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Effect of polyphenol-rich oil palm empty fruit bunch extract on *in vitro* rumen fermentation, fatty acid profile and microbial population

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Abstract: The aim of this study was to evaluate the effect of oil palm empty fruit bunch (OPEFB) extract on *in vitro* rumen fermentation, fatty acid profile, and microbial population. Rumen fluid was obtained from three female dairy goats fed a similar diet of 60% Napier grass and 40% commercial pellets. The substrate used for the fermentation was a Napier grass and commercial pellet mixture (60 : 40 in dry matter) and the treatment diets were: CON (substrate without OPEFB extract), OPEFB-5 (substrate with 5% of OPEFB extract), OPEFB-10 (substrate with 10% of OPEFB extract). The characteristics of rumen fermentation including pH, fermentation kinetics, total gas production, volatile fatty acid (VFA) production, fatty acid (FA) production, and microbial populations were examined. Results showed that OPEFB supplementation decreased rumen acetate concentration, increased isoacid concentrations as well as palmitic acid concentration. Meanwhile, the population of total bacteria, protozoa, and *B. fibrisolvens* decreased with the OPEFB supplementation. OPEFB-5 resulted in a moderate amount of acetate, isobutyrate, isovalerate concentration, insoluble fraction of gas production (but degradable), estimated potential gas production as well as *B. fibrisolvens* population when compared with the control and OPEFB-10 diet. Therefore it is suggested that the supplementation of OPEFB extract at 5% is suitable and practical to be used in ruminant feed without causing an imperative effect on rumen fermentation.

Keywords: *B. fibrisolvens*; empty fruit bunch extract; fermentation kinetics; protozoa population; volatile fatty acids; total gas production

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Oil palm empty fruit bunches (OPEFB) and fronds are two types of oil palm by-products from an oil palm plantation. In the palm oil mill, OPEFB production accounts for 23% of fresh fruit bunches (FFB) after being processed (Samadhi et al. 2020). The disposals of these waste products were either carried out by returning to the plantation or burnt off within nearby factory facilities, which is always a concern of every oil palm factory management during peak production seasons.

Feed interventions such as incorporating plant oil rich in linoleic acid (Almeida et al. 2013), and plant seeds (Schettino et al. 2017), among others, is a strategy to enhance beneficial fatty acids in animal products for human health benefits. Similarly, supplementation of plant phenolic compounds to the ruminant diet has shown promising results in reducing biohydrogenation in the rumen (Wanapat et al. 2014). Oil palm leaves, which contain high levels of polyphenols and tannin (Jaffri et al. 2011), can reduce biohydrogenation in ruminants (Cabiddu et al. 2009).

In the present study, OPEFB, a by-product of palm oil plantation, was utilised through extraction to obtain plant phytochemicals to incorporate it in the animal feed to further improve animal production. The extraction process of obtaining phytochemical compounds from the OPEFB can rule out the biggest predicament of using OPEFB in animal feed, high lignocellulosic content, while still benefiting from it through its bioactive compound content. The complexity of plant phytochemicals and the differences they would make towards the rumen microbial ecosystem were attractive, knowing the biological properties these compounds of interest would exhibit. A study by Al-Gorany et al. (2020) stated that the ethanolic extract of OPEFB contained natural compounds that have antioxidant, anti-inflammatory, antimicrobial, antifungal, antitumor, antiviral, anticonvulsant, analgesic and as well as antidiabetic properties. It was also reported that OPEFB extracts in both forms; wet and dried, exhibit radical scavenging activities which indicate the presence of antioxidant properties in the extracts (Han and May 2012). Despite the said extract having these potential beneficial biological properties, the ramification of using phytochemicals extracted from OPEFB on rumen fermentation in ruminants is still poorly understood. It is imperative to assess the potential of the OPEFB extract supplementation on *in vitro* rumen fermentation profile as well as microbial population of the rumen. Hence, this study aims to evaluate the effect of the OPEFB extract supplementation on *in vitro* rumen fermentation characteristics in goats.

MATERIAL AND METHODS

Preparation of substrate and treatment diets

In the present study a 60 : 40 (in dry matter) mixture of Napier grass and commercial pellets was used as the basal substrate. The dietary treatment was designed according to Kim et al. (2015) and Avila et al. (2020).

