EFFECTS OF OIL PALM EMPTY FRUIT BUNCH METHANOLIC EXTRACT ON RUMEN FERMENTATION, FATTY ACID PROFILES AND MILK COMPOSITION IN GOATS

By

NUR LIYANA AKMAL BINTI HARUN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair : Goh Yong Meng, PhD
Institute : Tropical Agriculture and Food Security

The oil palm empty fruit bunches (OPEFB) are an abundant and easily accessible agricultural by-product from the oil palm industry. The OPEFB has been investigated for its use as animal feed. However, the high fiber content in the spikelets and stalks that hinder digestion posed a challenge. On the other hand, OPEFB is also known to contain a broad range of bioactive polyphenols that could improve rumen fermentation, or even change the greenhouse gas emission profiles of rumen fermentation. These characteristics are highly relevant and would raise the commercial value of OPEFB once proven. The current study investigated the use of OPEFB extracts in animal feed, and to determine the effects of OPEFB extract on rumen fermentation characteristics, milk composition attributes, and fatty acid profile in the rumen, blood plasma, and milk in goats. Four studies were conducted to achieve the above objectives. In the first study, a characterization study was done on OPEFB extract to identify plant phytochemical bio-active compounds. The OPEFB extract was extracted using methanol and was analyzed for total phytochemical content and identified its chemical content through gas chromatography-mass spectrometry (GC-MS). Results showed that OPEFB methanolic extract contained 70 phytochemical compounds, of which twelve are major phytochemical compounds with hexadecanoic acid, methyl ester as the main phytochemical compounds of interest. Methanolic extract OPEFB revealed a total polyphenol content of 3.98%, total tannin content of 2.37%, and total flavonoid content of 1.30% with 7.17ml MDA per L extract for lipid oxidation activity. The next study focused on the *in-vitro* rumen fermentation characteristics in goats using three levels of OPEFB extracts (0%, 5% and 10% OPEFB extract) added to the diet. Results showed fermentation characteristics were not affected by the OPEFB extract levels, but by the rumen microbial population and rumen fatty acid content of palmitic acid. The total bacteria, protozoa, and *B. fibrisolvens* population decreased significantly while palmitic acid increased. Hence, 5% OPEFB extract

diets were chosen for further *in-vivo* study as results suggest that its effects lies mid-range in the acetate, iso-butyrate, iso-valerate concentration, insoluble fraction of gas production (but degradable), estimation potential gas production and in the population of *B. fibrisolvens* as compared with control and 10% OPEFB extract diet. In the third study, a crossover experimental design was employed where animals underwent two feeding trial sessions, which consisted of a 10-day adaptation period followed by a 7-day experimental period, with a 12-day washout period in between both feeding trial sessions. The result showed a reduction in the population of total methanogens (7.1%), methanobacteriales (7.26%), *R. Albus* (6.49%), *R. flavefaciens* (5.16%), *F. succinogenes* (7.98%), *B. fibrisolvens* (9.26%) while an increment in total protozoa content (17.46%) along with iso-butyrate (30.8%) and iso-valerate (38.4%) production in the rumen of OPEFB dietary feed. The milk protein content increased (4.13%) while milk lactose content decreased (2.79%). In summary, the current study showed that OPEFB extract could be incorporated into dairy goat diets at 5 % without adversely affecting rumen fermentation while improving the milk quality, and alleviating oxidative stress in the animal.

Keywords: Oil palm empty fruit bunch (OPEFB), plant phenolic extract, rumen fermentation, fatty acid profile, rumen microbial population, milk composition.

SDG: GOAL 2: Zero Hunger, GOAL 3: Good Health and Well-Being, GOAL 13: Climate Action

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN EKTRAK METANOL TANDAN KOSONG KELAPA SAWIT TERHADAP FERMENTASI RUMEN, PROFIL ASID LEMAK DAN KOMPOSISI SUSU PADA KAMBING

Oleh

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Tandan kosong kelapa sawit (OPEFB) merupakan hasil sampingan pertanian yang banyak dan mudah diperoleh daripada industri kelapa sawit. OPEFB telah dikaji untuk kegunaannya sebagai makanan haiwan. Walau bagaimanapun, kandungan serat yang tinggi dalam spikelet dan tangkai yang menghalang penghadaman jelas menimbulkan cabaran. Sebaliknya, OPEFB juga diketahui mengandungi pelbagai jenis polifenol bioaktif yang boleh meningkatkan fermentasi rumen, atau bahkan mengubah profil pelepasan gas rumah hijau bagi fermentasi rumen. Ciri-ciri ini sangat relevan dan akan meningkatkan nilai komersial OPEFB setelah terbukti. Kerja semasa menyiasat penggunaan ekstrak OPEFB dalam makanan haiwan, dan untuk menentukan kesan ekstrak OPEFB pada ciri fermentasi rumen, sifat komposisi susu, dan profil asid lemak dalam rumen, plasma darah dan susu dalam kambing. Empat kajian telah dijalankan untuk mencapai objektif di atas. Dalam kajian pertama, kajian pencirian telah dilakukan terhadap ekstrak OPEFB untuk mengenal pasti sebatian bio-aktif fitokimia tumbuhan. Ekstrak OPEFB diekstrak menggunakan metanol dan dianalisis untuk jumlah kandungan fitokimia dan mengenal pasti kandungan kimianya melalui kromatografi gas-spektrometri jisim (GC-MS). Keputusan menunjukkan bahawa ekstrak metanol OPEFB mengandungi 70 sebatian fitokimia, di mana dua belas daripadanya adalah sebatian fitokimia utama dengan asid heksadekanoik, metil ester sebagai sebatian fitokimia utama. Ekstrak metanol OPEFB mendedahkan jumlah kandungan polifenol sebanyak 3.98%, jumlah kandungan tannin sebanyak 2.37%, dan jumlah kandungan flavonoid sebanyak 1.30% dengan 7.17ml MDA per L ekstrak untuk aktiviti pengoksidaan lipid. Kajian seterusnya memfokuskan kepada ciri-ciri fermentasi rumen *in-vitro* dalam kambing menggunakan tiga tahap ekstrak OPEFB (0%, 5% dan 10% ekstrak OPEFB) yang ditambahkan ke dalam diet. Keputusan menunjukkan ciri-ciri fermentasi tidak dipengaruhi oleh tahap ekstrak OPEFB, tetapi oleh populasi mikrob rumen dan kandungan asid lemak di dalam rumen, iaitu asid palmitik. Jumlah populasi bakteria, protozoa dan *B. fibrisolvens*

menurun dengan ketara manakala asid palmitik meningkat. Oleh itu, diet 5% ekstrak OPEFB telah dipilih untuk kajian *in-vivo* selanjutnya kerana keputusan menunjukkan bahawa kesannya terletak pada julat pertengahan untuk kepekatan asetat, iso-butirat, iso-valerat, produksi gas daripada bahagian tidak larut (namun terurai), anggaran potensi pengeluaran gas dan juga populasi *B. fibrisolvens* berbanding dengan diet kawalan dan diet 10% ekstrak OPEFB. Dalam kajian ketiga, reka bentuk eksperimen secara silang telah digunakan di mana haiwan menjalani dua sesi percubaan pemakanan, yang terdiri daripada 10 hari tempoh penyesuaian diikuti dengan 7 hari tempoh eksperimen, dengan 12 hari tempoh pembersihan di antara kedua-dua sesi percubaan pemakanan. Hasil menunjukkan pengurangan populasi jumlah metanogen (7.1%), methanobacteriales (7.26%), *R. Albus* (6.49%), *R. flavefaciens* (5.16%), *F. succinogenes* (7.98%), *B. fibrisolvens* (9.26%) manakala peningkatan dalam jumlah kandungan protozoa (17.46%) bersama-sama dengan produksi iso-butirat (30.8%) dan iso-valerat (38.4%) dalam rumen diet OPEFB. Kandungan protein susu meningkat (4.13%) manakala kandungan laktosa susu menurun (2.79%). Ringkasnya, kajian semasa menunjukkan bahawa ekstrak OPEFB boleh dimasukkan ke dalam diet kambing tenusu pada 5% tanpa menjejaskan fermentasi rumen, sambil meningkatkan kualiti susu, dan mengurangkan tekanan oksidatif dalam haiwan.

Kata kunci: Tandan kosong kelapa sawit (OPEFB), ektrak fenolik tumbuhan, fermentasi rumen, profil asid lemak, populasi rumen mikrob, komposisi susu.

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DM	Dry matter
SSL	Self-sufficiency level
OPEFB	Oil palm empty fruit bunch
OPLE	Oil palm leaves extract
OPFF	Oil palm fresh fruit
TP	Total phenol
TT	Total tannin
TF	Total flavonoid
TCA	Trichloroacetic acid
MCP	microbial crude protein
PUFA	Polyunsaturated fatty acid
LDL	Low-density lipoprotein
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
PVPP	Polyvinyl poly pyrrolidone
NH_3N	Ammonia nitrogen
VFA	Volatile fatty acid
SCFA	Short-chained fatty acid
MCFA	Medium-chained fatty acid
LCFA	Long-chained fatty acid
OBCFA	Odd branched-chain volatile fatty acid
BCVFA	Branched-chain volatile fatty acid
BCAA	Branched-chain amino acid
OPLE	Oil palm leaf extract

CLA	Conjugated linoleic acid
CLnA	Conjugated linolenic acid
EFB	Empty fruit bunch
CON	Control
HDL	High density lipoprotein
MDA	Malondialdehyde
TBARS	Thiobarbituric acid reactive substances
MUFA	Monounsaturated fatty acid
UFA	Unsaturated fatty acid
SFA	Saturated fatty acid
PRISMA	Reporting Items for Systematic reviews and Meta-Analyses
SMD	Standard mean difference
CI	Confidence interval
I_2	Heterogeneity
CVD	Cardiovascular disease

CHAPTER 1

INTRODUCTION

The world's total population of ruminants (cattle, goats, and sheep) was estimated more than 3.9 billion in 2021 with a population of goat was more than 1.1 billion goats amounting to 28.3% of the total world ruminant's population (FAOSTAT, 2023). More than half of the goats in the world populate in the Asia region (51.5%), closely followed by Africa (43.3%) while the other region's goat population was less than 5% as depicted in Figure 1 (FAOSTAT, 2023). Meanwhile, the total ruminant population in Malaysia was only 1.1 million head in 2022 and decreased steadily by 10% from 2015 to 2022 as shown in Table 1.1 below. Whereas, the goat population in Malaysia decreased by 24% in total from 2015 to 2022.

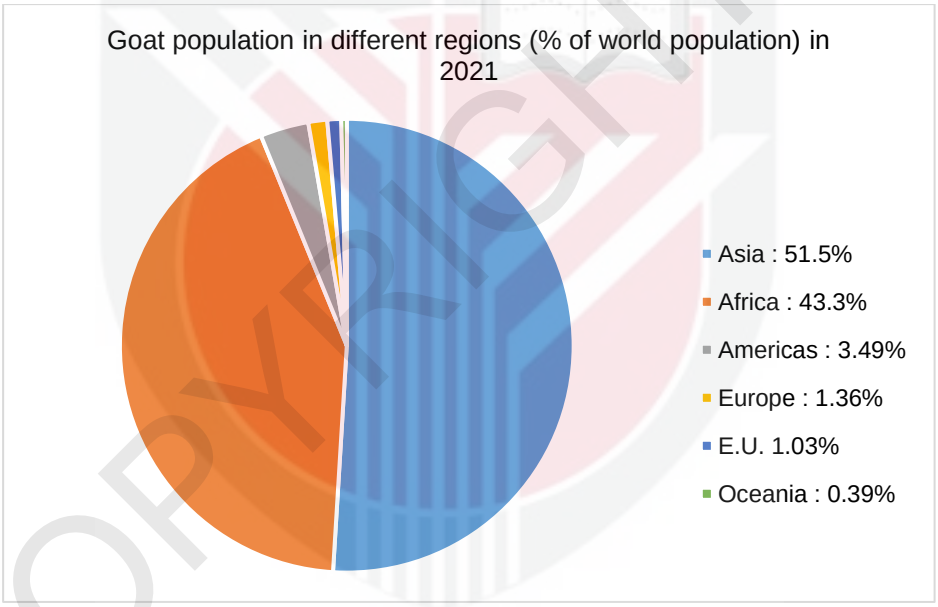

Figure 1 : **The population of goats in different regions in 2021.**
(FAOSTAT, 2023)

Table 1.1 : Ruminant populations in Malaysia from 2016-2023.

Item (heads)	Years							
	2016	2017	2018	2019	2020	2021	2022	2023
Cattle	737,827	703,832	676,686	657,407	699,424	717,431	733,655	737,754
Goat	416,529	385,304	359,200	312,571	324,355	323,994	332,306	338,361
Sheep	138,479	130,658	128,298	121,677	124,674	136,769	137,963	136,625
Total	1,292835	1,219794	1,164184	1,091655	1,148453	1,178194	1,203924	1212740

(Department of Veterinary Services, 2023)

In developing countries, milk and milk products from goats are important sources of protein, phosphate, and calcium due to the limitation of bovine milk availability (Park, 1991; Haenlein and Caccese, 1984). Milk consumption in Malaysia has seen a steady increase throughout the years. From 2015 to 2022, the per capita consumption of milk in Malaysia saw a 16.7% increase (Table 1.2) to 2.1 liters. Meanwhile, Malaysia's milk output only saw a 6.85% increase over the years (2015 to 2022), leading to a decrease of 11.02% in Malaysia's milk self-sufficiency level (SSL) (Table 1.2). The decrement was due to an increase in milk consumption nationwide as increasing health awareness in the Malaysian population has stimulated the increase in the demand for milk. Since the livestock population status in Malaysia kept decreasing in amount over the years, it is concerning if local livestock producers cannot keep up with the demand thus leading to importation reliance. Hence, to drive the livestock subsector further, National Agrofood Policy 2.0 was established by the Malaysian government to address issues that impede the growth of the livestock subsector. Among the livestock strategies related to the current study was the enhancement of livestock productivity and production (MAFI, 2021).

Table 1.2 : Milk outputs, capita consumptions, and self-sufficiency level (SSL, %) in Malaysia from 2015-2022.

Items	Years							
	2015	2016	2017	2018	2019	2020	2021	2022
Output (Mil litres)	36.5	36.7	36.6	38.5	40.6	41.8	38.7	39.0
Per capita consumptions (Litre/year)*	1.8	1.8	1.9	1.9	2.1	2.0	2.1	2.1
SSL (%)*	64.4	66.0	58.9	61.3	59.3	64.2	56.7	57.3

*Refer to fresh and imported liquid milk.
(Source: Department of Veterinary Services, 2023)

Malaysia's oil palm industry as well as Indonesia, contributes 80% of the total world palm oil production and export (Ofori-Boateng and Lee, 2013). The production of oil palm empty fruit bunch (OPEFB) was 23% of the oil palm fresh fruit (OPFF) after being processed in the palm oil mill (Schmidt, 2007). The OPEFB is an oil palm agricultural waste with limited capability to be included in

the animal feed industry due to its high fibre content (67% crude fibre) (Asoka, Abu, and Agwa, 2021). It is in the same fate as other oil palm biomass by-products such as oil palm fronds (OPF), oil palm trunks (OPT), palm press fibres (PPF), and shells, which it composed of mainly cellulose, hemicellulose and lignin (Tan, 2006). Currently, most of it is used in fibre and particle board production (EFB, OPF, OPT, PPF) and as charcoal briquette (shells) (Tan, 2006). Another application such as incorporating in the ruminant feed as a roughage source, OPF in pelleted form for example, has been reported to meat quality betterment and reduce saturated fatty acid (SFA) (Ebrahimi *et al.,* 2012). On the other hand, there are other study on fungi-treated empty fruit bunch replacement diet at 25% conducted in dairy goats showed a decreased in milk lauric acid (C12:0) without adverse effect on milk yield and milk protein (Zailan *et al*., 2023). Hence, these few studies indicates that there are potential of oil palm agricultural waste to be better exploited into other downstream product, such as animal feed, and further reinforce the terms of sustainable oil palm in Malaysia.

