplementation are increase fiber digestibility, VFA, LCFA and USFA, as well as
 434 decrease methane production in the rumen. Further investigation is required to test the
 435 ZPOS on dairy cows *in vivo*.

Commented [mp32]: In conclusion, Which level suggested the best treatment: 5% ZPOS or Partial ZPOS??
 Already did.

436

437

CONFLICT OF INTEREST

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

441

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Commented [mp33]: Please add the contract number and the year of the project.
 Already done

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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: ¹Total 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	<i>p</i> -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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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	0.850	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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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

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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682**Table 5.** In vitro proportion of fatty acids in rumen liquid with supplementation of zinc 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.

686
 687

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.

696

Commented [mp34]: There are not SA, LA, and UFA in the table

Please use the same abbreviations between the table and the notes

Corrected already done



Anis Muktiani <anis.muktiani@gmail.com>

Due date reminder

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Fri, Aug 16, 2024 at 1:01 PM

To: Anis Muktiani <anis.muktiani@gmail.com>

Dear Dr. Anis Muktiani,

Thank you for submitting the Copyediting revision of your manuscript. We will first check the submitted file. After the copyediting stage, we will send you the PROOF of your manuscript and ask you to check the final version.

Regards,

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Participants

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Dr Anis Muktiani (anismuktiani)

Messages

Note

From

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

komang

2024-08-20 09:14

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).

AM

Submission No. TASJ-56357

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Thank you for your article submission, and we are looking forward to receiving your incoming articles.

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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 of 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 (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 °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 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: ¹Total 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 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 samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

Nutrient digestibility (%) = $\frac{[(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})) / \text{Nutrient sample, g}] \times 100$

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 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% H₂SO₄ 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:

$E (\%) = \frac{[(0.622 pA + 1.091 pP + 1.558 pB) / (pA + pP + 2pB)] \times 100$

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: $[1 / (0.1 \times 0.0625 \times 16 \times 5)] \times 1000 \times DF \times C$, 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: ^{ab} 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 MCFAs, 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: ^{ab} 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: ^{ab} 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 of 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 (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 °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 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: ¹Total 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 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 samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

$$\text{Nutrient digestibility (\%)} = \frac{[\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})]}{\text{Nutrient sample, g}} \times 100$$

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 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% H₂SO₄ 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:

$$E (\%) = \frac{[(0.622 pA + 1.091 pP + 1.558 pB)]}{(pA + pP + 2pB)} \times 100$$

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: $[1 / (0.1 \times 0.0625 \times 16 \times 5)] \times 1000 \times DF \times C$, 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: ^{ab} 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 MCFAs, 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: ^{ab} 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: ^{ab} 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 of 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 (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 °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 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: ¹Total 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 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 samples, which are called blanks. Nutrient digestibility samples are calculated by the formula:

Nutrient digestibility (%) = $\frac{[(\text{Nutrient sample, g} - (\text{Nutrient residue, g} - \text{Nutrient blank, g})) / \text{Nutrient sample, g}] \times 100}{}$

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 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% H₂SO₄ 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:

$E (\%) = \frac{[(0.622 pA + 1.091 pP + 1.558 pB) / (pA + pP + 2pB)] \times 100}{}$

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: $[1 / (0.1 \times 0.0625 \times 16 \times 5)] \times 1000 \times DF \times C$, 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: ^{ab} 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 MCFAs, 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: ^{ab} 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: ^{ab} 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 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).

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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