Each of the basal substrate materials was dried separately in a 60 °C oven for 24 h and was ground to pass through a 1 mm sieve. As for the OPEFB extract, the empty fruit bunches were obtained from an oil palm processing plant in Banting, Selangor, Malaysia (Eng Hong Palm Oil Mill Sdn. Bhd.) and they were soaked in methanol at the ratio of 1 : 3 w/v for 72 h, and shaken for an hour prior to the collection of the methanolic extract and strained through a 4-layer muslin cloth before storage at room temperature (26 ± 1 °C). The methanolic extract was centrifuged at 4 000 × *g* for 20 min before storage at room temperature if the methanolic extract still contained large particles from the empty bunches. The methanolic extract was then evaporated under partial vacuum using a rotor evaporator (Heidolph, Germany). The OPEFB extract is then included in the weighed mixed substrates at their respective inclusion percentage (0%, 5% and 10% w/w) prior to the start of the *in vitro* gas production study.

Dry matter, ash, crude protein, and ether extract of the substrates were determined according to AOAC (1999). Neutral detergent fibre, acid detergent fibre and acid detergent lignin were determined according to Van Soest et al. (1991). While the phytochemical content of OPEFB extract was analysed according to Ismail et al. (2010) for the total phenolic content, Quettier-Deleu et al. (2000) for the total flavonoid content, and Hiai et al. (1976) for the total tannin content. The component and chemical composition of the basal diet as well as the phytochemical content of OPEFB are presented in Table 1.

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Table 1. Nutrient composition of basal diet and phytochemical content of OPEFB extract

Items	Basal diet (g/kg DM)
Component of the basal diet	
Napier grass	600.00
Commercial pellet	400.00
Chemical composition of basal diet	
Dry matter	96.69
Organic matter	86.85
Crude protein	15.20
Neutral detergent fibre	59.47
Acid detergent fibre	31.40
Acid detergent lignin	4.60
Ether extract	4.05
Phytochemical content of OPEFB	OPEFB extract (mean % of DM)
Total phenolic	3.98
Total flavonoid	2.37
Total tannin	1.30

DM = dry matter; OPEFB = oil palm empty fruit bunch

Animal management and rumen fluid collection

The experiment protocol was approved by the Animal Care and Ethics Committee of the university (UPM/IACUC/AUP-R0048/2017) before the start of the experiment. Three female Saanen dairy goats were used as rumen fluid donors. The animals were fed a 60 : 40 (in dry matter) mixture of Napier grass and commercial pellets, at 3.0% of body weight (BW), twice a day. The animals were kept in individual cages where water and mineral blocks were always available. Rumen fluid was obtained from each of the does at 8 a.m., before morning feeding through stomach tubing. Then, the rumen fluid was strained through a 4-layer muslin cloth and kept in a pre-warmed thermos flask that was pre-flushed with carbon dioxide and transported to the laboratory for subsequent *in vitro* gas production protocols.

In vitro gas production study

The gas production study was conducted by using the OPEFB extract at three levels of inclusion (0%, 5%, or 10%). The incubation for the gas production study was done according to Fievez et al. (2005). The incubation was repeated thrice with 5 replicates per treatment. An amount of 250 mg of substrates was weighed and placed in 100 ml calibrated glass syringes (Model Fortuna; Haberle Labortechnik, Lonsee-Ettlenschieb, Germany). Next, the said extract was placed in the randomly assigned glass syringes. Then, 30 ml of buffer (bicarbonate and phosphate buffers, 50 : 50 v/v) and rumen mixture at a 1 : 4 ratio was dispensed into the calibrated pre-warmed syringes under constant carbon dioxide flushing. The syringes were gently shaken and immediately incubated in a water bath of 39 °C. Two blanks were included in the incubation that acted as a correction. Gas production at 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation was recorded. Upon termination of incubation at 24 h, rumen pH was recorded. An amount of 2 ml of the content from each syringe was sampled for the microbial population study, and 10 ml of the content was acidified with 2 ml of 25% meta-phosphoric acid for volatile fatty acid and fatty acid analysis. Both samples were kept in a –20 °C freezer until further analyses.