1.1 Problem statement

The usage of empty fruit bunches in animal feeding has been found lacking due to its high fibre content. The composition of EFB where it is high in cellulose, hemicellulose and lignin content, makes it unlikely to be incorporated in animal feeding as it is. Hence, it remains a challenge in logistics and transportation due to its bulkiness. The same challenges were encountered by other studies with oil palm fronds in incorporating it as animal feed. However, Jaffri (2009) showed that the extraction of beneficial compounds such as polyphenols and anti-oxidants contained in the OPF, may provide an added value in addition to the possibility of resolving the logistics and transportation challenges. Phytochemical contained in oil palm wastes such as polyphenols has interesting biological properties such as anti-oxidant, and anti-microbial, among others (Ofori-Boateng and Lee, 2013). Previous findings by Al-Jumaili (2018), also reported reduced SFA and better-quality meat, with better colour stability, reduced lipid oxidation, and cholesterol levels when using oil palm leaves methanolic extract in feeding the goats. It brought to attention whether the methanolic extract of different parts of oil palm may cause the same outcome as previous studies. Since Malaysia produces a huge amount of EFB as a by-product of the huge oil palm industry, it remains underutilised. Hence, in tackling the logistic issues, it inadvertently tackles environmental issues as well since in its former years, EFB was burnt-off. Exploring the EFB's potential used as animal feed to improve animal production is considered vital, then. Aside from that, information related to the usage of EFB in animal feed to improve the quality of animal products such as meat and milk is found to be lacking. Hence, this study would provide another option in utilising underutilised oil palm waste, EFB as animal feed, which may resolve another environmental concern in Malaysia.

1.2 Hypothesis

It is hypothesized that incorporating oil palm empty fruit bunch extract may change the rumen fermentation profile along with the fatty acid profile in the rumen, blood, and milk. It will also improve the anti-oxidant capacity of milk and produce healthier milk.

1.3 Objectives

The general objective of this study was to assess the effect of oil palm empty fruit bunches methanolic extract on the rumen fermentation profile, fatty acid profiles and milk compositions in dairy goats. Therefore, the specific objectives of this study were;

1. To determine the bioactive phytochemical constituents in the oil palm empty fruit bunch methanolic extract.
2. To evaluate the effect of OPEFB extract on *in vitro* rumen fermentation characteristics in goats.
3. To determine the effect of oil palm empty fruit bunch methanolic extract on *in-vivo* rumen fermentation characteristics, rumen microbial populations, and fatty acid profile of rumen, blood, and milk in Saanen goats.
4. To determine the effect of oil palm empty fruit bunch methanolic extract on milk composition, blood chemistry profile, and lipid oxidation in the rumen, blood, and milk of Saanen goats.

CHAPTER 2

LITERATURE REVIEW

2.1 Ruminant and its digestive system

Ruminant, according to Merriam-Webster (2024) is an herbivorous, even toed, hoofed mammal is an herbivorous, even-toed, hoofed mammal (under the suborder of *Ruminantia* and *Tylopoda*) that has a complex 3- or 4-chambered stomach. The digestion process of ruminants starts with the ingestion of the feed from mouth to rumen via the esophagus, oftentimes the feed is swallowed to ensure less time is used to procure the feed. In the first chamber, the rumen, the feed is softened and turned into cud before being regurgitated to the mouth in the process called rumination via the esophagus again, as the esophagus of the ruminant is bidirectional. In the mouth, the cud is properly chewed and then swallowed again further into the reticulorumen, a collective term for rumen and reticulum, since both organs have the same function divided by a simple fold in the middle. There, the larger cud materials into the rumen for further fermentation, and the liquid portion and smaller cud material passed into the omasum. The rumen is mainly a chamber of anaerobic digestion which is 95% populated by bacteria, archea, fungi, and protozoa, with viruses and bacteriophages included (Cholewińska, Górniak, and Wojnarowski, 2021). Their role was to convert ingested feed into energy resulting in short-chain fatty acids, commonly called volatile fatty acids (VFA) mainly acetate, propionate, and butyrate as these are the animal's main energy sources where it has an immediate physiological impact, including production parameters (De Nardi *et al.,* 2016).

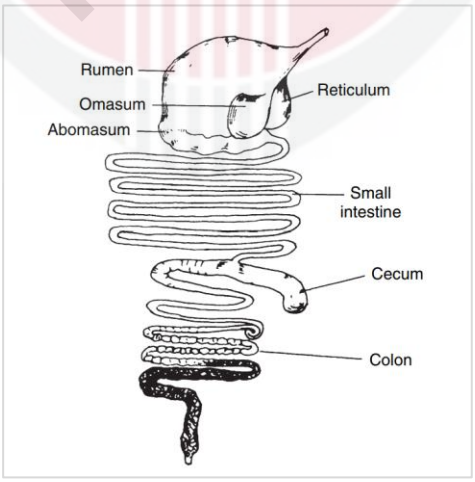

Figure 2.1 : Ruminant digestive tract and its compartments (Russel and Bruckner, 1991).

It was estimated that the rumen volume in small ruminants was approximately 13% of the body weight, accounting for 5.3 litre in estimation (Owen and Goestsch, 1993) whereas other studies estimated the rumen volume was approximately 4-6kg, although it may vary in accordance to the animal diets (Philipson, 1999). Rumen pH was kept constant at the range of 6-7, with potassium bicarbonate and urea in saliva as main buffers to maintain rumen pH level. This is crucial to ensure sustained and continual digestive functions. Ammonia from rumen fermentation was also considered as a buffer and plays a part in promoting rumen microbial growth in the digestive system (Matthews *et al.,* 2019). The reticulum of the ruminant features a smaller pouch anterior to the rumen and has a vital role in the separation of digesta as previously stated with the additional function of trapping other heavy objects or material in the reticulum. In the omasum, water is absorbed along with other substances before the cud or digesta enter the abomasum next, where it is a compartment that is called the 'true stomach' and resembles a tubular organ connecting the omasum with the small intestines (Harfoot, 1981). From this point, the digestion processes are similar to the simple-stomached animal, where the nutrients are further absorbed in the small intestines.

2.1.1 Rumen microbial ecosystem

The rumen microbial community is a diverse and dynamic ecosystem inhabited by a symbiotic population of anaerobic bacteria, protozoa, fungi, and phages (Forsberg and Cheng, 1992). The ability of the ruminant to utilize high lignocellulosic material as feed as well as in the conversion of non-protein nitrogen to microbial protein relies on the rumen microbial community. Although rumen fermentation plays a vital role in the usage of highly fibrous material as feed, the ramifications of the said activities associated with emission of the greenhouse gases as well as nitrogen excretion by manures, may adversely impact animal product nutritional values (Scollan *et al*., 2011). Low-quality, fibrous, plant material can be converted by the rumen microbes into a usable energy for the host ruminant (Cammack *et al.* 2018). It was suggested by a previous study that microbial digestion may contribute up to 70% of total dietary energy (Flint *et al.,* 2008). The rumen microbial community consists of bacteria (1010 to 1011 organisms/ml), but also archaea (108 to 109 organisms/ml), protozoa (105 to 106 organisms/ml), fungi (103 to 104 organisms/ml), and another uncharacterized virome (Weimer, 2015).

Bacteria can be described as solid-associated, liquid-associated, rumen epithelium-associated, and eukaryote-associated (Cammack *et al*., 2018), which composed of 95% of the rumen microbial populations (McAllister *et al*., 1994). Further classification of rumen bacteria is through substrate preference and end-product formation, where the substrates are mainly cellulose, hemicellulose, pectin, starch, and amino acid (Puniya *et al*., 2015). In ruminant, primary cellulose-degrading bacteria is *Fibrobacter succinogenes*, *Ruminococcus flavefaciens,* and *Ruminococcus albus* (Forsberg and Cheng, 1992; Schroeder, 2013). Other common hemicellulose-degrading bacteria that are omnipresent in forage diet are *Butyrivibrio fibrisolvens, Prevotella ruminocola, Ruminococcus*

flavefaciens, and *Ruminococcus albus* (Puniya *et al*., 2015). Rumen archea consist of methanogens, which are methane-producing microbes that utilize hydrogen and sometimes formate in the reduction process to produce methane (Janssen and Kirs, 2008). Methanogens utilize hydrogen produced by other microbes thus avoiding the inhibitory effect posed by the accumulation of hydrogen for subsequent fermentation (Hungate, 1966; Puniya *et al*., 2015). Rumen archea constitute a small amount, 2-4% of the total microbial mass (Millen *et al*., 2016) but play a vital role as hydrogen sinks in the rumen. Hydrogen produced by cellulolytic bacteria and anaerobic fungi was utilized by methanogens to generate ATP and methane (Stewart *et al*., 1997). Anaerobic fungi are considered as influential fiber degraders, particularly when ruminants are fed with poor quality forages (Krause *et al*., 2003). It was reported by Huws *et al*., (2018) that anaerobic fungi constitute 12-20% of the rumen microbiome. Protozoa representation in the rumen although small, the large size of protozoa (10–200 μm) make up 50% of the total microbial mass in the rumen (Williams, 1986). Protozoa's mode of action was to engulf bacteria, feed particles, and digest carbohydrates, protein, and fats (William and Coleman, 1992). Rumen fermentation was stabilized by protozoa through possibly increasing the pH of the rumen (Williams and Coleman, 1992) as protozoa seem to consume lactate faster than bacteria (Newbold *et al*., 1986).

2.1.2 Biohydrogenation in rumen

Rumen biohydrogenation is a conversion process of unsaturated fatty acid to saturated fatty acid by rumen bacteria. A previous study stated *Fusocillus spp.* was identified as a bacteria that carried out the formation of stearate (Kemp *et al*., 1975). Then, other study discovered a bacterium that manages to form stearate from linoleate, which found it was phenotypically similar to *Fusocillus spp*, but closer phylogenetically to *Butyrivibrio hungatei* (Van de Vossenberg and Joblin, 2003). Past studies classify biohydrogenation-related bacteria into two groups which Group A (bacteria that hydrogenate linoleic acid and linolenic acid to vaccenic acid) and Group B (bacteria that hydrogenate linoleic acid and linolenic acid to stearic acid) (Harfoot and Hazlewood, 1997). However, a recent study suggests classifying biohydrogenation-related bacteria according to their appropriate taxonomy (Lourenco, Ramos-Morales and Wallace, 2010). There are few biohydrogenation pathways that in recent years, through various *in-vitro* and *in-vivo* studies, make it more understandable. According to Dewanckele *et al*. (2020), 18:2n-6 is mainly isomerized to cis-9, trans-11 CLA, and hydrogenated further to trans-11 18:1 and lastly to 18:0, under normal conditions. Meanwhile, 18:3n-3 major biohydrogenation pathways involve cis-9, trans-11, cis-15 conjugated linolenic acid (CLnA), trans-11, cis-15 18:2 and trans-11 18:1 as intermediates while biohydrogenation pathway of cis-9 18:1 is directly hydrogenated to 18:0 in the rumen. Nevertheless, other than these biohydrogenation pathways depicted in Figure 2-4 below, there are other alternative biohydrogenation pathways that possibly exist but yet to be discovered. The presence of biohydrogenation intermediates in rumen and animal products, might provide insight on the extent of biohydrogenation activities occurred.

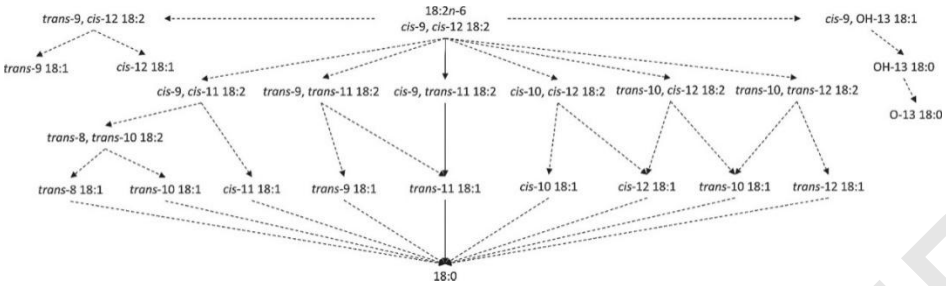

Figure 2.2 : Rumen 18:2n-6 metabolism pathway. Solid line arrows (⟶) show the major biohydrogenation pathway, and dashed line arrows (⇢) show the formation of minor intermediates, under physiologically normal conditions in the rumen. (Source: Shingfield and Wallace, 2014).

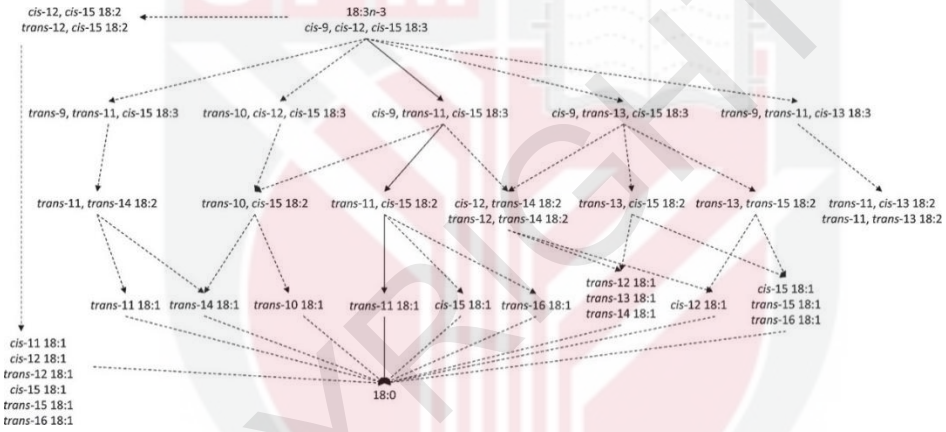

Figure 2.3 : Rumen 18:3n-3 metabolism pathway. Solid line arrows (⟶) show the major biohydrogenation pathway, and dashed line arrows (⇢) show the formation of minor intermediates, under physiologically normal conditions in the rumen. (Source: Shingfield and Wallace, 2014).

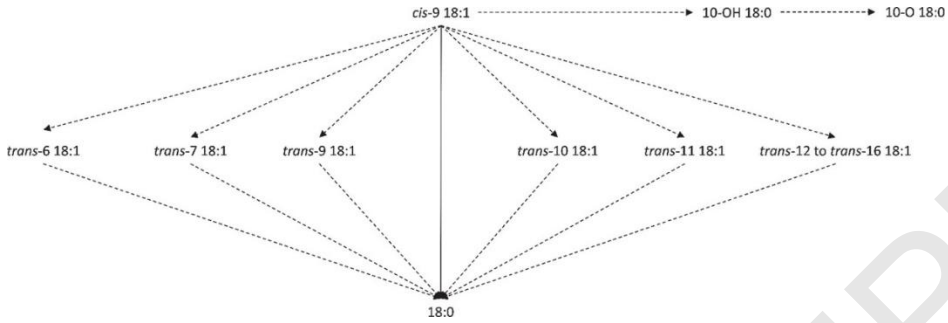

Figure 2.4 : Rumen cis-9 18:1 metabolism pathway. Solid line arrows (⟶) show the major biohydrogenation pathway, and dashed line arrows (⇢) show the formation of minor intermediates, under physiologically normal conditions in the rumen. (Source: Shingfield and Wallace, 2014).

2.2 Phytochemicals and bioactive in animal feed

Generally, phytochemicals are chemicals plants produce as a defense mechanism against predation, and bioactive are compounds that naturally occur in plant or animal that benefits human health and well-being. Having said that, certain bioactive are also considered as phytochemicals depending on the sources. The occurrence of phytochemicals and bioactive in animal feed is regular, as it takes on different forms, in serving different industries, whether ruminant or non-ruminant. In ruminants, phytochemicals existed more in the form of roughages, as-is, or extract. The extract of the plant is a concentrated fluid that contains plant compounds of interest through the process of extraction. The complexity of an extract depends on the plant sources used. The variability of phytochemical composition of a given variety or species of plant attributes on the geographical, especially (Nigg and Seigler, 2013; Tiwari *et al*., 2013). An example of geographic differences affecting the compound composition of the plant was studied in earlier studies which indicated a higher proportion of flavonoids were found in the tropical and high–altitude plant compared to the temperate, among others (Chappell & Hahlbrock, 1984; Olsson *et al*., 1998; Hofmann *et al*., 2000; Logemann *et al*., 2000; Kolb *et al*., 2001). Other studies also suggest that differences may exist in the molecular structure of condensed tannin between tropical and temperate forages as it affects the condensed tannin metabolism in the ruminant digestive system (Min and Solaiman, 2018; Min *et al*., 2015; Min *et al*., 2015). Therefore, the uniqueness of plant phytochemical composition would render the properties of plant compounds exhibited useful for it to be incorporated in animal feed.

2.2.1 Effect of supplementing plant phytochemicals and bioactive on rumen microbial ecosystem

The chemodiversity of plant phytochemicals and bioactive are too enormous, hence diagram below was illustrated by Tiwari, Brunton, and Brennan (2013) to show the phytochemical classifications into a few major groups.

