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EFFECT OF DIFFERENT UNSATURATED FATTY ACIDS SOURCES ON *IN VITRO* FERMENTABILITY AND DIGESTIBILITY OF RATION IN DAIRY CATTLE

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ABSTRACT: Supplementation of oil rich in unsaturated fatty acids (FAs) such as canola, soybean, and palm oils improved the quality of milk fatty acids. However, the unprotected unsaturated oil might impair rumen fermentation, feed, and fiber digestibility. A study was conducted to determine the best type of oil supplementation (factor A) including canola (A1), soybean (A2), or palm (A3) and level oil supplementation (factor B) including B0 = 0%, B1 = 1%, B2 = 2% or B3 = 3%) on the *in vitro* feed fermentation and digestibility. The study used a 3 x 4 factorial block design. Two-stages were used to measure the pH, ammonia (NH3), volatile fatty acids (VFAs), protozoal number, dry matter (DMD), organic matter (OMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD), digestibility. The results showed that oil type did not significantly influence the fermentability (pH, NH3, VFAs, and protozoa) and feed's digestibility (DMD, OMD, NDFD, and ADFD) but oil level influence the fermentability and digestibility significantly. In addition, an increase above 1% in oil levels reduced protein fermentability, protozoal number, DMD, and OMD, but increased VFAs. It is concluded that the addition of unprotected canola, soybean, or palm oil in dairy cattle ration could be applied in a concentration not more than 1%.

Keywords: Canola oil, Milk fatty acid, Palm oil, Ration, Soybean oil.

INTRODUCTION

Milk fatty acids (FAs) have become increasingly important in consumer awareness due to their link with human's health (AbuGhazaleh, 2008; Bauman et al., 2006; Despal et al., 2021a). They have positive and negative effects (Chen and Liu 2020). About 400 FAs were identified in milk with 4 to 26 carbons chain length with different degrees, positions, and configurations of unsaturation (Amores and Virto, 2019). However, not more than 30 FAs can be detected in tropical dairy cattle milk (Anzhany et al., 2021; Despal et al., 2021a; Riestanti et al., 2021) due to their small quantity. Saturated FA increases the risk of some diseases (Despal et al., 2021b). Actually, not all SFA harm human's health. Only the C12:0, C14:0, and C16:0 were considered unhealthy. While the C4:0, C6:0, C8:0, C10:0, C18:0 have been reported to have beneficial effects (González-Martín et al., 2020). In contrast, polyunsaturated fatty acids (PUFAs) have a beneficial effect since they decrease both the low-density lipoprotein cholesterol (LDL-C) and the serum cholesterol levels (Chen and Liu 2020).

In most human's diet, unsaturated fatty acids (UFAs) are found in a *cis* configuration. However, *trans* fatty acid (TFA) configurations were found in milk. The TFA has been linked to a negative effect on human health. It resulted from partial hydrogenation of UFAs (Amores and Virto 2019). Although conjugated linoleic acids (CLAs) have a TFA configuration resulting from partial biohydrogenation in the rumen, it has been separated from TFA (Chen and Liu 2020). Furthermore, CLAs may have different health benefits from TFAs, such as anti-cancer and anti-atherosclerosis activities (Despal et al., 2021a). Therefore, many strategies have been planned to increase the CLA content in milk (AbuGhazaleh, 2008; Oliveira et al., 2018; Prieto-Manrique et al., 2018; Pi et al., 2019). One of the strategies is supplementation with oil rich in UFA such as fish oil, sunflower rubber seed oil (AbuGhazaleh, 2008), flaxseed oil (Pi et al., 2019), safflower oil (Shi et al., 2015), palm oil prill fat (Riestanti et al., 2021), canola oil and soybean oil (Loor and Herbein, 2003).