The gas production data were fitted to the following model of Orskov and McDonald (1979):

$$Y = a + b\,(1 - \exp^{-ct}) \quad (1)$$

where;

Y – volume of gas produced at time t;

a – gas production from the immediately soluble fraction (ml);

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b – gas production from the insoluble fraction (ml);
c – gas production rate constant for the insoluble fraction (*b*);
t – incubation time (h).

Thawed samples were centrifuged at 3 000 × *g* for 10 min at 25 °C, and 0.5 ml of the supernatant was filtered (0.45 µm) and added to 0.5 ml of 4-methyl-*n*-valeric acid (20 mM) before being analysed by using a 6890N Network GC System gas chromatograph (Agilent Technologies, Palo Alto, CA, USA).

Volatile fatty acid analysis

Rumen samples were left thawed at room temperature. Then, the subsequent procedure was done according to Filipek and Dvorak (2009). The samples were analysed by using gas chromatography (Agilent 69890N Series Gas Chromatography System from Agilent Technologies, USA) fitted with Flame Ionisation Detector (FID). The column used was 30 m × 0.25 mm × 0.25 µm (DB-FFAP) with oven temperature of 200 °C, FID temperature of 230 °C and nitrogen gas as the carrier gas with a flow rate of 60 µl per minute. The volatile fatty acid concentrations were expressed as mol/100 mol of the identified volatile fatty acid. As internal standard, 4-methyl-*n*-valeric acid (Sigma, St. Louis, MO, USA) was used.

Rumen ammonia-nitrogen (N) analysis

Rumen fluid samples were thawed and centrifuged at 6 000 × *g* for 10 minutes. The supernatant was collected and ammonia-N determination was done according to Parsons (2013). The stock standard solution of ammonium chloride was prepared to give a 1 000 mg/l of solution. Standards of 0.2, 0.5, 1.0 and 2.0 ppm solutions were prepared from the stock solution for the construction of the regression equation. The regression equation was constructed from blank and standard samples and ammonia-N levels in the samples were determined. The absorbance of the intensity was read at 640 nm by using a spectrophotometer (Spectronic GENESYS 20 Spectrophotometer 4001 Series; Thermo Electron Corp., USA). The rumen ammonia-nitrogen concentrations were expressed in mg/dl.

Quantification of rumen microbial population by qPCR

The population of total bacteria, total protozoa, total fungi, total methanogens, Methanobacteriales, *Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes,* and *Butyrivibrio fibrisolvens* were quantified by qPCR. Genomic DNA was obtained from a rumen fluid sample and total DNA extraction was carried out by using the QIAamp® DNA stool mini kit (Qiagen Ltd, Crawley, West Sussex, UK) following the manufacturer's instructions. An absolute quantification of rumen microbes was achieved based on the standard curve method in real-time PCR. Standard curves were constructed from the amplification of known amounts of target microbe DNA. The qPCR master mix was prepared for a total volume of 25 µl using the QuantiFast® SYBR® Green PCR kit (Qiagen Inc., Valencia, USA) consisting of 12.5 µl of 2 × SYBR Green Master Mix, 1 µl of 20 µM forward primers, 1 µl of 20 µM reverse primers, 2 µl of DNA samples and 8.5 µl of nuclease-free water for each reaction. Each sample was analysed in duplicate. Primers used in the PCR amplifications for targeted rumen microbial populations are detailed in Table 2. Real-time PCR quantification was done by using a BioRad CFX96 Real-Time PCR System (BioRad, USA) with optical grade plates. The qPCR cycling conditions consisted of initial 5 min of denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 20 s each cycle, annealing for 30 s and extending at 72 °C for 20 seconds. The annealing temperatures for the primers of total bacteria, total fungi, total protozoa, total methanogens, *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Fibrobacter succinogenes* were 55 °C, while those for *Ruminococcus flavefaciens* and Methanobacteriales were 58 °C. The rumen population concentrations were expressed as $\log_{10}$ copy number/ml of the identified target group of rumen microbial population.