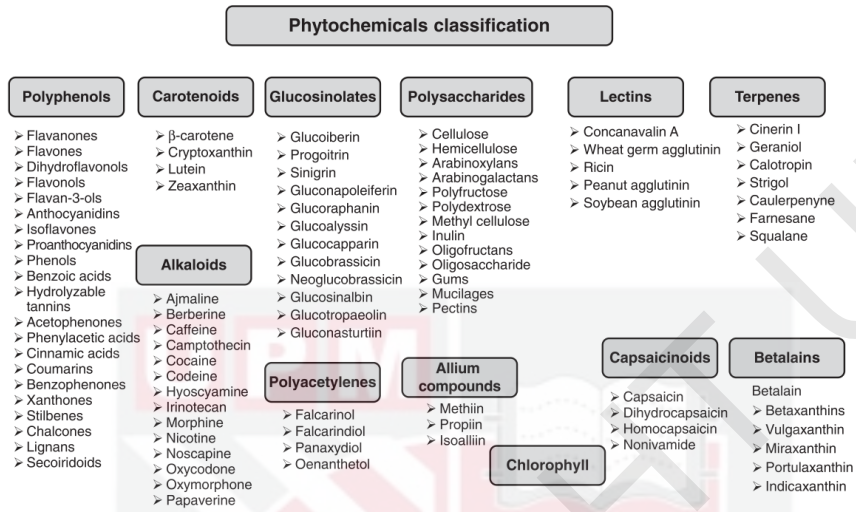

Figure 2.5 : The classification of plant phytochemicals. (Source: Tiwari, Brunton, and Brennan, 2013)

As each of the plant compounds exhibits biological properties that would benefit animal health and production, there has been an increasing effort in exploiting phytochemicals in animal feed in many recent researches. The differences it would make toward the rumen microbial ecosystem were interesting, especially knowing the biological properties such as anti-microbial, and anti-fungal, among others, exhibited by the compound of interest. A study of supplementing garlic and citrus extract on dairy cows, resulted in increases in the population of *Succinivibrionaceae* and *Methanobrevibacter*, which related to methane reduction (Khurana *et al*., 2023). The combination of organosulphur compound from garlic and polyphenol compound from citrus extract efficiently reduces methane production in Rusitec system without affecting rumen fermentation (Eger *et al.,* 2018). An example of selective anti-microbial activity by plant phytochemicals was reported by Harlow *et al.,* (2017b) and (2018), in which the growth of *F. succinogenes, R. flavefaciens,* and *R. albus* was inhibited while increasing *Lactobacilli* population by utilizing biochanin A, an isoflavone phytoestrogen found mainly in the leguminous plant, in an *ex-vivo* study. Another *in-vitro* study utilizing walnut leaves ethanolic extract in the barley and corn diet substrates observed that *F. succinogenes, R. flavefaciens,* and *R. albus* population was reduced. These studies showed the potentiality of plant extract to be used as a tool to manipulate rumen microbial populations. Among recent studies that reported on the effect of plant phytochemicals on the rumen microbial population which can be seen in the table below (Table 2.1).

Table 2.1 : The effect of plant phytochemical on rumen microbial population.

Items	Phytochemical sources and levels	Total bacteria	Total methanogenes	Total protozoa	others
Aiman-Zakaria *et al.,* (2017)	Oil palm leaves extract (OPLE) in *in-vitro* study; (2.5-10% of OPLE)	-	9.1-12.78% reduction in population	-	Methanobacteriales:7.31-11.76% reduction in population
Al-Jumaili (2018)	Oil palm leaves extract (OPLE) in *in-vivo* study; (300-600mg of OPLE/kg diet)	-	5.5-13.6% increase in population	7.7-13.4% increase in population	*R. albus*: 5.4-7.1% decrease in population. *F. succinogenes*: 19.3-24.3% decrease in population *B. fibrisolvens*: 115-134% increase in population
Huang *et al.,* (2022)	Paulownia leaves and silage in *in-vitro* study; (20-60 g/kg DM)	5.2-11.04% decrease in population	-	-	*F. succinogenes*: 159-334% increase in population *B. fibrisolvens*: 179-382% increase in population
Yu *et al.,* (2023)	Citrus flavonoid extract (CFE) in *in-vivo* study; (50-150g/day)		32.3-77.2% reduction in population	-	*R. flavefacien*: 14.5-103% increase in population
Belanche *et al.,* (2023)	Plant extract blend of turmeric, thymol and yeast cell wall component (PEY); (0.03, 0.45, 18, and 30 mg/kg of turmeric, thymol, mannan oligosaccharides, and β-glucan)	-	-	11% reduction in population	Anaerobic fungi: 27.3% decrease in population.
Ramos-Morales *et al.,* (2018)	Liquorice extract (LE) in *in-vitro* study; (1-2g/L)	-	1.66% increase in population	19-35.6% decrease in population	Anaerobic fungi: 6.5-41.1% decrease in population.
Salami *et al.,* (2018)	Condensed tannin extract (CT) in weaning lamb diet; (40g/kg of CT extract)	-	11.5-12.8% decrease in population	15.1-20.8% decrease in population	-

2.2.2 Effect of supplementing plant phytochemicals and bioactive on rumen fatty acid profile and biohydrogenation

Studies have suggested that plant phytochemical and bioactive may help in reducing rumen biohydrogenation through ingesting compounds that contain interesting biological properties, such as anti-oxidant and anti-microbial activities. Dietary polyphenol supplementation can be considered as a way to preserve polyunsaturated fatty acid (PUFA) from a complete biohydrogenation in the rumen. In the study of cumin seed extract, it was reported that biohydrogenation was inhibited by the reduction in stearic acid and the increment of linoleic and linolenic acid concentration in the rumen (Miri *et al.,* 2015). It was also suggested that tannin had shown a consistent trend in the decline of the microbe population associated with rumen biohydrogenation (Patra and Saxena, 2011). Indeed, tannin from a variety of sources namely tropics and subtropics (Jayanegara *et al.,* 2011; Jayanegara, Kreuzer, and Leiber, 2012), may repress biohydrogenation and stearic acid efficiently (Carreño *et al.,* 2015). Earlier studies showed a decrease in the biohydrogenation process due to the presence of phenolic acid (Canini *et al.,* 2007) in an *in-vitro* study with papaya leaf (Jafari *et al*., 2016), however, other study reported that biohydrogenation increases in the study of different sources of lipid supplementation where linseed increases the rumen linolenic acid concentration while hemp increases the rumen linoleic acid concentration (Cremonesi *et al*., 2018). Hence, Table 2.2 below showcases the summarisation of plant phytochemicals in other studies and the responses on rumen fatty acid profile and biohydrogenation.

Table 2.2 : The effect of plant phytochemicals on rumen fatty acid profile and biohydrogenation.

Items	Phytochemical sources and compound	LA (Linoleic acid)	LNA (Linolenic acid)	Total PUFA
Miri *et al*., (2015)	Cumin seed extract; (cuminaldehyde)	1.28% increase in concentration	3.95-4.52% increase in concentration	10.2-16.4% increase in concentration
Cieślak *et al*., (2013)	Grape oil and black current oil; (Tocopherol and anthocynin)	59.2-110% increase in concentration	8.6-11% decrease in concentration	29-65% increase in concentration
Buccioni *et al*., (2017)	Chestnut and quebracho tannin; (tannin)	29.7-127.9% increase in concentration	19.01-53.1% increase in concentration	-
Huang *et al.,*(2022)	Paulownia leaves; (Phenylpropanoid glycosides)	17.1% increase in concentration	9.76% increase in concentration	5.62% increase in concentration
Bryszak *et al.,* (2019)	Berry seed residue; (anthocyanins)	133% increase in concentration	~	6.4% increase in concentration
Thanh *et al.,* (2022)	Grape seed tannin; (tannin)	2.8-7.4% increase in concentration	0.45-1.82% increase in concentration	2.06-6.2% increase in concentration
Yusuf *et al.,* (2017)	*Andrographis paniculata* leaves; (Andrographolide)	147-278% increase in concentration	183-350% increase in concentration	~

2.2.3 Effect of polyphenol supplementation on milk composition and fatty acid of dairy animal through a systematic review

Milk production and quality can be enhanced by supplementing animal feed with phytochemical and bioactive. It has been demonstrated through earlier studies the benefits posed by the phytochemical and bioactive-rich diet. Polyphenols, essential oils, saponins, and polysaccharides, are among plant bio-active compounds that have been used to reduce methane production by manipulating ruminal microbial feed fermentation without interfering with ruminant production performance (Patra and Saxena, 2010). High-yielding dairy animals are prone to oxidative stress, which can be exacerbated by certain environmental, physiological, and dietary factors (Castillo *et al.,* 2005). Phenolic compounds, including stress-related phytochemicals, have been linked to beneficial effects caused by fruit and vegetable consumption, particularly due to their antioxidant activity (Heim *et al.,* 2002). A study by Winkler *et al.,* (2015) found that an increase in milk performance with a fat content reduction in the liver was found when green tea extract was fed to the cow. However, Oliveira *et al.,* (2010) findings suggest that digestibility of fat and protein was decreased in immunocompetence calves when fed with polyphenol-rich pomegranate extract.

As of late, many reviews on plant extracts or plant components, such as saponins, tannins, and essential oils, as rumen modifiers have recently been published. However, they have mostly focused on the alterations in ruminal fermentation (Wallace *et al.*, 2002; Castillejos *et al.*, 2006; Hart *et al.*, 2008; Kamra *et al.*, 2008; Dai and Faciola, 2019; Torres *et al.,* 2020), ruminal microbial population (Patra and Saxena, 2009; Dai and Faciola, 2019), and their association with methanogenesis (Patra, 2010). Lipids supplementation was also evaluated and compared in cattle and sheep for their effects on methane emission, digestibility, ruminal fermentation, and lactation performance. According to a recent meta-analysis study conducted by Khiaosa-ard and Zebeli (2013), the benefits of essential oil supplementation vary amongst ruminant species. Beef cattle, according to these authors, are more sensitive to essential oil than dairy cattle and small ruminants. Meanwhile, according to Gessner *et al.*, (2015), milk performance was improved with the supplementation of dairy cow feed with polyphenol-rich grape seed and marc extract. Despite significant knowledge gained in recent years on the potential application of plant extract or plant components on rumen microbial fermentation modification and animal performances, the effects of plant extract supplementation to ruminant diets on their dairy performances such as milk compositions and fatty acid profiles are still lacking. Very few studies have reviewed and evaluated the changes in milk composition and milk fatty acid content when the animals have been fed with phytochemicals and lipids in the feed. Hence, in this part of the literature review, a systematic review was done to evaluate and summarize the extent of plant polyphenol supplementation effects on milk composition and fatty acid profile in ruminants. Further details of the systematic review methodologies can be found in the Appendices of this thesis. Onwards, the result and summary of the systematic review will be discussed further.

2.2.3.1 Effect of polyphenol supplementation on milk fat content

Nineteen research articles reporting 44 studies, were eligible to be included in the analysis to evaluate the effects of polyphenol supplementation on milk fat content. Subgroup analyses were conducted to see whether different sources of polyphenols affect the fat content in the milk. The different polyphenol sources were grain, forages and agro-industrial by-products, which constitute six, seventeen and twenty-one studies, respectively. As depicted in Figure 6.2, the pooled standard mean differences showed did cross the line of no effect, and thus it can be concluded that the overall effect is not significant statistically when supplemented with polyphenols in the diet ($P>0.05$). Meanwhile, significant heterogeneity was observed for this outcome measure ($P<0.05$) ($I^2=93\%$). Sub-group analysis was conducted to evaluate different sources of polyphenol supplementation (grain, forages, or agro-industrial by-products) effects on milk fat content. The analysis showed there are no significant differences ($P>0.05$) in milk fat content between different sources of polyphenol supplementations with significantly lower heterogeneity ($I^2=15.6\%$).

It is clearly shown in the forest plot of the grain group that the 95% confidence intervals of four studies crossed the line of no effect. These studies, individually were not significant however the 95% confidence intervals of two studies by Mitsiopoulou *et al.* (WSS10) and Santos *et al.* (FO) did not cross the line of no effect, which means that the results from these individual studies were significant. Meanwhile, 95% confidence intervals of five studies crossed the line of no effect and twelve other studies did not cross the line of no effect as shown in the forest plot of the forages group. Among these twelve studies, the studies by Abbeddou *et al.* (LS) (2011) showed the highest standard mean difference and 95% confidence interval [3.91 (2.30, 5.52)] and the study by Cabiddu *et al.* 4May showed the lowest standard mean differences and 95% confidence intervals [-6.58 (-8.77, -4.39)] in the forages group, which signalled these are the possible outliers for this group. Whereas, in the agro-industrial by-product group, 95% confidence interval of ten studies crossed the line of no effect and eleven studies did not. The possible outliers for this group are shown by studies of Santos *et al.* PBP-E, where it is at the highest value of standard mean difference and 95% confident interval [6.02 (4.3, 7.74)] and the lowest value of standard mean difference and 95% confident interval is depicted by Silva *et al.* (WMM100) in his study with -4.12 (-5, -3.23).

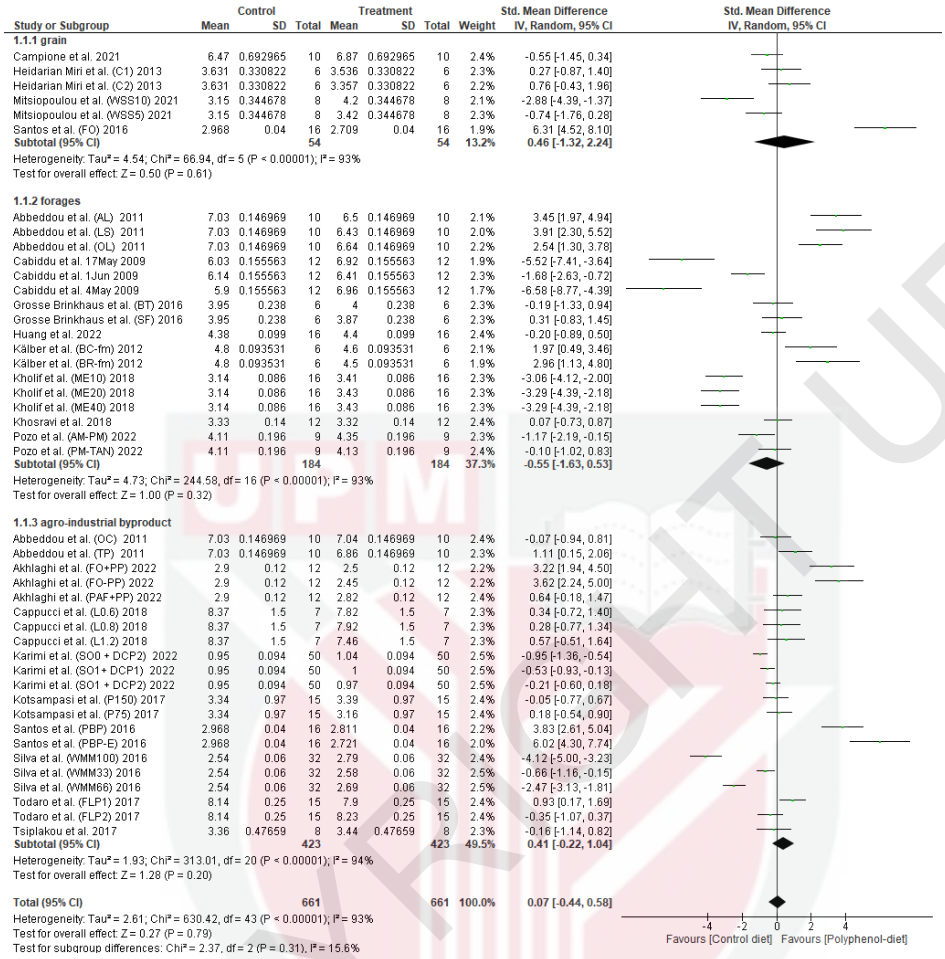

Figure 2.6 : Forest plots for the links between milk fat content and polyphenol.

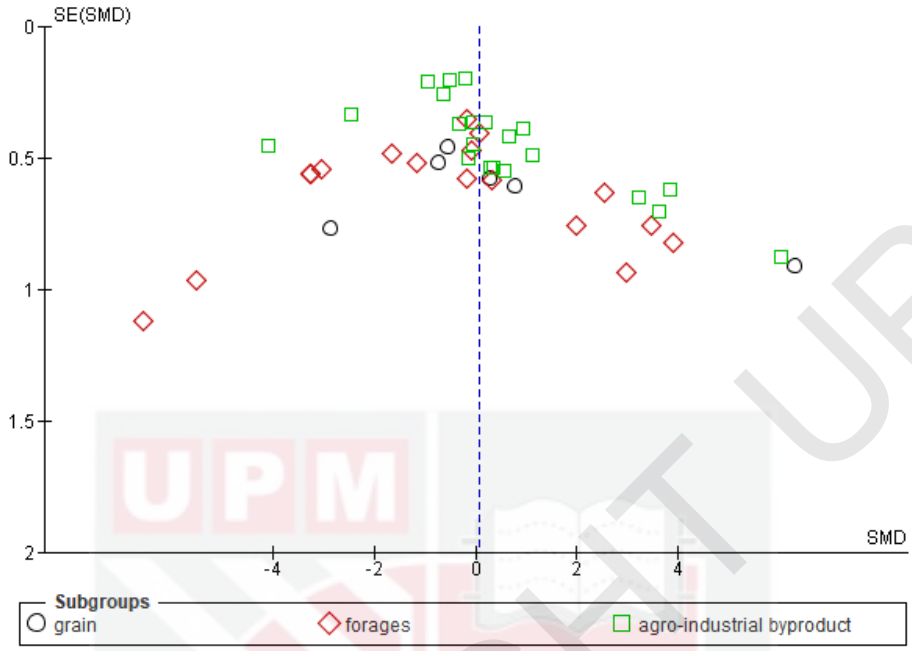

Figure 2.7 : Funnel plots for the links between milk fat content and polyphenol.