Studies showed that addition of unprotected UFA to increase milk CLA content impaired rumen fermentation, feed and fiber digestibilities that induced milk fat depression (Hussein et al., 2013; Baldin et al., 2014; Pi et al., 2019). Unsaturated lipid supplements affect the hydrogenation process and result in different intermediate products in the rumen. Identifying precursors leading to the production of 18:1 and 18:2 isomers with a *trans*10 double bond in the

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rumen is of interest because they depress milk fat synthesis (Loor and Herbein, 2003). Canola oil contains about 12% α-linolenic acid (omega-3) and 65% oleic acid (Ghazani and Marangoni 2016). It contains high *cis*9-18:1, an omega-3. Soybean oil meal contained 10% palmitic acid (16:0), 4% stearic acid (18:0), 18% oleic acid (18:1), 55% linoleic acid (18:2), and 13% linolenic acid (18:3). It also contains high 18:2n-6. Supplementation of fat high in triglyceride-bound 18:2n-6 produced high *trans*11-18:1 and *cis*9, *trans*11-18:2 accumulations that could alter the profiles of intermediates product in the rumen, thereby affecting the amounts available for absorption in the small intestine. Palm oil contained less UFA than soybean and canola oils. It contained 50% SFA (80% palmitic (C16:0), 10% stearic (C18:0) and myristic (C14:0), 40% oleic (C18:1), and 10% polyunsaturated linoleic (C18:2) and linolenic acid (C18:3). However, this oil is more available and affordable to dairy farmers. This study aimed to determine the optimum canola, soybean, and palm oil supplementation level that does not impair the dairy ration's fermentation and digestibility.

MATERIALS AND METHODS

Ethical regulation

As a source of inoculant in the *in vitro* study, rumen liquor was collected from two fistulated Holstein Frisian bulls kept in the field of laboratory Dairy Nutrition, Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, IPB University. Licensed veterinarians conducted the surgery for fistula implantation. The surgery- and animal handling and care followed the IPB University Animal Ethics Committee regulation.

Dairy cattle ration preparations

This experiment's basic dairy cattle ration includes 58.28% Napier grass, 33.62% concentrate, and 8.10% tofu waste. In addition, oils were added at 0% (B0), 1% (B1), 2% (B2), and 3% (B3) of canola, soybean, or palm oils, respectively. The composition of each ration and their nutrient content are shown in Table 1.

Table 1 - Ingredient and nutrient content of experimental rations

Ingredients and Nutrient contents	Rations			
	B0	B1	B2	B3
Ingredients				
Napier grass	58.28	57.7	57.14	56.58
Concentrate	33.62	33.29	33.96	32.64
Tofu waste	8.10	8.01	7.94	7.86
Oils (canola, soybean or palm)	0	1	2	3
Nutrient contents				
Ash	9.98	9.88	9.78	9.68
Crude protein	12.03	11.91	11.79	11.67
Ether extract	2.54	3.47	4.45	5.37
Crude fiber	22.47	22.25	22.02	21.81
Calcium	0.667	0.660	0.653	0.646
Phosphorus	0.481	0.476	0.471	0.467
TDN	57.17	58.42	59.67	60.80

TDN = total digestible nutrients, B0, B1, B2, and B3 = ration with addition 0, 1, 2, and 3% of oils.

Fermentability measurement

Two fistulated Holstein Frisian dairy bulls breed were used as an inoculant source. The rumen liquid was taken in the morning before feeding by filtering the content with two-fold cheese cloths. It was then kept in a warm container and transported into the laboratory. Feeds fermentability measurements were conducted following Tilley and Terry's first stage (Tilley and Terry, 1963). First, the 0.5 g sample was placed in a 100 ml fermentor tube, and 40 ml McDougall buffer and 10 ml rumen liquid were added. The tube was then aerated with CO_2 for 30 seconds to build an anaerobic condition, closed with ventilated rubber stopper, and placed in a 39°C water shaker bath. The fermentation lasted for 4 hours and was stopped by adding two drops of saturated $HgCl_2$. Afterwards, the tube was centrifuged at 3000 rpm for 15 minutes, and then the supernatant was collected and stored chills until observation of fermentability parameters. The fermentability parameters including pH, ammonia (NH3), and volatile fatty acid (VFA) concentrations were observed. The pH was measured using the Hanna HI98191 pH meter. Ammonia was measured using the Conway method, while VFA was measured using the steam distillation method.