Rumen fatty acid analysis

The amount of fatty acids in syringe content 24 h post incubation was determined as described by Ebrahimi et al. (2015) using a chloroform/methanol (2 : 1) (v/v) mixture containing butylated hydroxytoluene to prevent oxidation during the sample preparation. The transmethylation was

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Table 2. Primers used for qPCR reactions

Target groups	Primer sequence 5' to 3'	Product size (bp)	References
Total bacteria	R: CCATTGTAGCACGTGTGTAGCC F: CGGCAACGAGCGCAACCC	145	Koike and Kobayashi (2001)
Total protozoa	R: GCTTTCGWTGGTAGTGTATT F: CTTGCCCTCYAATCGTWCT	223	Sylvester et al. (2004)
Total fungi	R: CAAATTCACAAAGGGTAGGATGATT F: GAGGAAGTAAAAGTCGTAACAAG GTTTC	121	Lane (1991)
Total methanogens	R: CGGTCTTGCCCAGCTCTTATTC F: CCGGAGATGGAACCTGAGAC	160	Zhou et al. (2009)
Methanobacteriales	R: TACCGTCGTCCACTCCTT F: CGWAGGGAAGCTGTTAAGT	343	Yu et al. (2005)
Fibrobacter succinogenes	R: CGCCTGCCCCTGAACTATC F: GTTCGGAATTACTGGGCGTAAA	122	Lane (1991)
Ruminococus albus	R: CCTCCTTGCGGTTAGAACA F: CCCTAA AAGCAGTCTTAGTTCG	175	Koike and Kobayashi (2001)
Ruminococus flavefaciens	R: CCTTTAAGACAGGAGTTTACAA F: TCTGGAAACGGATGGTA	259	
Butyrivibrio fibrisolvens	R: CCAACACCTAGTATTCATC F: GYGAAGAAGTATTTCGGTAT	417	Boeckaert et al. (2008)

bp = base pair; F = forward primer; R = reverse primer

conducted by using 0.66 N KOH in methanol and 14% methanolic boron trifluoride (Sigma Chemical Co., St. Louis, MO, USA). The fatty acid methyl ester was separated using Agilent 7890A gas-liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) using a 100 m × 0.25 mm ID (0.20 µm film thickness) Supelco SP-2560 capillary column (Supelco, Inc., Bellefonte, PA, USA). The fatty acid concentrations were expressed as g/100 g of the identified fatty acid. A reference standard (Supelco 37 Component FAME Mix; Sigma-Aldrich, Inc., St. Louis, MO, USA) was used to determine recoveries and correction factors for the determination of individual FA composition.

Statistical analysis

All statistical analyses were carried out using SPSS v22 (IBM, USA), through the one-way analysis of variance (ANOVA) procedure in a completely randomised design, to examine the effect of OPEFB extract supplementation on *in vitro* rumen fermentation characteristics. The differences between treatment means were compared using Duncan's multiple range test. The differences between means were considered significant at *P*-values ≤ 0.05.

RESULTS

In vitro gas production

In vitro total gas production and rumen fermentation kinetics are presented in Table 3. The present study shows that CON diet has significantly higher values of *b* [gas production from the insoluble (but degradable) fraction] ($P < 0.05$) and $a + b$ (potential gas production) ($P < 0.05$) as compared to the other diets. Whereas OPEFB-10 showed a significantly higher value of *c* ($P < 0.05$), which is the gas production rate constant for the insoluble fraction. On the other hand, no significant differences were found in the total gas production or in the gas production from the immediate fraction (*a*) ($P > 0.05$).