2.2.3.2 Effect of polyphenol supplementation on milk protein content

A total of 44 studies from 19 research articles were eligible to be included in the study to evaluate milk protein content as affected by polyphenol supplementation in the diets. Subgroup analyses were conducted to see whether different sources of polyphenols affect the milk protein content. The different polyphenol sources were grain, forages, and agro-industrial by-products, which constitute six, seventeen, and twenty-one studies, respectively. Figure 2.8 shows the forest plot of the effect of three different sources of polyphenol on protein content in milk. The pooled standard mean differences shown above did cross the line of no effect, which concludes that the overall effect is statistically insignificant when supplemented with polyphenols in the diet ($P>0.05$). However, it was observed that the heterogeneity for this overall outcome measure was significant ($P<.0001$) where $I^2=88\%$. When the analysis was done to evaluate different sources of polyphenol supplementation (grain, forages, or agro-industrial by-products) effects on milk protein content, the results showed that there are no significant differences ($P=0.18$) in milk protein content between different sources of polyphenol supplementations with considerably low heterogeneity, $I^2=42\%$.

In the forest plot of the grain group, 95% confidence intervals of three studies crossed the line of no effect while the other three did not cross the line of no effect. Since all of the confidence intervals of these studies falls within the range of pooled results of 95% confidence interval of this group (-1.60, 0.53), there are no outliers identified in the grain group of polyphenol sources. As for the forages

group of polyphenol sources, 95% confidence intervals of ten studies crossed the line of no effect and seven other studies did not cross the line of no effect as shown the forest plot. Among these seven studies, Huang *et al*. 2022 showed the highest standard mean differences [4.26 (2.95, 5.58)] and the lowest value of standard mean differences [-2.05 (-3.07, -1.03)] was by the study of Cabiddu *et al*. 4May 2009, and thus also represent the potential outliers under forages group. Meanwhile, the forest plot of the agro-industrial by-product group showed 95% confidence interval of ten studies crossed the line of no effect and eleven studies did not cross the line of no effect. However, the result for polyphenols from agro-industrial by-products showed significant differences (P=0.04) with significant heterogeneity at I^2=91% (P<.00001). As for this group, Akhlaghi *et al*. (FO+PP) showed the highest value of standard mean differences at [4.83 (3.13, 6.52)] and the lowest value of standard mean differences is by the study of Santos *et al*. (PBP) at [-2.37 (-3.3, -1.44)], in which also represented as possible outliers for this group.

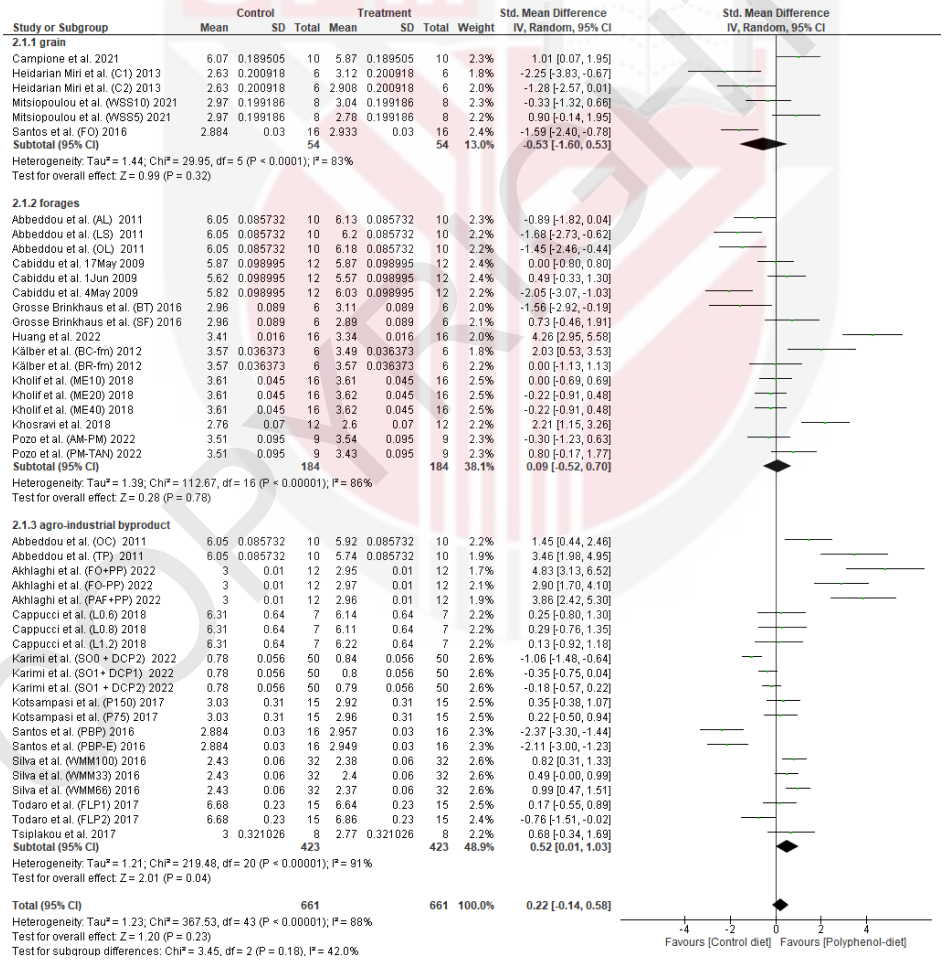

Figure 2.8 : Forest plots for the links between milk protein content and polyphenol.

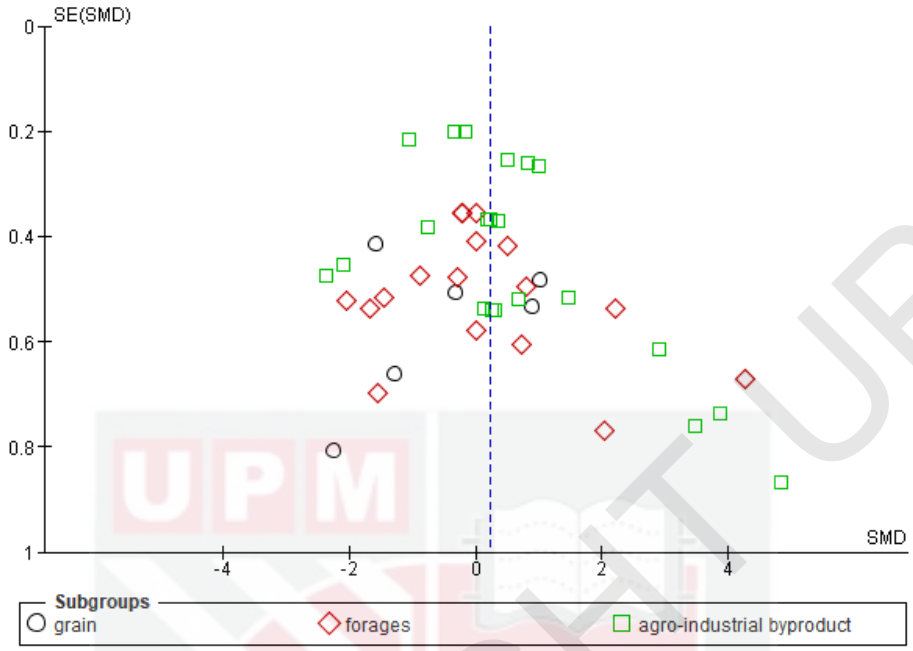

Figure 2.9 : Funnel plots for the links between milk protein content and polyphenol.

2.2.3.3 Effect of polyphenol supplementation on milk lactose content

In the study to evaluate the effect of polyphenol supplementation on milk lactose content, thirty-seven studies from seventeen research articles were eligible to be included. Among the eligible publications, the number of studies belonging to grain, forages, and agro-industrial by-products are only six, fourteen, and seventeen studies, respectively. Forest plots of the effect of three different sources of polyphenol on lactose content in milk are shown in Figure 2.10. From the plot, it was observed that the pooled standard mean differences for overall effect did not cross the line of no effect, which concurs that the supplementation of polyphenol in the diet insignificantly affects lactose content in milk ($P>0.05$). However, it was noted that the heterogeneity for this overall outcome measure was significant ($P<.0001$) where $I^2=86\%$. There are significant differences ($P<0.05$) found when assessing different sources of polyphenol supplementation (grain, forages or agro-industrial by-products) effects on milk lactose content. Results showed that there are significant differences ($P=0.02$) in milk lactose content from grain source of polyphenol supplemented in the diet, with considerably moderate heterogeneity, $I^2=43\%$.

In the grain group, the forest plot shown in Figure 2.10 shows 95% confidence intervals of four studies crossed the line of no effect while the other two did not cross the line of no effect. However, there were no outliers detected since the 95% confidence intervals of all the studies included in the grain group overlap with the pooled standard mean differences of the grain group. Concurrently, 95%

confidence intervals of eleven studies crossed the line of no effect while three other studies did not cross the line of no effect as shown in the forest plot of the forages group. These three studies were the outliers for the forages group since it does not overlap with the pooled standard mean difference of the forages group. The highest value of the study standard mean difference and 95% confidence interval is from the studies of Khosravi *et al*. [2.25 (1.19, 3.31)] and the lowest value of the study standard mean difference and 95% confidence intervals are from the studies of Abeddou *et al.* (LS) with -2.84(-4.16, -1.53) and Abeddou *et al*. (OL) with -6.22(-8.54, -3.9). As for the agro-industrial by-product group, the forest plot showed 95% confidence interval of ten studies crossed the line of no effect and seven studies did not cross the line of no effect. Among the individually significant studies, the highest value of standard mean difference and 95% confidence interval was found in the study of Akhlagi *et al*. (FO+PP) with 3.86(2.42, 5.3), and the lowest value was shared with the study of Abeddou *et al*. (OC) and Abeddou *et al*. (TP) with -4.62(-6.44, -2.4).

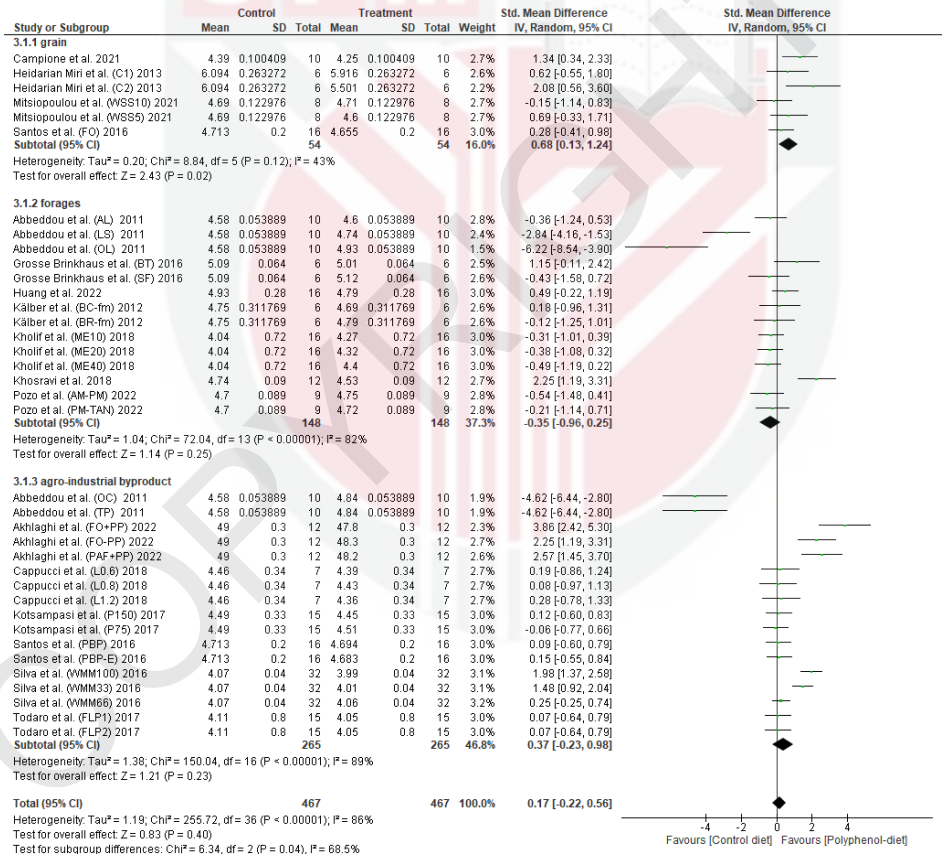

Figure 2.10 : Forest plots for the links between milk lactose content and polyphenol.

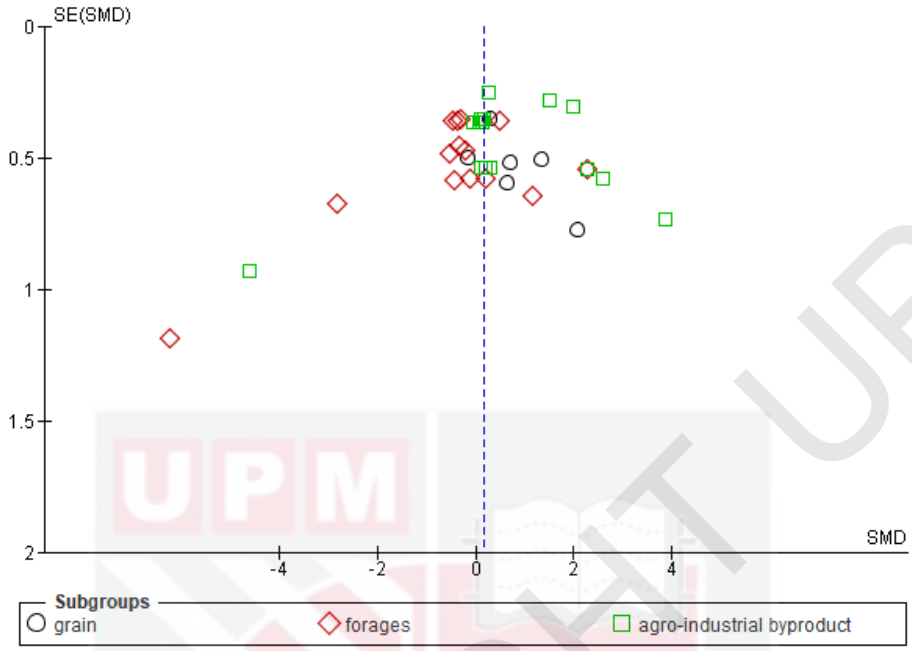

Figure 2.11 : Funnel plots for the links between milk lactose content and polyphenol.

2.2.3.4 Effect of polyphenol supplementation on milk saturated fatty acid content

In the evaluation of milk saturated fatty acid content as affected by the supplementation of polyphenol, there are seventeen publications where the data from thirty-nine studies were incorporated in the review. Figure 6.8 depicts the forest plots of the effect of three different sources of polyphenol on saturated fatty acid content in milk. It was found that polyphenol supplementation significantly affects ($P<0.05$) the saturated fatty acid content in milk with significantly high heterogeneity ($P<0.05$) at $I^2=96\%$. In the analysis to evaluate different sources of polyphenol supplementations, it was also found that different polyphenol sources (grain, forages, or agro-industrial by-products) significantly affect ($P<0.05$) the content of saturated fatty acid in milk with high heterogeneity at $I^2=75.7\%$. Results showed that there are significant differences ($P<0.05$), with a heterogeneity of $I^2=89\%$ in milk saturated fatty acid content from grain source of polyphenol supplemented in the diet, and significant differences ($P<0.05$) with a heterogeneity of $I^2=91\%$ in milk saturated fatty acid content from forages source of polyphenol supplemented diet. The polyphenol from agro-industrial by-product sources also showed a significant difference ($P<0.05$) in milk saturated fatty acid content at high heterogeneity of $I^2=97\%$.