Digestibility measurement

Digestibility measurement was conducted following a two-stage method (Tilley and Terry, 1963). The first is fermentative digestion, which followed a procedure similar to the fermentability measurement above but lasted 48 hours. After cancelling the fermentation activity, the tube was centrifuged at 3000 rpm for 15 minutes and the supernatant was removed. In the second stage, 50 ml 2% HCl-pepsin was added to the tube and incubated aerobically in a 39°C shaker water bath for 48 hours. Afterwards, the tube was filtered with a predetermined weight of Whatman paper no 41. The

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residue was dried at 60°C oven for 48 hours. Part of the residue was used for NDF and ADF analysis, and the rest was dried in a 105°C oven to determine dry matter (DM) residue. Incineration was then carried out in a 600°C oven for 6 hours to determine ash residue. NDF and ADF feed and residues were determined using fiber analyzer ANKOM 200. The measurement followed the AOCS standard procedure Ba 6a-05 (AOCS, 2005). The digestibility of DM, OM, NDF, and ADF was calculated by subtracting the residue from the sample and expressed as percentages.

Study design and data analysis

The experiment used a 3 x 4 factorial block design with four replications. Factor A is oil types consisting of A1 = canola oil, A2 = soybean oil, and A3 = palm oil. Factor B is oil supplementation levels consisting of B0 = 0%, B1 = 1%, B2 = 2% and B3 = 3%. The SPSS version 20 was adopted for data analysis using analysis of variance (ANOVA) followed by the Tukey test from SPSS version 20 (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Feed fermentability

Feed fermentability that measured from pH, ammonia (NH_3), and VFA parameters is shown in Table 2. The results showed that oil type did not significantly influence fermentability, but its level influenced the ammonia and VFA concentrations as well as the number of protozoa. Moreover, addition of oil significantly ($P<0.05$) reduced the ammonia concentration and protozoal number, but increased VFA concentration significantly ($P<0.05$). The pH value in ranges 6.25 to 7.3 was considered to be suitable for rumen microbial growth (Satter and Slyter, 1974). The normal pH value was found in this study due to the forage was used as the main composition in the ration. The addition of oils slightly reduced the pH from 7.02 to 6.7. Riestanti et al. (2020) reported consistent results, but Pi et al. (2019) showed higher results. The difference in the pH between the two studies is attributed to the ration and oils type used.

Table 2. Fermentability of feeds as the impact of different types and levels of oil supplementation

Items	Levels	Canola	Soybean	Palm	Average
pH	0	7.02±0.30	7.02±0.30	7.02±0.30	7.02±0.30
	1	6.66±0.27	6.69±0.26	6.60±0.28	6.65±0.05
	2	6.65±0.36	6.80±0.27	6.75±0.40	6.73±0.09
	3	6.70±0.27	6.80±0.27	6.74±0.25	6.75±0.06
	Average	6.76±0.18	6.83±0.14	6.78±0.18	
NH_3 (mM)	0	6.51±1.03	6.51±1.03	6.51±1.03	6.51±1.03^{a}
	1	3.75±0.34	3.75±1.03	4.32±0.53	3.94±0.33^{b}
	2	3.30±0.20	4.43±0.36	3.86±0.40	3.86±0.57^{b}
	3	3.53±0.86	4.66±1.61	3.97±0.52	4.05±0.57^{b}
	Average	4.28±1.51	4.84±1.19	4.67±1.25	
VFA (mM)	0	68.11±3.29	68.11±3.29	68.11±3.29	68.11±3.29^{b}
	1	105.1±28.6	92.16±6.47	140.3±70.5	112.5±24.9^{ab}
	2	145.6±35.1	130.7±22.0	152.9±52.8	143.0±11.3^{a}
	3	138.6±53.1	138.2±24.0	162.0±33.4	146.2±13.6^{a}
	Average	114.4±35.5	107.3±33.0	130.8±42.8	
Protozoa (log/ml)	0	6.63±0.10	6.63±0.10	6.63±0.10	6.63±0.10^{a}
	1	6.32±0.38	6.49±0.08	6.52±0.06	6.44±0.11^{b}
	2	6.37±0.02	6.38±0.16	6.47±0.14	6.40±0.06^{bc}
	3	6.25±0.02	6.26±0.10	6.19±0.05	6.23±0.05^{c}
	Average	6.40±0.17	6.44±0.16	6.46±0.19	

NH_3 = ammonia, VFA = volatile fatty acids, different superscript at different rows in the same parameter, expressed a significantly different ($P<0.01$) between the level of oils. The different superscripts at the different columns in the same parameters expressed a significantly different ($P<0.05$) between oil types.