Rumen fermentation parameters

Table 4 shows the effect of OPEFB extract supplemented diet on *in vitro* rumen fermentation parameters after 24-hour incubation. Acetic acid was significantly decreased by the increasing amount of OPEFB extract in the diet ($P < 0.05$) as shown in Table 4. A significant amount of isobutyric acid ($P < 0.05$) and isovaleric acid ($P < 0.05$) was found in OPEFB-10, followed

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Table 3. Total gas production and rumen fermentation kinetics of the substrate without CON and with extract supplementation at different inclusion levels incubated in the ruminal fluid of goat (mean ± SD)

Items	Treatment			SEM	*P*-value
	CON	OPEFB-5	OPEFB-10		
Total gas production	35.0	31.7	36.5	0.930	0.097
a	4.07	5.66	5.52	0.390	0.179
b	90.3[a]	61.5[b]	41.3[c]	3.82	<0.001
a + *b*	94.3[a]	67.2[b]	46.8[c]	4.17	<0.001
c	0.039[a]	0.051[a]	0.079[b]	0.004	<0.001

a + *b* = potential gas production; *a* = gas production from the immediate soluble fraction (ml/250 mg DM); *b* = gas production from the insoluble (but degradable) fraction (ml/250 mg DM); *c* = gas production rate constant for the insoluble fraction (ml/h); CON = 0%, basal diet: concentrate + Napier grass (40 : 60); DM = dry matter; OPEFB = oil palm empty fruit bunch; OPEFB-10 = 10% OPEFB extract per DM of basal diet; OPEFB-5 = 5% of OPEFB extract per DM of basal diet; SEM = standard error of the mean

[a–c]Values on the same row with different superscripts differ ($P < 0.05$)

Table 4. The effect of OPEFB extract supplemented diet on *in vitro* rumen fermentation parameters of goats (mean ± SD)

Items	Treatment			SEM	*P*-value
	CON	OPEFB-5	OPEFB-10		
pH	6.94	6.95	6.93	0.006	0.707
VFA (mol/100 mol)					
Acetic acid	44.7[a]	40.1[b]	39.1[b]	0.944	0.015
Propionic acid	31.8	33.9	33.9	1.08	0.718
Isobutyric acid	1.56[a]	1.68[a]	2.83[b]	0.183	<0.001
Butyric acid	15.5	18.4	19.6	0.865	0.130
Isovaleric acid	3.44[a]	3.64[a]	8.43[b]	0.856	0.008
Valeric acid	3.40	3.47	3.63	0.114	0.737
Total VFA (mM/ml)	42.7	39.5	34.4	1.821	0.172
A/P ratio	1.20	1.36	1.24	0.034	0.148
Ammonia N (mg/dl)	18.5	24.6	16.3	1.880	0.180

A/P = acetic acid/propionic acid; CON = 0%, basal diet: concentrate + Napier grass (40 : 60); DM = dry matter; OPEFB = oil palm empty fruit bunch; OPEFB-10 = 10% OPEFB extract per DM of basal diet; OPEFB-5 = 5% of OPEFB extract per DM of basal diet; SEM = standard error of the mean; VFA = volatile fatty acid

[a–c]Values on the same row with different superscripts differ ($P < 0.05$)

by OPEFB-5 and CON (Table 4). Meanwhile, there were no significant differences between diets in the rumen pH, and in the concentration of propionic acid, butyric acid, valeric acid, total VFA, ammonia N or in the A to P ratios ($P > 0.05$).

Rumen microbial population

The quantification of the *in vitro* rumen microbial population at three levels of OPEFB extract supplementation by qPCR is presented in Table 5. The study shows that OPEFB extract significantly affected the populations of total bacteria, total protozoa, and *B. fibrisolvens* (Table 5) ($P < 0.05$). CON diet showed the highest population of total bacteria, total protozoa, and *B. fibrisolvens*. Nevertheless, the populations of total methanogens, total fungi, Methanobacteriales, *R. albus, R. flavefaciens*, and *F. succinogenes* in the rumen were not significantly affected by the inclusion of OPEFB extract in the diets ($P > 0.05$).

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Table 5. The *in vitro* rumen microbial population (log_{10} copy No./ml) after application of three diets with different supplementation levels of OPEFB extract (mean ± SD)

Microbial population, log_{10} copy No. per ml of DNA extract	Treatment			SEM	*P*-value
	CON	OPEFB-5	OPEFB-10		
Total bacteria	11.7[a]	11.2[b]	11.0[b]	0.101	0.001
Total protozoa	7.71[a]	6.87[b]	7.27[ab]	0.203	0.041
Total methanogens	5.94	5.60	5.67	0.080	0.208
Total fungi	5.23[a]	6.88[b]	5.46[a]	0.318	0.053
Methanobacteriales	5.57	5.78	5.76	0.058	0.267
Ruminococus albus	10.9	10.2	9.49	0.314	0.185
Ruminococus flavefaciens	9.94	8.92	8.42	0.291	0.079
Fibrobacter succinogenes	7.36	7.07	6.49	0.241	0.245
Butyrivibrio fibrisolvens	6.23[a]	5.91[a]	5.72[b]	0.082	0.018