In the grain group of polyphenol sources, only one study showed the 95% confidence interval that crossed the line of no effect (Figure 6.8) while the other five did not cross the line of no effect. The study that crossed the line of no effect

also showed the lowest value of standard mean difference and 95% confidence interval at 0.51(-0.38,1.41) which is a study by Campione *et al*. (2021). Meanwhile, the study that recorded the highest value of standard mean difference and 95% confidence interval was by Santos *et al*. (FO) with 7.05(5.08, 9.02). In the forages group of polyphenol sources, the amount of studies in this group was evenly split with the 95% confidence interval of seven studies crossed the line of no effect while the other seven did not cross the line of no effect. The highest value of standard mean differences and 95% confident interval was recorded by the study of Kholif *et al*. (ME40) with 7.22 (5.21,9.23) and the lowest value of standard mean differences and 95% confident interval was found by Kalber *et al*. (BC-fm) with -0.49(-1.65,0.66). The forest plot of the agro-industrial by-product group showed the 95% confidence interval of all studies in the group did not cross the line of no effect and thus showed all studies in the group were significant, individually. Among the results, Abbeddou *et al*. (TP) (2011) showed the highest value of standard mean difference and 95% confidence interval with 13.07(8.47,17.67) whereas Todaro *et al*. (FLP2) (2017) recorded the lowest value of standard mean difference and 95% confident interval with -3.22 (-4.35, -2.09) in this analysis.

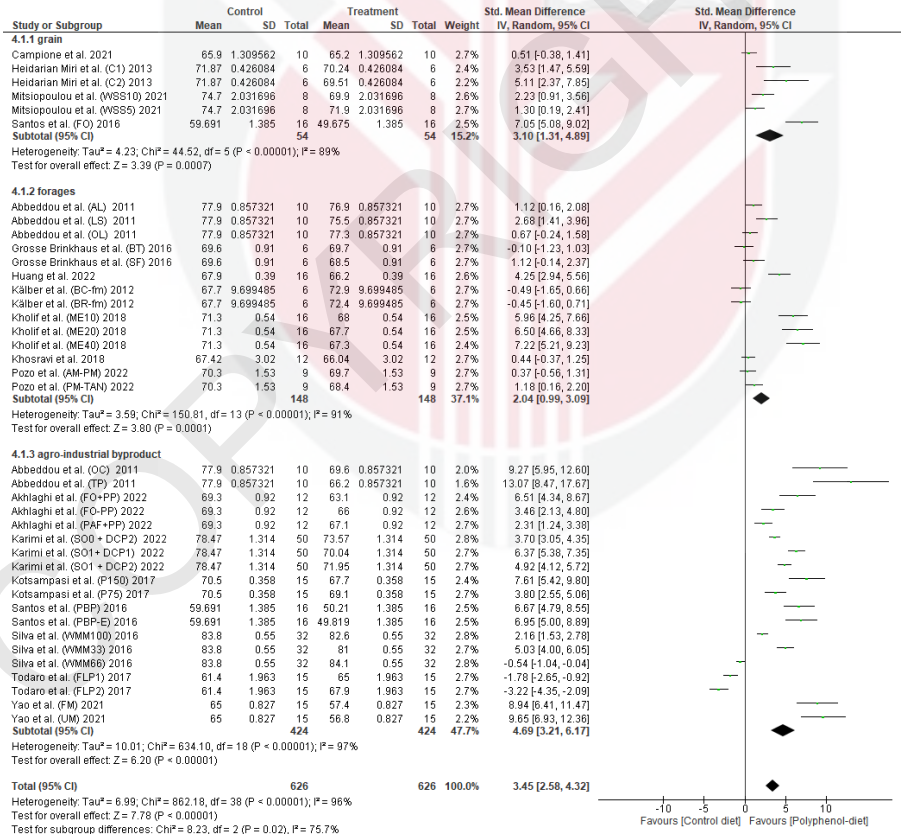

Figure 2.12 : Forest plots for the links between saturated fatty acid content in milk and polyphenol.

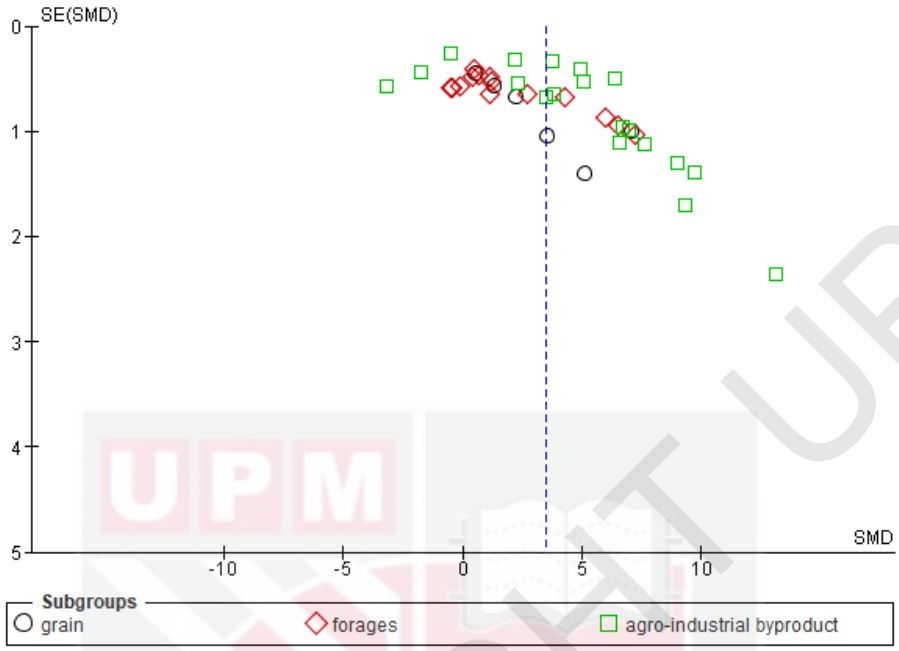

Figure 2.13 : Funnel plots for the links between milk saturated fatty acid content and polyphenol.

2.2.3.5 Effect of polyphenol supplementation on milk medium-chained fatty acid content

In this study, the data from twenty-one studies were extracted from nine publications to evaluate the content of medium-chained fatty acid in milk when the animals were fed with a polyphenol-supplemented diet in the feed. The forest plot on the effect of polyphenol supplementation on medium-chained fatty acid content in milk is depicted in Figure 6.10. The pooled standard mean differences shown in the above figure recorded significant differences ($P<0.05$) with the heterogeneity of $I^2=96\%$ which concurred that polyphenol supplementation significantly affects the medium-chained fatty acid content in milk. It was worth noting that different polyphenol sources (grain, forages or agro-industrial by-products) significantly affect ($P<0.05$) the content of medium-chained fatty acid in milk with high heterogeneity at $I^2=87.7\%$. Results from the analyses show significant differences were noted in grain and agro-industrial by-product ($P<0.05$) polyphenol sources with the heterogeneity of $I^2=87\%$ and $I^2=97\%$, respectively.

It was shown in the forest plot of grain group (Figure 6.10) that 95% confidence intervals of all studies did not cross the line of no effect which indicates that the studies were individually significant where the highest value of standard mean difference, and 95% confident interval was recorded by Coreddu *et al*. (MIX) (2016) with 11.25 (5.64, 16.85) and the lowest value of standard mean difference

and 95% confident interval was by Mitsiopoulou *et al*. (WSS5) (2021) with 1.09 (0.02, 2.16). The outlier among the studies in this group was also by Mitsiopoulou *et al*. (WSS5) (2021) since the 95% confidence interval of this study does not overlap with the group pooled standard mean differences. Meanwhile, the forest plot of the forages group (Figure 6.10) showed that 95% confidence intervals of two studies crossed the line of no effect while five other studies did not cross the line of no effect. There were no outliers detected in this group (Figure 6.10) since all 95% confidence intervals fall within the range of the pooled standard mean difference of this group. The highest value of standard mean difference and 95% confidence interval was found by Cabiddu *et al*. 4May (2009) with 3.67 (2.28, 5.06) whereas Cabiddu *et al*. 17May (2009) recorded the lowest value of standard mean difference and 95% confidence interval with -4.66 (-6.31, -3.01). As for the agro-industrial by-product group, the forest plot showed 95% confidence interval of two studies crossed the line of no effect while ten other studies did not cross the line of no effect. It was observed that the highest value of standard mean difference and 95% confidence interval was by Abbeddou *et al*. (TP) (2011) with 12.65 (8.19, 17.11) while Todaro *et al*. FLP2 (2017) recorded the lowest value of standard mean difference and 95% confidence interval with -2.43 (-3.40, -1.46). Both of these studies were also among the outliers since their 95% confidence intervals did not overlap with the pooled standard mean differences of this group.

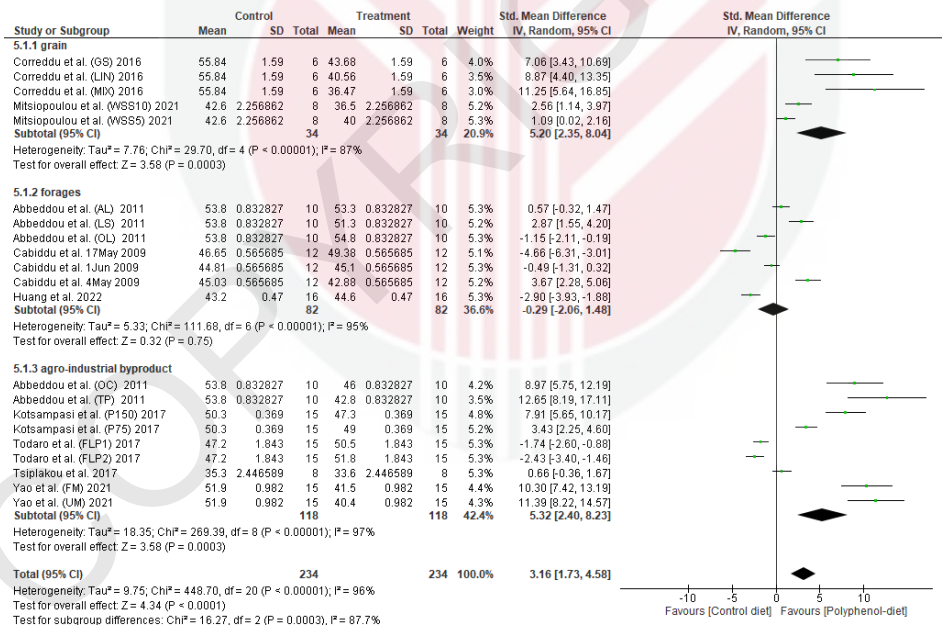

Figure 2.14 : Forest plots for the links between medium-chain fatty acid in milk and polyphenol.

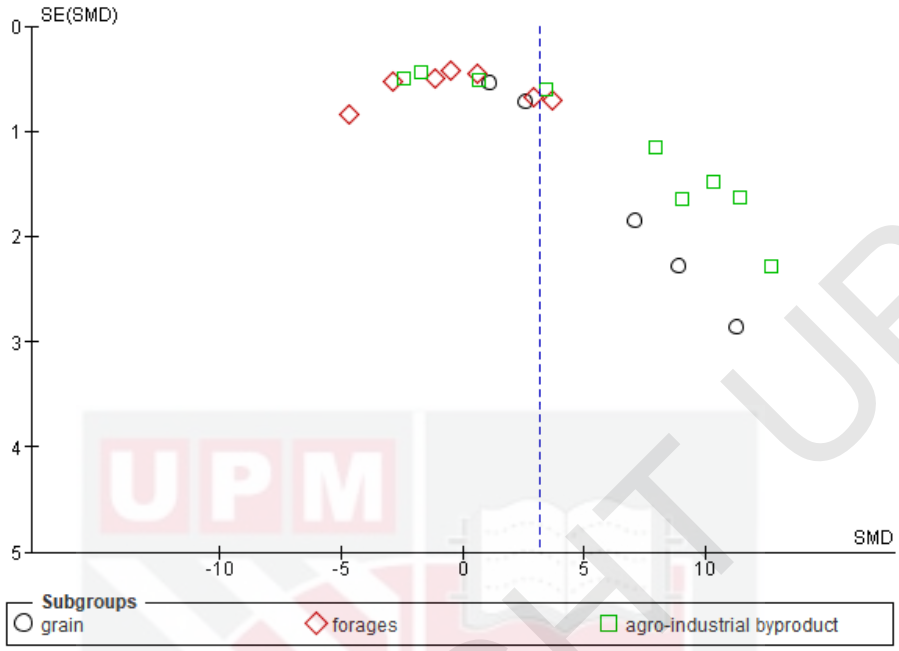

Figure 2.15 : Funnel plots for the links between medium-chain fatty acid in milk and polyphenol.

2.2.3.6 Systematic review discussions on the effect of polyphenol supplementation on milk composition and fatty acid of dairy animal

Generally, plant polyphenol has been given to dairy animals to improve health and productivity. Dairy animal health and productivity can be enhanced through feeding feeds that contain plant components that have attractive biological properties such as antioxidant, anti-inflammatory and anti-microbial (Qin and Hou, 2017). In this study, the effects of plant polyphenols in dairy feeds on milk compositions and fatty acid content in milk were explored.

In the present analysis, no clear difference was observed in milk fat content of animals fed with a polyphenol-supplemented diet compared with feed without polyphenol supplementation. Among the dietary supplementation throughout the reviewed study, the highest value of standard mean difference was found in the study of Santos *et al*. (FO) (2016) where flaxseed oil was included in the diet of dairy cows with total phenolic content of 0.392% in DM whereas the lowest value of standard mean difference is showed in the study of Cabiddu *et al*. 4May (2009) where polyethylene glycol was orally fed to the ewes during grazing the flowering-stages of pastures on 4May with total phenolic content of 3.21% in DM. From the observations in this study, the number of studies which favour the control diet is 13 while the number of studies which favour the polyphenol diet is 12, where it can be concluded that the fat content in the milk is not affected significantly by the supplementation of polyphenols in the diet, despite having it

from different sources. It is interesting to note that the pooled effect estimate for the forages favour the control diet but the pooled effect estimate for grain and agro-industrial by-products favour the polyphenol diet.

The same observations can be seen in the evaluation of milk protein content when supplemented with polyphenols in the diet where there are no significant effects were found on the overall effect of polyphenol supplementation in the diet. It is also found true when tested against different polyphenol sources, where there is no significant difference was found which suggests that milk protein content was similar throughout different polyphenol sources. Throughout the reviewed studies, Akhlaghi *et al*. (FO+PP) (2022) showed the highest value of standard mean difference with total phenolic content of 0.357% of DM, and Santos *et al*., (PBP) (2016) showed the lowest value of standard mean difference with total phenolic content of 2.83% of DM. According to the forest plot, the total number of studies that milk protein content favour the control diet is ten while the total number of studies that milk protein content favour the polyphenol diet is eleven. These findings lead to the insignificant results of this overall outcome effect, and only polyphenol source from agro-industrial by-product is found significant in this study. It is worth highlighting that the pooled effect estimate for the grain group favour the control diet but the pooled effect estimate for forages and agro-industrial by-products group favour the polyphenol diet and the overall pooled effect estimates favour the polyphenol diets with 0.22 value of the standard mean difference, although insignificant.

In the evaluation of milk lactose content changes when supplemented with polyphenol diets, an insignificant result was reported in the overall effect estimates, on the other hand, a significant difference was found only in the grain group of polyphenol sources diet. Throughout the dietary supplementation in the reviewed studies, Akhlaghi *et al*. (FO+PP) (2022) again showed the highest value of the standard mean difference in this outcome with total phenolic content of 0.357% of DM and the lowest value of the standard mean difference in this outcome was by Abbeddou *et al*. (OL) (2011) with total phenolic content of 2.25% of DM. In this study, the number of studies that milk lactose content favours the control diet amounts to four, and the number of studies that milk lactose content favours the polyphenol diet amounts to eight. Meanwhile, it is worth mentioning that the milk lactose content of the studies in the grain groups favours the polyphenol diet compared with the control diet while the rest, although insignificant, milk lactose content of the studies in the forages group mostly favours the control diet and the agro-industrial by-product group favours polyphenol diet. This is in line with the reported study by Modarasi *et al*., (2011) where milk lactose content increased with pomegranate seed pulp supplementation, an agro-industrial by-product, in the feed and similarly, an increase in milk lactose content in the study by Caroprese *et al*., (2011) when lactating ewes were supplemented with flaxseed (grain group) as fat supplement under high temperature-humidity index value averaging 77 during daytime.

In the effect of polyphenol supplementation in the diet on milk saturated fatty acid content, significant differences were observed in this study, in the overall pooled

estimate effect as well as all three different polyphenol sources (grain, forages and agro-industrial by-product). Among the reviewed studies, dietary supplementation by Abbeddou *et al.* (TP) (2011) showed the highest amount of the standard mean differences with total phenolic content of 0.6% of DM while the lowest value of the standard mean differences was by Todaro *et al.* (FLP2) (2017) with total phenolic content of 1.322% of DM. Interestingly, the total number of studies that milk saturated fatty acid content favours the polyphenol diet is 28 while the total number of studies that milk saturated fatty acid content favours the control diet is only three which concludes most result from individual studies in this outcome favours the polyphenol diets compared to control diet. The differences from the three studies mentioned previously might lies in its diet where they use fruit wastes such as pulp, peel and seed; in this cases mango and lemon to replace part of concentrates portion of the diet. The common content of phytochemical in these fruits are p-coumaric acid and ferulic acid, (Pieracci *et al.,* 2022; Maldonado-celis *et al.* 2019) among others.