The addition of oils at 1% reduced ammonia concentration to 4 mM. However, the concentration was still sufficient to support microbial growth (Satter and Slyter, 1974). The alteration of rumen fermentation after the addition of unprotected oils was reported by Pi et al. (2019). However, the ammonia concentration in their study was higher (8.99 - 9.10 mM after adding 4% rubber seed and flaxseed oils). The higher ammonia concentration reported by Pi et al. (2019) is due to the *in vivo* study used. Riestanti et al. (2021) reported ammonia concentration in the range of 8 - 10 mM in an *in vitro* study conducted after adding 2 - 6% protected oil. Furthermore, Jayanegara et al. (2021) reported an increasing ammonia concentration after adding 1 - 5% maggot oil, which contained short-medium fatty acids (SMFA). The short-chain fatty acid (SCFA) supplementation effect on rumen fermentation is not well documented. In contrast, medium-chain fatty acids (MCFA) were shown to disrupt rumen metabolism by decreasing the number of protozoa, depressing fiber

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degradability (Dohme et al., 2004). Also, 4% MCFA supplementation level has been reported to disrupt microbial populations (Hristov et al., 2009).

Here, oils addition in a concentration of up to 3% could increase the VFA concentration up to 146 mM which is similar to the *in vitro* study of Riestanti et al. (2021). In contrast, Jayanegara et al. (2021) showed no influence of maggot oil supplementation up to 5% on VFA concentration. In the *in vivo* study of Pi et al. (2019), it was demonstrated that ammonia and VFA decreased with oil supplementation. The addition of fat in dairy ration to increase the energy content in high producing cattle is commonly conducted to prevent high concentrate usage and milk fat depression (Hristov et al., 2009). However, the result varies depending on the saturation of FAs (Avila et al., 2000), fat level, and the diet (Palmquist and Jenkins, 1980).

Protozoal found in this study was 6.23 - 6.63 log/m, and the oil supplementation reduced this number significantly at $p<0.05$ but still in the normal range of the rumen protozoal population (McDonald et al., 2010). This result is similar to Riestanti et al. (2021) and Jayanegara et al. (2021) who reported a slight increase in the protozoal number as maggot oil level increased. Furthermore, Jayanegara et al. (2021) reported that C12:0 fatty acid (rich in maggot oil) was toxic for methanogens, partially eliminating ciliate protozoa, and depressed fiber fermentation by cellulolytic microbes (Machmüller et al., 2002).

Feed digestibility

The digestibility of feed and fiber is shown in Table 3. The DMD and OMD ration found in this experiment was more than 50%, except for the high level of Canola oil supplementation that produced below 50%. However, the DMD and OMD here were lower than the normal ration digestibility for dairy cattle (67 - 71%) (Zahera et al., 2015; Hasanah et al., 2017) and dairy goat's ration (60 - 65%) (Despal et al., 2017). Moreover, low DMD and OMD were due to the addition of oil (Riestanti et al., 2021) and the *in vitro* method used in the assessment. According to the *in vivo* conditions, lower digestibility means higher nutrient excess to the environment.

Table 3 - Digestibility of feeds as an impact of different types and levels of oil supplementation