CON = 0%, basal diet: concentrate + Napier grass (40 : 60); DM = dry matter; OPEFB = oil palm empty fruit bunch; OPEFB-10 = 10% OPEFB extract per DM of basal diet; OPEFB-5 = 5% of OPEFB extract per DM of basal diet; SEM = standard error of the mean

[a–c]Values on the same row with different superscripts differ ($P < 0.05$)

Table 6. The *in vitro* rumen fatty acid profiles of three diets with different supplementation levels of OPEFB extract (mean ± SD)

Fatty acid (g/100 g of total fatty acid)	Treatment			SEM	*P*-value
	CON	OPEFB-5	OPEFB-10		
Capric acid (C10:0)	1.12	1.05	0.990	0.068	0.754
Lauric acid (C12:0)	3.31	3.11	2.59	0.166	0.194
Myristic acid (C14:0)	3.55	4.02	3.50	0.339	0.809
Myristoleic acid (C14:1)	1.33	1.31	1.17	0.155	0.908
Pentadecanoic acid (C15:0)	1.73	1.86	1.47	0.119	0.422
Palmitic acid (C16:0)	31.2[a]	35.7[b]	38.9[b]	1.066	0.004
Margaric acid (C17:0)	9.13	6.71	7.84	0.968	0.622
Stearic acid (C18:0)	31.5	31.8	29.6	0.674	0.384
Oleic acid (C18:1n-9)	10.6	10.6	9.88	0.227	0.366
Linoleic acid (C18:2n-6)	6.53	3.85	4.00	0.626	0.146
Total SFA	81.6	84.3	85.0	0.706	0.166
Total UFA	16.8	16.2	15.5	0.349	0.375
Total MUFA	11.9	11.9	11.0	0.328	0.499
UFA/SFA	0.23	0.19	0.18	0.011	0.125
PUF/SFA	0.08	0.05	0.05	0.009	0.153

DM = dry matter; CON = 0%, basal diet: concentrate + Napier grass (40 : 60); MUFA = monounsaturated fatty acids (C14:1, C18:1n-9); OPEFB = oil palm empty fruit bunch; OPEFB-10 = 10% OPEFB extract per DM of basal diet; OPEFB-5 = 5% of OPEFB extract per DM of basal diet; PUFA = polyunsaturated fatty acids (C18:2n-6); PUFA/SFA = polyunsaturated fatty acids/saturated fatty acids; SEM = standard error of the mean; SFA = saturated fatty acids (C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0); UFA = unsaturated fatty acids (C14:1, C18:1n-9, C18:2n-6); UFA/SFA = unsaturated fatty acids/saturated fatty acids

[a,b]Values on the same row with different superscripts differ ($P < 0.05$)

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Rumen fatty acid profile

The profiles of *in vitro* rumen fatty acids of three different diets are listed in Table 6. The results show that rumen palmitic acid is the only fatty acid that is significantly different between the three diets ($P < 0.05$). The OPEFB-10 diet recorded the highest concentration of rumen palmitic acid, while the lowest concentration was found in CON diet. Nonetheless, the inclusion of OPEFB extract in the diet did not affect other fatty acids such as capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, stearic acid, oleic acid, linoleic acid, total saturated fatty acids, total unsaturated fatty acids, total monoenes, unsaturated to saturated ratio and polyunsaturated to saturated ratio ($P > 0.05$).