The changes in medium-chained fatty acid content in the milk-fed with polyphenol supplementation in the diet showed similar outcomes as observed for milk SFA content, previously but not forages group. Among the dietary supplementation in this study, the highest value of the standard mean differences comes from the agro-industrial by-product group which is Abbeddou *et al.* (TP) (2011), where lipid-rich tomato pomace, which contains 0.6% of DM in phenolic content were included in the ewe's diet. On contrary, the lowest value of standard mean differences in this study comes from the forages group, where Cabiddu *et al*. 17May (2009), in its study of polyethylene glycol oral supplementation to the ewes prior to grazing the flowering stages of Sulla pastures on 17May, in which the phenolic content of the pasture was 3.55% of DM. What stands out in this study was the total number of studies that favour the polyphenol diet was more than the total number of studies that favour the control diet, which is thirteen and five studies, respectively. The five studies that favour the control diets are considered outliers since it does not fall within the 95% of the confidence interval of overall effect estimates. Among the five studies were, both Todaro *et al.* (2017) study as previously mentioned in milk saturated fatty acid content, Cabiddu *et al*. 17May (2009) study, Abbeddou *et al*. (OL) (2011), where polyphenol-rich olive leaves, which contains 2.25% of DM in phenolic content were included in the ewes' diet, and the study by Huang *et al*. (2022) where paulownia leaves silage was used as alfalfa silage replacement in dairy cows' diet which contains 0.36% of DM in phenolic content. The most dominant phenolic acid in paulownia leaves silages as addressed by Huang *et al*. (2021), was phenylpropanoid glycosides meanwhile the most dominant phenolic acid in olives leaves are secoiridoids, oleuropein specifically, as secoiridoids is a family compound that is characteristic of Oleaceae plant (Hashmi *et al*., 2015).

2.2.3.7 Systematic review summary on the effect of polyphenol supplementation on milk composition and fatty acid of dairy animal

The current study shows milk fat, protein, and lactose content were unaffected by polyphenol supplementation, overall. However, when polyphenol supplementation was categorized into different sources (grain, forage, and agro-industrial by-product), the milk protein content of the agro-industrial by-product group as well as the milk lactose content of the grain group was significantly affected. Both SFA and MCFA content in milk, show a significant difference in all three groups but not in the forage group of milk MCFA where it is insignificant. In conclusion, milk protein content was affected by agro-industrial by-product-sources polyphenols, milk lactose content was influenced by grain-sources polyphenols, and both of these sources were impactful towards milk SFA content. On the other hand, milk MCFA was affected by all three polyphenol sources, significantly.

2.2.4 Inclusion of oil palm agriculture waste by-products in animal feed

Generally, lignocellulosic waste by-products and liquid waste by-products are the types of waste by-products produced by oil palm plantations and industry. Liquid waste such as palm oil mill effluent (POME) and lignocellulosic waste such as oil palm trunk (OPT), empty fruit bunch (EFB), fronds, palm pressed fibers, and shells. In Malaysia, oil palm frond has been incorporated as a substitute for tropical grasses in ruminants (Osman and Ishida, 1992). Several studies have been reported on the usage of oil palm fronds in ruminant feed (Goh, 2002; Ebrahimi *et al.,* 2015; Ghani *et al,* 2017) and further produced quality meat (Ebrahimi *et al.,* 2012) as well as milk (Lunsin *et al.,* 2021). The presence of polyphenols in the feed material especially forage and roughages may contribute to the success of oil palm waste by-products in feeds as it could enhance performances in production and food oxidative stability in products originating from farm animals (Starčević *et al.,* 2015). According to Neo *et al.* (2008), the phenolic of oil palm fruit contains high antioxidant activities along with high phenolic content and flavonoid content, which may help animals in combating oxidative stress. Farm animal are prone to oxidative stress due to heat stress especially in the tropical region. Hence, the usage of health-promoting bioactive compound present in the oil palm agricultural by-product may benefits the consuming animal.

2.2.4.1 Oil palm empty fruit bunch

Oil palm empty fruit bunch is an agricultural by-product of the oil palm plantation industry, which can be found in substantial amounts in Malaysia, as Malaysia is the second largest palm oil producer in the world. The term oil palm empty fruit bunch is the remaining biomass after the removal of fresh oil palm fruits from the fruit bunches, which is different from oil palm fresh fruit bunches and is characterised as newly, harvested oil palm fruit as shown in the Figure below.

After harvesting fresh fruit bunch in the plantation, the fruits are delivered to the oil palm mill within 24 hours for further processing to avoid palm oil quality being degraded due to the increased free fatty acid (FFA) formed (Osawa *et al*., 2007) as a result of lipase enzymatic degradation of triglycerides (Said *et al.,* 2020). In the oil palm mill, the process starts with the sterilisation of the fresh fruit bunch, to soften the palm bunches and release the palm fruit, enzyme inactivation to stop the formation of FFA, and further remove moisture content from the bunch (Wae-hayee *et al.,* 2022).

Oil palm empty fruit bunch capability to become a renewable source in generating bio-oil, which may converted into biofuel, was indeed proven by Mohamed *et al.* (2014). It was then found that through their extraction study by using the green extraction technique (super-critical fluid extraction), the obtained bio-oil from the OPEFB has identified palmitic acid (C16:0) and stearic acid (C18:0) as the major compound found in the bio-oil (Mohamed *et al.,* 2014).

Figure 2.16 : Oil palm fresh fruit bunch and oil palm empty fruit bunch.

2.3 Consumer concerns related to milk consumption

Milk is one of the animal products that is an excellent source of vitamins and minerals, particularly calcium and high-quality protein. Milk from ruminant also provide essential amino acid that is vital in the muscle protein synthesis. The importance of milk during childhood is widely known. It has been reported that undernourished children may experience stunting, and a high risk of short-term cognitive development, while undernourished adults may have hyperglycemia, hypertension, elevated blood lipids as well as obesity (de Onis and Branca, 2016). It was emphasized by Michaelsen (2013) that milk possessed certain

growth-promoting effects in children, which is seen in developed and developing countries. This suggests that children's growth may commence provided with adequate energy and nutrient intake.

It was argued that dietary saturated fatty acid consumption should be reduced in order to decrease metabolic syndrome and cardiovascular disease (CVD) risk, and milk, being a high-fat sourced food, was linked with the incidence of the diseases mentioned. According to WHO (2003), studies have established overindulgence in saturated and trans-fatty acids is related to the elevated risks of human CVD. The current level of trans fatty acid intake in the human diet from ruminant-derived food has shown no association with the increasing risk of cardiovascular diseases (Jakobson *et al*., 2006; Chardigny *et al*., 2008) it is becoming evident as clinical trial data tend to incriminate trans fatty acid with more than one double bond as harmful (Shingfield *et al*., 2008b). However, recent scientific studies did not support the relationship between biomarkers of dairy fat intake with the risk of diabetes mellitus or CVD, but further suggests that consumption of dairy fat within the typical dietary pattern is inversely related to previously mentioned risk (Alexander *et al*., 2016; Kratz, Baars, and Guyenet, 2013; Thorning *et al*., 2017).

In the past, the intake of C12:0, C14:0, and C16:0 saturated fatty acid in excessive amount were regards as unhealthy but due to recent approach of nutrient composition of milk, a term called dairy matrix, would change the responses of blood lipid to saturated fatty acid by the presence of few dairy matrix component such as calcium, peptides, phosphorus and the milk fat globule membrane (Thorning *et al*., 2017). This results in the hypercholesterolemic fatty acid (C12:0, C14:0, and C16:0) having no impact on the parameters of cardiovascular health when supplied in the dairy matrix. Thus, milk fat remains exclusively as the source of certain bio-actives compounds from animals, and the importance of milk in the human diet was inevitable.

2.4 Summary

Small ruminant dairy industry is a slow-growing agricultural sub-sector in Asia. Increasing awareness of milk consumption has put the industry back on the resurgence and as a result, milk prices increase. The usage of polyphenol-rich agriculture by- products in dairy feed as shortage of high-quality roughage supply occurs may provide a solution for farmer’s interest in small ruminant husbandry. Naturally rich anti- oxidants in milk as a result of utilizing high-polyphenol agro-byproducts in feed is expected as phytochemical compounds may absorbed and deposited in milk. To the best of our knowledge, empty fruit bunch, an agricultural waste from oil palm plantations, readily available in Malaysia, has never been used as part of animal feed in modifying milk nutrients and composition. Hence, current research is focusing on oil-palm empty fruit bunch utilization in dairy goats, and its response to fermentation and milk products. These are done with the aim of not only diversifying the feed sources for ruminants but also to further optimize and improve the resultant goat's milk for human health.

CHAPTER 3

CHARACTERISATION OF OIL PALM EMPTY FRUIT BUNCH METHANOLIC EXTRACT

3.1 Introduction

Oil palm empty fruit bunch (OPEFB) and fronds are two types of oil palm by-products from an oil palm plantation. In the palm oil mill, OPEFB production amounted to 23% of fresh fruit bunch after being processed (Sadi, 1992; Schmidt, 2007). The disposal of these waste products was either deposited back in the plantation or burnt within nearby factory facilities which is always a concern of every oil palm factory management whenever it is high in production season.

In Malaysia, cattle integration systems under oil palm plantations are easily implemented due to the shade provided by the trees. Oil palm wastes are usually used for livestock feed through various interventions in order to reduce wastage. Oil palm fronds were a popular choice among farmers to use as animal feed, by either fresh chopped OPF, silage, or pelleted/cubed. Meanwhile, OPEFB was an unpopular choice due to its high content of fibre. Early studies by Mat Rasol *et al.,* (1993) showed OPEFB fermented by inoculating *Pleurotus sajor-caju* was found to be palatable to beef cattle. OPEFB is regarded as high-quality organic matter material compared to other oil palm plantation by-products, and it is useful for soil properties improvement due to its nutrient-rich content, high loading rate and fast decomposition rate, despite its bulk (Sung, 2016). It is considered a loss if there is no investigation done on the chemical constituent residue in the OPEFB, and it is vital to explore OPEFB as a possible raw material source for future use which may increase its value.

In the present study, OPEFB chemical constituent residue was extracted by using a solvent system in order to further understand its content and benefits. A previous study by Jaffri *et al.,* (2011) showed that oil palm leaves are rich sources of polyphenols, meanwhile, Al-Jumaili (2018) identified 56 chemical constituents in his study of oil palm leaf methanolic extract characterization with linolenic acid, methyl ester was the major compound of interest contained in the oil palm leaf extracts. Whereas, n-hexadecanoic acid was the major compound of interest found in oil palm fruits, oleic acid came as the second major compound of interest (Odion *et al.,* 2010). As of this moment, there is a lack of literature published on the characterisation of OPEFB extract where phytochemicals and bioactive compounds was identified. Therefore, this study was done with the objective is to determine the bioactive phytochemical constituents in the oil palm empty fruit bunch methanolic extract.

3.2 Materials and methods

3.2.1 The preparation of the extract

Fresh fruit bunchs were collected from Eng Hong Palm Oil Mill Sdn. Bhd. (GPS coordinates: 2.8598918783, 101.476206779), an oil palm processing plant in Banting, Selangor, Malaysia. Upon retrieving the fresh fruit bunch of oil palm, bunches were shredded into small pieces approximately 2cm long and 1cm wide. Then, it was soaked in methanol at the ratio of 1:3 w/v for 72 hours, and was shaken for every 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). Methanol was chosen as a solvent in this extraction system due to its high polarity and is considered as universal solvent, as it can extract polar and non-polar component (Riyadi *et al.,* 2023). The methanolic extract was centrifuged at 4000rpm for 20 minutes before storage at room temperature if the methanolic extract still contain large particles from the empty bunches. The methanolic extract were then evaporated under partial vacuum using a rotor evaporator (Heidolph, Germany). The concentrate is then stored at -80°C freezer. The oil palm empty fruit bunch methanolic extract is then used to prepare the treatment diets for *in-vitro* and *in-vivo* animal trials.

Figure 3.1 : Chopped oil palm empty fruit bunches.

Figure 3.2 : The methanolic extract of oil palm empty fruit bunch at the ratio of 1:3 (w/v) kept within a month before further rotor-evaporated into concentrate. (From left: methanolic extract after centrifugation and methanolic extract before centrifugation).

3.2.2 Determination of plant secondary compound in OPEFB extract

3.2.2.1 Determination of total phenol (TP)

OPEFB extract were subjected to determine the content of total phenol (TP) according to the method reported by Ismail *et al.* (2010). Fifty µl of OPEFB extract were used in a test tube and the volume was made up to 1.0 ml with distilled water. Next, 0.5 ml of Folin Ciocalteu reagent was added and was mixed properly. Then, 2.5 ml of sodium carbonate solution (20% w/v) were added, mixed and kept for 40 min at room temperature (26 ˚C). The optical density were measured at 725 nm wavelength in a spectrophotometer (Spectro Sc 20, Labomed, Inc. USA). The phenol concentration in the OPEFB extract was estimated from a constructed standard curve (Ismail *et al.,* 2010). The TP was estimated as tannic acid equivalents and expressed on a dry matter (DM) basis.

3.2.2.2 Determination of total tannin (TT)

Total tannin was measured by substracting non-TP from TP (TT = TP – non-TP). The portion of non-TP was determined by precipitating tannins using polyvinyl poly pyrrolidone (PVPP), which binds tannins. To bind tannins, 200 mg of PVPP were placed in a test tube along with 2.0 ml of distilled water and then 2.0 ml of OPEFB extract were added. The mixture was filtered and 150 µl of the filtrate was placed in a test tube and the volume was made up to 1.0 ml with distilled water. The rest of the processes were the same as the determination of TP as mentioned previously. The concentration of the non-TP was calculated according to the standard curve and were expressed on dry matter (DM) basis as described by Hiai *et al.* (1976).

3.2.2.3 Determination of total flavonoid (TF)

The determination of the total flavonoids content of OPEFB extract was done according to the method of Quettier-deleu *et al.* (2000). Approximately, 1 ml of OPEFB extract was added to 1 ml of methanolic aluminum chloride hexahydrate ($AlCl_3$, $6H_2O$). The mixture was left for 10 minutes before the absorbance was measured at 430 nm by a spectrophotometer. The assay was done in triplicate and the results were expressed in mg rutin/100 g dry matter, calculated using a rutin standard curve (A_{430}=0.0056x + 0.1343, *x=rutin, r=0.9735*), estimated using 8 concentrations of rutin (25-1000 µg/ml).

3.2.3 Determination of lipid oxidation

Lipid peroxidation activity was measured through a modified thiobarbituric acid-reactive species (TBARS) assay by using egg-yolk homogenates as lipid-rich media (Ruberto *et al.,* 2000). Egg homogenate (250 µl, 10% in distilled water, v/v) and 50 µl of extract were mixed in a test tube and the volume was made up to 500 µl, by adding distilled water. Finally, 25 µl FeSO4 (0.07 M) was added to the above mixture and incubated for 30min, to induce lipid peroxidation. Thereafter, 750 µl of 20% acetic acid (pH 3.5) and 750 µl of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulphate) and 25 µl 20% TCA were added, vortexed, and then heated in a boiling water bath for 60min. After cooling, 3.0 mL of 1-butanol was added to each tube and centrifuged at 3000 rpm for 10min. The absorbance of the organic upper layer was measured against 3mL butanol at 532 nm. For the blank 50 µl of distilled water was used in place of the OPEFB extract.

3.2.4 Characterisation of OPEFB methanolic extract by GC-MS analysis

An analysis by gas chromatography-mass spectrometry (GC-MS) was done to identify the compound present in the OPEFB extract (Arunkumar and Muthuselvam, 2009; Delazar *et al.,* 2009). The OPEFB methanolic extract was filtered through a 0.45µm syringe filter straight into a 1.5ml embered glass autosampler GC vial for analysis. The analysis was performed on an Agilent 7890A gas chromatograph (GC) coupled directly to the mass spectrometer system (MS) of an Agilent 5975C inert MSD with a triple-axis detector. The column used was the DB-5MS UI, length 30 m, diameter 0.25 mm, film thickness 0.25 µm and 5% phenyl methylpolysiloxane as stationary phase (Agilent Technologies, Santa Clara, CA, USA). The MSD Chemstation was used to find, integrate and count all the peaks in the raw GC chromatogram. A library search was carried out for all the peaks using the NIST/EPA/NIH version 2.0, and the results were combined in a single peak table. The instrument used in this study has been supported by the Molecular Structure Characterization Laboratory, Centre for Research and Instrumentation Management (CRIM), Universiti Kebangsaan Malaysia (UKM).