Items	Level	Canola	Soybean	Palm	Average
DMD	0	63.55±4.13	63.55±4.13	63.55±4.13	63.55±0.00^{a}
	1	59.30±1.33	61.08±3.67	54.0 6±11.44	58.15±3.65^{ab}
	2	54.05±6.90	60.57±4.12	56.12±4.64	56.91±3.34^{ab}
	3	48.67±12.1	53.33±2.06	60.62±3.36	54.21±6.03^{b}
	Average	56.40±6.46	59.64±4.40	58.59±4.30	
OMD	0	62.32±2.49	62.32±2.49	62.32±2.49	62.32±0.00^{a}
	1	58.07±0.88	59.76±1.84	60.16±5.98	59.33±1.12^{ab}
	2	51.85±7.77	56.34±2.26	57.29±6.94	55.16±2.91^{bc}
	3	45.29±12.0	50.76±3.27	56.01±3.27	50.69±5.37^{c}
	Average	54.39±7.44	57.3±5	58.95±2.85	
NDFD	0	64.41±6.44	64.41±6.44	64.41±6.44	64.41±6.44
	1	52.50±13.7	58.53±4.46	59.35±5.66	56.79±3.75
	2	58.87±7.16	56.75±8.22	60.00±5.49	58.54±1.66
	3	55.38±6.34	60.31±5.63	57.02±8.70	57.57±2.51
	Average	57.79±5.13	60.00±3.28	60.20±3.09	
ADFD	0	50.70±11.4	50.70±11.4	50.70±11.4	50.70±11.4
	1	37.41±19.1	45.88±8.58	43.06±12.2	42.12±4.31
	2	46.66±16.9	42.73±13.8	43.94±11.6	44.45±2.02
	3	40.01±11.8	45.64±11.8	42.86±14.6	42.84±2.82
	Average	43.70±6.09	46.24±3.31	45.14±3.74	

DMD = dry matter digestibility, OMD = organic matter digestibility, NDFD = neutral detergent fiber digestibility, ADFD = acid detergent fiber digestibility. Different superscripts at different rows in the same parameter expressed a significantly different ($P<0.01$) between the level of oils. The different superscripts at the different columns in the same parameters expressed a significantly different ($P<0.05$) between oil types.

Oil types have no significant effect on digestibility, but their levels affect DMD and OMD. However, fiber digestibility was not significantly reduced. Furthermore, addition of 1% and 2% oils did not significantly reduce OMD and DMD, respectively. The insignificant difference in fiber digestibility is due to the high variation of data shown by the high standard deviations.

The negative effect of fat or oil supplementation on rumen fermentation does not apply to all conditions (Palmquist and Jenkins, 1980). Several reported studies showed decreasing in digestibility coefficient due to fat supplementation (Pi et al., 2019; Riestanti et al., 2020). It was reported that oil supplementation specifically reduced the fiber digestion due to depression in the cellulolytic bacterial population (Riestanti et al., 2021). The addition of oils set in this experiment comprised less than 5% of the total ether extract in the diet. However, the unprotected type of the used oil might impair the rumen fermentation.

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Furthermore, the oil's energy supplemented did not compensate for the declination of digestibility. It might be caused by the *in vitro* method that did not measure post ruminal digestibility (Tilley and Terry, 1963). Many studies also compared the different effects of the *in vitro* and *in vivo* oil and fat supplementation on digestibility (Riestanti et al., 2020; Riestanti et al., 2021). In the *in vivo* study, the dependency of ruminants on non-glucose metabolites for energy metabolism explains these results. In lactating cattle, daily FA output in the milk might exceed daily intake (Palmquist and Jenkins, 1980). Therefore, lipid metabolism plays an essential role in these different results. However, it is not easy to be imitated in an *in vitro* study.

CONCLUSION

The *in vitro* addition of unprotected canola, soybean, or palm oil as sources of UFAs to increase CLA content in milk did not influence rumen fermentability and feed digestibility. Oil levels could influence fermentability and digestibility. The addition of up to 1% of all of type oils did not impair feed digestibility. Therefore, it is recommended to protect the oil from biohydrogenation in the rumen to increase its utilization in the dairy ration. The use of *in vivo* study is also recommended.

DECLARATIONS

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Authors' contribution

Despal D. designed the study, searched for funding, supervised the laboratory work, wrote and reviewed the manuscript. Permana I.G. and Zahera R. designed the study, searched for funding, supervised the laboratory work, and reviewed the manuscript. Irmadani D. designed the study, conducted the laboratory work, and wrote the manuscript. Nuraina N. supervised the laboratory work, analyzed the data, edited and reviews the manuscript.

Conflict of interests

The authors have not declared any conflict of interest.

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