DISCUSSION

The *in vitro* rumen fermentation study in this experiment showed a decreasing trend of acetic acid production as the inclusion level of OPEFB extract in the diet increased (Table 1). The reduction in acetate production is a result of the high pressure of hydrogen and the high NADH/NAD^+ ratio in the rumen due to the inhibition of methanogenesis (Miller 1995). Furthermore, Noziere et al. (2011) reported that the acetate molar proportion positively correlates with NDF digestibility ($r = 0.95$) and both supplemental phytochemicals and lipids decreased total tract digestibility of NDF, which is in line with the result of the present study. Therefore, both phytochemicals and lipids contained in the OPEFB extract (Table 1) decreased the molar proportion of acetate.

A trend showed increasing levels of isobutyric acid and isovaleric acid (isoacids) production in the rumen parallelly with the increasing levels of OPEFB extract in the diet (Table 4). The concentration of isoacids in the rumen is the balance between the N produced from degradation and the N utilised by the bacteria to synthesize microbial protein in the rumen (Hume 1970). Although insignificant, the ammonia N concentrations for all the treatment diets were above the level limiting the *in vitro* ruminal microbial growth (5 mg/100 ml) (Satter and Slyter 1974). This suggested that the ruminal microbial growth is not restricted by especially cellulolytic bacteria which utilise isoacids in the rumen, since the concentration of isoacids increased along with the inclusion of OPEFB extract in the diet.

In Table 3, OPEFB-10 diets show the highest total gas production values among the diets while statistically insignificant. Meanwhile, OPEFB-5 diets show the highest value of gas production from the immediate soluble fraction, which suggests that they were digested faster compared to the other diets, albeit statistically insignificantly. In their study Salem et al. (2014) found that the addition of extracts obtained from some tree species to a high concentrate diet linearly increased the *in vitro* potential gas production ($a + b$) and decreased the gas production rate (c) while in the present study the situation was in reverse (Table 3). Where the volume of gas production from insoluble fraction (b), and potential gas production ($a + b$) were decreased, and the gas production rate (c) was increased, which can be explained by the high roughage diet consumed by the animals. This is in line with the study of Nathani et al. (2015), who reported that the diet has an impact on microbial diversity further resulting in the variability of carbohydrate-active enzyme families present in the rumen. The differences were prominent in the rumen solid phase at a higher roughage fraction meanwhile in the rumen liquid phase the presence of carbohydrate-active enzyme families was reduced at 75 : 25 (roughage to concentrate ratio), and it increased at 100% roughage diet. Furthermore, it was also observed in previous studies that more gram-negative bacteria were found compared to gram-positive bacteria in the case of a high forage diet in the rumen, and the situation was reversed in the case of a high concentrate diet (Matthews et al. 2019). Additionally, a previous study showed that feeds producing high amounts of propionate yielded lower gas volumes (Cone and van Gelder 1999), which is in line with the result of the present study where feed with the highest value of propionate concentration, which was OPEFB-5, produced the lowest value of total gas production (Table 4), although insignificant.

This study also highlighted the effect of different levels of OPEFB extract supplementation on the microbial population in the *in vitro* rumen fermentation (Table 5). The inclusion of OPEFB extract decreased the population of total bacteria, *B. fibrisolvens* and total protozoa as compared to the control diet. The reduced population of rumen protozoa in this study can be called partial rumen protozoa