3.3 Results

The yield of OPEFB methanolic extract in this study was 28.32 g/kg OPEFB DM. The bioactive content of OPEFB extract are shown in Table 3.1. This OPEFB methanolic extract yields are much lower than the methanolic extract in previous study by Al-Jumaili (2018), which is 60 g/kg DM extracted from oil palm leaves.

Table 3.1 Biochemical analysis of OPEFB (mean % DM ± SE).

Item	TP	TT	TF	Lipid peroxidation activity, ml MDA/L extract
OPEFB extract	3.98 ± 0.11	2.37 ± 0.11	1.30 ± 0.01	7.17 ± 0.24

OPEFB: oil palm empty fruit bunch; DM: dry matter; TP: total polyphenols; TT: total tannins; TF: total flavonoids; and MDA: Malondialdehyde.

The GC-MS analysis of OPEFB methanolic extract showed the extract contained a total of 70 compounds (Appendix C1) based on the number of peaks, peak area percentage, retention time, molecular formula, and molecular weight. Chemical constituents were identified using spectrum data-based software (NIST 11.0) installed in GC-MS. GC-MS analysis were conducted to identify major chemical compounds that are extracted from OPEFB and its biological activities. The major phytochemical compounds found in OPEFB methanolic extract are hexadecanoic acid, methyl ester, a linoleic acid ester (41.29%), 11-Octadecenoic acid, methyl ester, a methyl trans vaccenic acid (32.23%), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, a linolenic acid (9.55%), methyl stearate (2.55%), and n-hexadecanoic acid, a palmitic acid (1.94%).

Table 3.2 The potential activity of the major identified compound in OPEFB extract.

Items	Area (%)	Library ID	Potential activity
46, Appendix C1	41.2894	Hexadecanoic acid, methyl ester	Antioxidant, hypocholesterolemic, nematicide, pesticide, anti-androgenic flavor, haemolytic anti-inflammatory, hypocholesterolemic, hepatoprotective, insectifuge, antihistaminic, antieczemic, antiacne, antiandrogenic, antiarthritic, anticoronary
55, Appendix C1	32.2902	11-Octadecenoic acid, methyl ester	Antioxidant and antimicrobial
54, Appendix C1	9.5539	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Antioxidant, anti-inflammatory, antibacterial, hypocholesterolemic and hepatoprotective activities,
47, Appendix C1	1.9438	n-Hexadecanoic acid	Antioxidant, hypocholesterolemic nematicide, pesticide, anti androgenic flavor, hemolytic, 5-Alpha reductase inhibitor, antimicrobial
57, Appendix C1	0.9587	trans-13-Octadecenoic acid	Good anti-inflammatory activity, antimicrobial
29, Appendix C1	0.8521	Methyl tetradecanoate	Antiplatelet activity
44, Appendix C1	0.4767	7-Hexadecenoic acid, methyl ester, (Z)-	Anti-inflammatory, antimicrobial
49, Appendix C1	0.4422	Hexadecanoic acid, 15-methyl-, methyl ester	
6, Appendix C1	0.4321	2-Methoxy-4-vinylphenol	Antimicrobial, antioxidant, anti-inflammatory
39, Appendix C1	0.3404	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	Antimicrobial, antioxidant, antiviral
43, Appendix C1	0.3351	Pentadecanoic acid, 14-methyl-, methyl ester	Antioxidant, antifungal, and antimicrobial activities.
13, Appendix C1	0.1308	Butylated Hydroxytoluene	Antioxidant

Source: Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 1994).

Library Search Report

Data Path : D:\DATA\sample 2018\
Data File : 050221.D
Acq On : 5 Feb 2018 16:27
Operator :
Sample : L
Misc :
ALS Vial : 2 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0
C:\Database\NIST05a.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

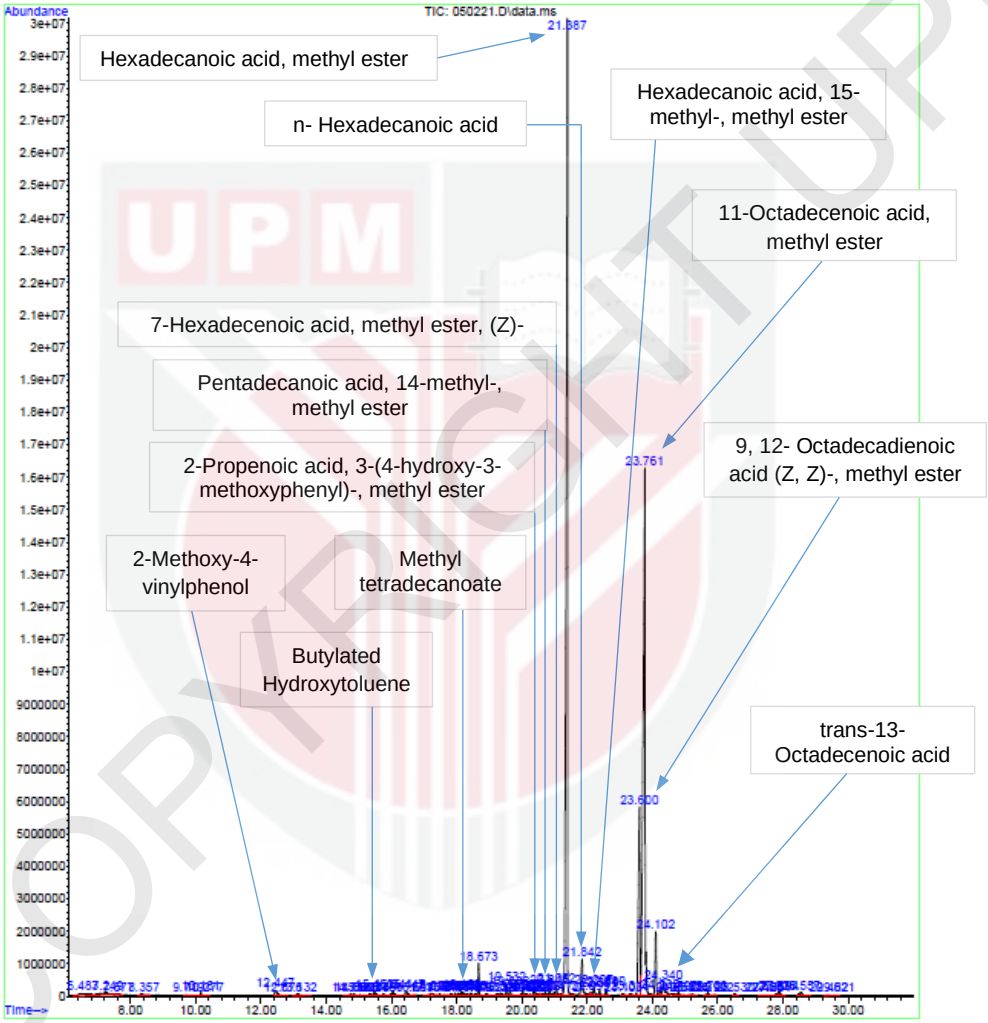

Figure 3.3 : GC-MS chromatogram of oil palm empty fruit bunch methanolic extract.

3.4 Discussion

The yield of OPEFB methanolic extract in this study was 28.32 g/kg OPEFB DM, which is much lower compared to the yield of OPLE methanolic extract of 60 g/kg oil palm leaves reported by Al-Jumaili (2018), conducted with the same procedures.

Current study reported that OPEFB extract contain 3.98% of total polyphenol content which is lower than the OPEFB extracted with dichloromethane, at 4.2% (Sabrina *et al*., 2012) and 4.54% in OPLE (Al-Jumaili, 2018), but higher compared to OPEFB extraction with ethanol; 1.79% (Tsouko *et al.,* 2019). The differences between the amount of polyphenolic content obtained in current study with result reported by Al-Jumaili (2018) were due to the different parts of the oil palm used, which are empty fruit bunch and leaves, respectively. This finding are in line with previous studies that reported total polyphenol content of leaf extract was higher than other plant part (Pyo *et al.,* 2004; Wong and Kitts, 2006). In present study, the amount of tannin in the OPEFB methanolic extract are lower than tannin extracted from OPEFB acetonic extract, which is 2.37% and 4.54% (Ibrahim *et al.,* 2005), respectively. OPEFB extract in this study contain higher tannin content when compared to the tannin content of the leaf extract as reported by Al-Jumaili (2018) which is 2.37% and 1.52%, respectively. The differences of the tannin content may be due to the parts of the plant used to extract from, as copious amount of plant tannins are in susceptible parts of plants to predation (Terrill *et al*., 1992), such as new leaves, buds and fruits. It was also reported by previous study that tannin extracted by methanol resulting in more yield as compared to water extraction (Duraisamy *et al*., 2020; Medini *et al*., 2014). Meanwhile, total flavonoid content of the OPEFB extract showed in this study were higher; 1.30% DM as compared to the content in OPLE extract by Al-Jumaili, (2018); 0.28% DM and another study performed by Ahmad *et al*. (2018) where they investigate phytochemical content in oil palm leaves extract through a few ethanol extraction systems, in which the highest content were 1.16% DM. As mentioned before, the differences may lie on the fact that different part of plant used for extraction. Plant flavonoid has been associated with pollination, plant-pathogen interaction, light screening, seed development and allelopathy (Winkel Shirley, 2001) which suggests flavonoid are abundant in flower (Tuyen *et al*., 2017) and fruits (Zoratti *et al*.,2014). Therefore, it is clear that the current work reflects to the accumulation of select flavonoid in OPEFB.

In the present study, extracted polyphenol from OPEFB were characterised. Through GC-MS characterisation studies, 12 components were identified and chosen according the highest area peak percentage or with interested activity. The identified component showed interesting activities such as anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer, and anti-fungal, among others. Hexadecanoic acid, methyl ester was found as the highest constituent (41.3%) in the OPEFB extract in this study, which followed by 11-Octadecenoic acid, methyl ester (32.3%). This finding corroborated with Odion *et al*. (2020) in their study on compound identification of *Elaeis guineensis* fruit where hexadecanoic acid, methyl ester was in the highest quantity (10.53%), as well as oleic acid

(25.92%). n-hexadecanoic acid (31.41%), cis-vaccenic acid (22.82%) and octadecanoic acid (13.16%), in which are also present in our extract although it was in different amount.

According to the current study, the extract also detected to have anti-microbial properties (Table 3.2). Previous study by Al-Jumaili (2018) showed a decrease in the population of *Butyrivibrio fibrisolvens* and methanobacteriales while *R. albus* population increases with the inclusion of oil palm leaves extract in the substrates of the *in-vitro* study. This shows that there are compounds in the OPLE extract that have selective anti-microbial effect towards microbial population in the rumen. As the extract possess selective anti-microbial effect against rumen microbial population, it would inadvertently affect fibre digestibility as *R. albus* population increases, fibre degradation may increase as well due to earlier studies reported that *R. albus* are one of the three species that plays a key role in fibre degradations (Kala *et al.,* 2017; Wanapat and Cherdtong, 2009). The compound contained in EFB extract that have anti-microbial properties in the current study are 11-Octadecenoic acid, methyl ester, n-Hexadecanoic acid, trans-13-Octadecenoic acid, 7-Hexadecenoic acid, methyl ester, (Z)-, Hexadecanoic acid, 15-methyl-, methyl ester, 2-Methoxy-4-vinylphenol, 2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester, and Pentadecanoic acid, 14-methyl-, methyl ester. As most of these compounds was fatty acid methyl esters, it is well known that long chain unsaturated fatty acids are to inhibit Gram-negative bacteria such as *Escherichia coli* (Dilika, Bremner, and Meyer, 2000; Sun, O'Conner and Roberton, 2003). These differences in the gram-positive and gram-negative bacterias sensitivities to fatty acids may due to the impermeability of the Gram-negative bacteria outer membrane, which is an effective barrier against hydrophobic substances (Galbraith and Miller, 1973; Sheu and Freese, 1973; Sheu *et al.,* 1975).

The present study also showed that OPEFB extract possesses compounds that may exhibit antioxidant properties (Table 3.2). It was reported that phenolic play a vital role in antioxidant activity and stronger antioxidant activity are results of a higher content in phenolic (Sumczynski *et al*., 2016), especially in flavonoid content (Ismail *et al*., 2010). This was due to its scavenging ability resulting in the hydroxyl group directly contributing to antioxidative action (Vadnere *et al*., 2012, Bendary *et al*., 2013). It was established that there is positive correlation between antioxidant potential with the bacteriostatic activity of plant extract (Nikitina *et al*., 2007). In the current study, components that exhibit anti-oxidant activities in OPEFB extract are higher amounted as compared with the result by Al-Jumaili (2018) where Hexadecanoic acid, methyl ester, component in the OPEFB extract in which is the highest amount (41%), while 9, 12, 15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)- is the component in OPLE that is the highest amount (16.5%) in which contain antioxidant activities. Henceforth, in this study, the compounds contained in OPEFB extract that have anti-oxidant properties are Hexadecanoic acid, methyl ester (41.3%), 11-Octadecenoic acid, methyl ester (32.3%), 9,12-Octadecadienoic acid (Z, Z)-, methyl ester (9.6%), n-Hexadecanoic acid (1.94%), 2-Methoxy-4-vinylphenol (0.43%), 2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester (0.34%), Pentadecanoic acid, 14-methyl-, methyl ester (0.34%), and Butylated Hydroxytoluene (0.13%).

Since most of the interested compounds in OPEFB extract exhibit antioxidant activity, it shows that OPEFB extract can be considered as a potential natural antioxidant source. Therefore, OPEFB extract that has compounds with antioxidant and antimicrobial properties would modify the dinamics of rumen microbial community as well as fortified antioxidant capacity in the rumen while avoiding oxidative stress especially during high concentrate feeding (Bezerra *et al*., 2020).

Present study shows Hexadecanoic acid, methyl ester or can be called methyl palmitate or margaric acid, with a molecular weight of 270.4507 (Appendix C1) represented the highest amount, 41.3% of total identified amount in OPEFB extract. In leaves extract of palm oil as previously studied by Al-Jumaili (2018), methyl palmitate only amounted to 3.87% of total identified compound which is much lower than detected in current study. Methyl palmitate or margaric acid (C17) is an odd-chain fatty acid, along with branch-chain fatty acid were closely related to VFA, microbial crude protein (MCP) and bacterial population in the rumen (Xin *et al*., 2021). It shows positive correlations between margaric acid concentration in the rumen with total VFA and MCP, while total C17 which includes iso-C17 and anteiso-C17, positively correlates with butyrate concentration in the in-vitro pure carbohydrate mixture incubation study. This suggested that the presence of species-specific microbes (Marinšek Logar *et al*., 2001; Kopečný *et al*., 2003 in relation to the amount of odd branch-chain fatty acid concentration in the rumen. The changes of rumen bacterial population shifts can be reflected by the amount of OBCFA (anteiso-C15 and iso-C17, especially), where cellulolytic bacteria (*R. flavefaciens* and *B. fibrisolvens*) are enriched in iso-C17:0, meanwhile the amylolytic bacteria (*S. ruminantium, R. amilophilus*, and *S. dextrinosolvens*) contain low iso-C17:0 (Vlaeminck *et al*., 2006).

Apart from major fatty acid esters in the extract, 2-Methoxy-4-vinylphenol and butylated hydroxytoluene are among other compounds detected its presence in the EFB extract. 2-Methoxy-4-vinylphenol are an interesting volatile compound. It falls under flavonoids of natural compound and according to Jeong and Jeong (2010), it demonstrated to have potent anti-carcinogenic activity by specifically blocking the hyper-phosphorylation of retinoblastoma protein *in vitro*. Plants from different region may contribute in the differences in the phytochemical presence in the plant extract. This is due to the evolution of plant defences, the tropical tree leaves contained higher phenolic diversity (Peguero *et al*., 2021). *Elaeis* genus belong to the Palm family or in correct term is *Arecaceae,* (Dransfield *et al*., 2008) which is the one of oldest family of flowering plant, dating to Cretaceous period (Purseglove, 1972). Henceforth, showing that palm tree successfully survived over a long period of time, through the plant structural and chemical evolution (Dransfield *et al*., 2008; Windsor-Colins, 2007).

3.5 Conclusion

This study revealed the presence of at least 70 phytochemical constituents in oil palm empty fruit bunch methanolic extract. There are at least twelve major phytochemical constituents, with the highest content being hexadecanoic acid, methyl ester, and 11-Octadecenoic acid, methyl ester as the second highest content in OPEFB extract. Identification of these compounds in the oil palm empty fruit bunch extract serves as the basis for discovering new novel compounds in waste products of oil palm plantations and determining future possible downstream products.