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inhibition. Although the lowest population of protozoa at 5% extract inclusion was unexpected, it is likely that the rumen microorganisms are able to use the plant metabolites in the extract as an energy source (Salem et al. 2014), hence leading to an increase in the protozoa population at 10% extract inclusion. A previous study suggested that supplemental phytochemicals and lipids can be effective in reducing methane production when rumen protozoa numbers are below 7 $\log_{10}$ cells/ml, especially by supplemental saponins, tannins, and medium-chained fatty acids (Dai and Faciola 2019), which is consistent with the results in the present study where the 5% OPEFB extract diet shows the rumen protozoa population lower than 7. Meanwhile, a decrease in cellulolytic bacteria and methanogenic bacteria was often observed with most fat sources included in the diet (Nur Atikah et al. 2018). The *B. fibrisolvens* population in this study was decreased by the inclusion of OPEFB diets. It also suggests that there are substances in the OPEFB extract that affect *B. fibrisolvens* populations in the rumen. It has been reported that condensed tannins and saponins were able to enhance the bacterial population and increase fermentation but suppress the biohydrogenation bacteria population (Khiaosa-Ard et al. 2009). It was in line with the study of Zhu et al. (2014), where the *Butyrivibrio* group in the study was reduced when garlic oil was supplemented to the rumen. The decrement in the *B. fibrisolvens* populations might be related to the increasing concentration of fatty acids in the rumen as supported by Tyagi et al. (2015), whose study revealed that an increase in the concentration of fatty acids suppressed the growth of *B. fibrisolvens* and its cell density reached the maximum. Another study also showed that *B. fibrisolvens* was affected by tannin from sainfoin leaves by causing bacterial morphology alterations (Jones et al. 1994). Lesser populations of *B. fibrisolvens* also suggest that the degradation of cellulose was impeding as it plays an important role in cellulose degradation (Rodriguez Hernaez et al. 2018). Increasing the inclusion of OPEFB extract in the diet reduced the population of total bacteria in this study. Beneficial effects of the plant extract on the animals such as antioxidant, anti-inflammatory, immune and antimicrobial ones have been shown against a wide variety of microorganisms (gram-positive or gram-negative bacteria, fungi, viruses, and protozoa), but they are more effective against the gram-positive bacteria. This was due to most active compounds present being lipophilic. The presence of aromatic hydrocarbons in the extract may destroy the external membrane in gram-negative bacteria (Ugbogu et al. 2019). It was also found that some polyphenols may produce cell apoptosis through breaking up the cell membranes of microorganisms (Tocci et al. 2018).

It has been shown in the present study that rumen fatty acid profiles were altered by the inclusion of OPEFB extract in the diet (Table 6). As the level of OPEFB inclusion increases, the fat content of the feed increases. The present study also showed rumen palmitic acid increases as the dietary OPEFB increases. It was because the extract from OPEFB has the same main fatty acids as the crude palm oil (palmitic acid, oleic acid, linoleic acid, and stearic acid) as the residual crude palm oil remains on the OPEFB after the stripping and threshing process of sterilised FFB where palm oil fruitlets are removed from the branches (Ng et al. 2020). The temperature during OPEFB sterilisation is 140 °C, which caused the leakage of palm oil from the fruitlets (Abdullah and Sulaiman 2013) and thus formed a coating over OPEFB surfaces. Apart from that, the accumulation of mixed rumen bacteria in the rumen also contributed to the increase in the concentration of palmitic acid since the cell membrane of the bacteria is rich in palmitic acid (Or-Rashid et al. 2007). The increment in this study was in line with a study of Vasta et al. (2010), where the content of palmitic acid in the rumen was increased when a tannin-added diet was fed, compared with the diet without tannin addition. The increase in rumen palmitic acid does not, however, imply that the study's rumen bacterial population has increased as well (Vargas-Bello-Perez et al. 2016). It was found that the circumstances were inverted. A study by Sears et al. (2020) showed that feeding palmitic acid-enriched supplement increased fibre digestibility while nitrogen metabolism remained unaffected. Conversely, another study by Piantoni et al. (2013) found that the supplementation of palmitic acid increased milk yield and milk fat concentration without lowering fibre digestibility and dry matter intake. Rumen UFA were decreased as the dietary OPEFB increased, although it was found insignificant in this study, which signifies an increase in rumen biohydrogenation. It was in line with the reduction in the population of *B. fibrisolvens* in the present study as it was known that *B. fibrisolvens* participates in the biohydrogenation processes in the ruminants (Rodriguez Hernaez et al. 2018).

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CONCLUSION

Supplementation of OPEFB extract to the animal feed did not affect rumen fermentation activity, which is reflected by the insignificant gas production and total VFA production. The OPEFB extract diet increased rumen isobutyric acid, isovaleric acid, and palmitic acid concentration while decreasing the concentration of acetic acid as well as the population of total bacteria, protozoa, and *B. fibrisolvens*. However, the rumen fermentation characteristics were moderate in the supplementation of OPEFB-5 compared with the other two diets. Hence, this suggests that 5% OPEFB extract supplementation is suitable to be used in ruminant feed without any adverse effect on the rumen fermentation dynamics of the animal.

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Conflict of interest

The authors declare no conflict of interest.

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