CHAPTER 4

EFFECT OF OIL PALM EMPTY FRUIT BUNCH METHANOLIC EXTRACT ON *IN-VITRO* RUMEN FERMENTATION CHARACTERISTICS IN GOAT

4.1 Introduction

The *in-vitro* rumen fermentation technique is a beneficial tool that enables wide screening of potential feedstuff within a relatively short time scale by simulating the incubation of an anaerobic environment inside the rumen. McBee (1953) and Hungate (1966) were the first who develop the principle of potential rumen feed degradability or fermentability through the measurement of gas production from a batch culture. Over the years, the techniques have been well developed and discussed and it is still widely used to predict the digestibility of feedstuffs, evaluate the effects of secondary compounds and feed additives on rumen microbial activity, determining fermentation kinetics, and analysing the fermentation products of various feedstuffs (Getachew *et al*., 2005).

Alterations in feed ration lead to modifications in the rumen microbial community that modify fermentation patterns and affect changes in the product of nutrient digestion in the rumen; such as rumen ammonia nitrogen (NH_3N), volatile fatty acid (VFA) or specifically short-chained fatty acid, fatty acid (medium-chained fatty acid and long-chained fatty acid) and to such extent, lactic acid to produce better ruminant product. Feed interventions such as incorporating plant oil rich in linoleic acid (Almeida *et al*., 2013), marine lipid (Toral *et al*., 2014), 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 in the ruminant diet has shown promising results in reducing biohydrogenation in the rumen (Jayanegara *et al*. 2012, 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 current study, oil palm empty fruit bunch, a by-product of palm oil plantation was utilized through methanolic extraction to obtain plant bio-actives to incorporate it in the animal feed to further improved animal produce. The extraction process of obtaining bio-actives from the empty fruit bunch can rule out the biggest predicament of using empty fruit bunch in animal feed, high lignocellulosic content, while still benefiting from it through its bio-actives content. In the previous chapter, it was recorded that OPEFB extract contains hexadecanoic acid, methyl ester or methyl palmitate as the highest bio-active component in the extract. Methyl palmitate has antibacterial activity as stated by Astiti and Ramona (2021) where the mechanism of the antibacterial activity was through impairing the walls and membranes of bacterial cells. In addition, methyl palmitate from banana corm was also recorded to have anti-bacterial activity against *Escherichia coli, Salmonella typhi, Staphylococcus aureus,* and *Bacillus cereus* (Fajrih *et al.,* 2022). Another study of feeding oil palm vegetation liquor extract to rat increase *Ruminococcus sp.* in ceacal digesta

while total bacteria in ceacal digesta remains unaffected (Conlon *et al*., 2020). Meanwhile, it was reported that methyl palmitate contained in mint (*Mentha spicata*) has antioxidant potential (Abdel-Hady *et al*., 2018) and in *Cirsium arvense* plants have antimicrobials, pesticides, and nematicides potential (Hema *et al*., 2011; Banaras *et al*., 2017). Hence, it is imperative to assess the potential of OPEFB extract which contains a substantial amount of methyl palmitate, on the rumen fermentation, *in-vitro*, especially the microbial population of the rumen. Thus, the objective of this study is to evaluate the effect of OPEFB extract on in vitro rumen fermentation characteristics in goats.

4.2 Materials and methods

4.2.1 Preparation of substrate

The substrate used in the present study was a 60:40 mixture of napier grass and commercial concentrates. The dietary treatment was designed according to Kim *et al*., (2015) and Avila *et al*., (2020) Before mixing, both materials were dried separately in a 60°C oven for 24 hours and ground to pass through a 1mm sieve. The OPEFB extract is then included in the weighted, mixed substrate at their respective inclusion percentage (0%, 5% and 10% w: w) prior to the start of 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).

Table 4.1 : Feed composition (%) of dietary treatments.

Feed composition	CON	5% OPEFB	10% OPEFB
Napier grass	60	60	60
Commercial pellet	40	40	40
OPEFB methanolic extract	-	5	10
Gross energy (MJ/kg)	3.681	3.701	3.681
Dry matter	90.33	89.76	87.93
Crude protein	13.77	12.91	13.36
Neutral detergent fibre	59.40	58.33	57.92
Acid detergent fibre	38.60	40.53	41.39
Acid detergent lignin	4.73	5.40	5.08
Ether extract	1.74	4.14	6.20

Control (0 %, basal diet: concentrate + napier grass (40:60 %)), OPEFB extract diet (5% and 10% OPEFB extract per DM diet).

4.2.2 Animal management and rumen fluid collection

The experiment protocol was approved by the Animal Care and Ethics Committee of the Universiti Putra Malaysia (UPM/IACUC/AUP-R0048/2017). Three female Saanen dairy goats were used as rumen fluid donors. Animals were fed a 60:40 mixture of napier grass and commercial pellet, where the

amount of feed was given to the animal as per dry matter basis at 3.0% of BW, twice a day. The animals were kept in individual cages where water and mineral blocks were available at all times. Rumen fluid was obtained at 0800 h, before morning feeding through stomach tubing as described by Harun (2015) from each of the does. The animals was manually restrained by a handler while the rumen fluid was obtained. The amount of rumen fluid taken from each doe was around 50ml, amounting to 150ml of rumen fluid. Then, rumen fluid was strained through 4 layers of muslin cloth and kept in a pre-warmed thermos flask that was pre-flushed with carbon dioxide and transported to the laboratory.

4.2.3 *In-vitro* gas production

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

The gas production data were fitted to the following model of Ørskov and McDonald (1979) where Y is the volume of gas produced at Time t, a is the gas production from the immediately soluble fraction (mL), b is the gas production from the insoluble fraction (mL), c is the gas production rate constant for the insoluble fraction (b) and t is the incubation time (h).

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

Thawed samples were centrifuged at 3000 × g for 10 min at 25°C, and 0.5ml of the supernatant was filtered (0.45µm) and was added to 0.5ml of 4-methyl- n-valeric acid (20 mM) before being analyzed by using a 6890N Network GC System gas chromatograph (Agilent Technologies, Palo Alto, CA, USA).

4.2.4 Determination of rumen volatile fatty acid

Rumen samples were left thawed at room temperature. Then, the procedure afterward was done according to Filipek and Dvorak (2009). The mixture was centrifuged for 10 min at 6000 × g. The supernatants (0.5 ml) were collected and added with 0.5 ml of internal standard before being stored in vials. Preparations of the standards were explained in Appendix A. The samples were then analyzed by using gas chromatography (Agilent 69890N Series Gas Chromatography System from Agilent Technologies, USA) fitted with Flame Ionization Detector (FID). The column used was 30 m × 0.25 mm × 0.25 µm (DB-FFAP) with oven temperature of 200ºC, FID temperature of 230ºC and nitrogen gas as the carrier gas with a flow rate of 60 µl per minute.

4.2.5 Determination of rumen ammonia nitrogen

Rumen fluid samples were thawed and centrifuged at 6000 × g for 10 min. The supernatant was collected and ammonia-N determination was done according to Parsons (2013). Stock standard solution of ammonium chloride (NH_4Cl) was prepared to give a 1000 mg/L of solution. A standard 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.

Approximately, 5 ml of sample or standards were added to 0.2 ml of phenol solution and swirled. Approximately, 0.2 ml of 0.02 M sodium nitroprusside solution and 0.5 ml of oxidizing solutions were added in sequenced. The mixtures were swirled, stopped, and allowed to stand for one hour. The absorbance of the intensity was read at 640 nm by using a spectrophotometer (Spectronic GENESYS 20 Spectrophotometer 4001 Series by Thermo Electron Corp., USA). The regression equation was constructed from blank and standard samples and ammonia-N levels in the samples were determined.

4.2.6 Quantification of rumen microbial population by qPCR

The population of total bacteria, total protozoa, total fungi, total methanogens, methanobacteriales, *Ruminococcus albus, Ruminococcus flavefacien, Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens* were quantified by qPCR. Genomic DNA was obtained through 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's 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) consisted of 12.5 µL of 2 × SYBR Green Master Mix, 1 µL of 20 µM forward primers, 1 µL of 20 µM reverse primer, 2 µL

of DNA samples and 8.5 µL of nuclease-free water for each reaction. Each sample was analysed in duplicates. Primers used in the PCR amplifications for targeted rumen microbial populations are detailed in Table 4.2. Real-time PCR quantification was done by using BioRad CFX96 Real-time PCR system (BioRad, USA) with optical grade plates. The qPCR cycling conditions consisted of an initial 5 min of denaturation at 94°C, and is 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 s. The annealing temperatures for primers of total bacteria, total fungi, total protozoa, total methanogens, *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Fibrobacter succinogenes* were 55°C, while that for *Ruminococcus flavefaciens* and methanobacteriales was 58°C.

Table 4.2 : Primers used for qPCR reactions

Target groups	Primers sequence 5' to 3'	Product size	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:GAGGAAGTAAAAGTCGTAACAAGGTTTC	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)

4.2.7 Rumen fatty acid profile

The amount of fatty acid in syringe content at 24 hours post incubation was determined as described by Ebrahimi *et al*. (2015) using chloroform: methanol (2:1) (v/v) containing butylated hydroxytoluene to prevent oxidation during sample preparations. The transmethylation was 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 are 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.

4.2.8 Statistical analyses

All statistical analyses were carried out using SPSS (IBM version 22), through the One-way analysis of variance (ANOVA) procedure in a completely randomized design, to examine the effect of EFB feed on in-vitro rumen fermentation characteristics. Differences between treatment means were compared by using Duncan's multiple range test. The means differences were deemed significant at P values ≤ 0.05.

4.3 Results

Table 4.3: Effect of OPEFB extract supplemented diet on *in-vitro* rumen fermentation parameters of goats (mean ± SD).

Items	CON	5% OPEFB	10% OPEFB	SEM	P value
pH	6.94 ± 0.03	6.95 ± 0.03	6.93 ± 0.03	0.006	0.707
VFA (mol/100mol)					
Acetate	44.71 ± 3.37[a]	40.11 ± 0.31[b]	39.13 ± 2.00[b]	0.944	0.0153
Propionate	31.83 ± 2.96	33.87 ± 4.61	33.85 ± 4.21	1.081	0.718
Isobutyrate	1.56 ± 0.27[a]	1.68 ± 0.21[a]	2.83 ± 0.24[b]	0.183	.00006
Butyrate	15.50 ± 2.02	3.47 ± 0.34	3.63 ± 0.61	0.865	0.130
Isovalerate	3.44 ± 1.53[a]	3.64 ± 1.21[a]	8.43 ± 2.68[b]	0.856	0.008
Valerate	3.40 ± 0.22	3.47 ± 0.34	3.63 ± 0.61	0.114	0.737
Total VFA (Mm/ml)	42.72 ± 7.33	39.51 ± 5.34	34.37 ± 4.03	1.821	0.172
A/P	1.20 ± 0.07	1.36 ± 0.13	1.24 ± 0.11	0.034	0.148
NH_3N, mg/dL	18.49 ± 7.46	24.62 ± 3.67	16.33 ± 6.08	1.880	0.180
Total gas production	34.95 ± 5.96	31.73 ± 5.38	36.53 ± 6.69	0.930	0.097
Fermentation kinetics estimation, (ml/200mg of DM feed)					
a	4.07 ± 2.85	5.66 ± 1.97	5.52 ± 2.49	0.390	0.179
b	90.26 ± 19.8[a]	61.52 ± 21.1[b]	41.31 ± 14.7[c]	3.820	<.001
a+b	94.33 ± 21.1[a]	67.18 ± 20.6[b]	46.83 ± 14.6[c]	4.170	<.001
c	0.039 ± 0.02	0.051 ± 0.02[a]	0.079 ± 0.02[b]	0.004	<.001

CON (Control: 0 %, basal diet: concentrate + napier grass (40:60 %)), OPEFB extract diet (5% and 10% OPEFB extract per DM diet), *a*, estimation of gas production from immediate soluble fraction (ml/200 mg DM); *b,* estimation of gas production from the insoluble (but degradable) fraction ((ml/200 mg DM); *a+b,* estimation of potential gas production; *c,* estimation of gas production rate constant for the insoluble fraction (ml/h); SEM=standard error of the mean.

Table 4.3 shows *in-vitro* rumen fermentation characteristics after 24h incubation as affected by OPEFB extract in the diet. There are no significant differences between treatment diets on rumen pH, the content of propionate, iso-butyrate, butyrate, valerate, iso-valerate, and total VFA. On the other hand, significant differences ($p<0.01$) were found in the content of acetic acid, iso-butyric acid, and iso-valeric acid. The highest acetate production was recorded in the 0% OPEFB diet, while the 10% OPEFB extract diet showed the highest amount of iso-butyric and iso-valeric acid production in the rumen. Further, in Table 4.3, significant differences were found in the gas production estimation from the insoluble fraction (b), potential gas production estimation (a+b), and estimation

of the gas production rate constant for the insoluble fraction (c) ($p<0.05$). It was observed that there was no significant difference in total gas production at 24 hours of incubation as well as the *in-vitro* gas production from an immediate soluble fraction (a) for all treatment diets. Meanwhile, 0% OPEFB treatment diets showed the lowest value of c, which is in line with Figure 4.1, as it shows 0% OPEFB diet is the lowest value during the earlier incubation period.

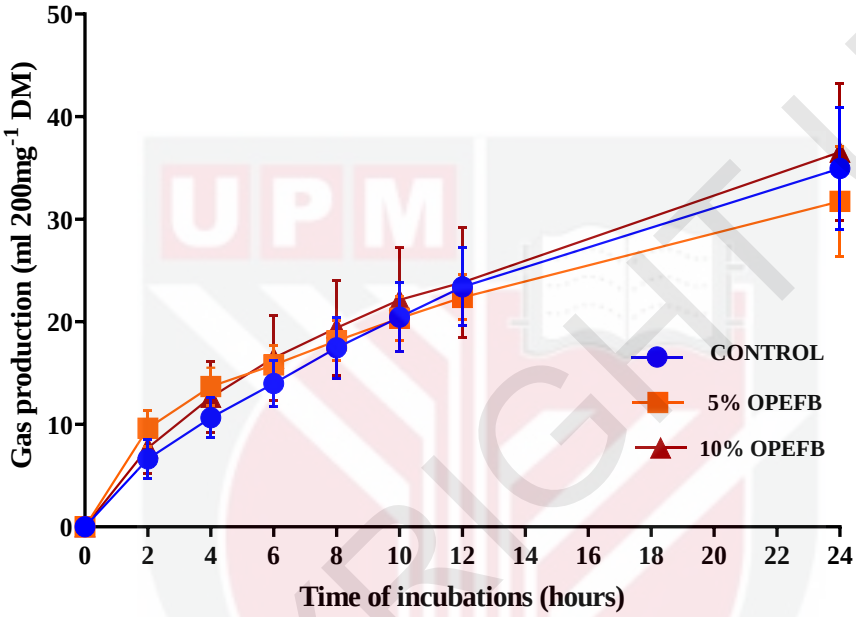

Figure 4.1 : Cumulative gas production of different levels of OPEFB extract in the goat's diet (means ± 1SD).

Table 4.4 : Rumen microbial population (log_{10} copy number/mL) of goat fed with extract supplementation in different inclusion levels (mean ± SD).

Microbial population, Log_{10} copy no. per ml of DNA extract	CON	5% OPEFB	10% OPEFB	SEM	P value
Total bacteria	11.70±0.07^{a}	11.19±0.07^{b}	11.00±0.11^{b}	0.1012	0.001
Total protozoa	7.71±0.15^{a}	6.87±0.13^{b}	7.27±0.27^{ab}	0.2030	0.041
Total fungi	5.94±0.20	5.60±0.09	5.67±0.04	0.0797	0.208
Total methanogens	5.23±0.50^{a}	6.88±0.39^{b}	5.46±0.42^{a}	0.3180	0.053
Methanobacteriales	5.57±0.13	5.78±0.09	5.76±0.05	0.0579	0.267
Fibrobacter succinogenes	10.92±0.41	10.20±0.39	9.49±0.65	0.3135	0.185
Ruminococcus albus	9.94±0.04	8.92±0.41	8.42±0.60	0.2908	0.079
Ruminococcus flavefaciens	7.36±0.32	7.07±0.42	6.49±0.50	0.2411	0.245
Butyrivibrio fibrisolvens	6.23±0.14^{a}	5.91±0.02^{a}	5.72±0.10^{b}	0.0817	0.018

CON (Control: 0 %, basal diet: concentrate + napier grass (40:60 %)), OPEFB extract diet (5% and 10% OPEFB extract per DM diet).

In this study, OPEFB extract influenced populations of total bacteria, total protozoa, and *B. fibrisolvens* ($p<0.05$). Control diet showed the highest population of total bacteria, total protozoa, and *B. fibrisolvens*. 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.

Table 4.5 : *In-vitro* rumen fatty acid profile of goat fed with extract supplementation in different inclusion levels (mean ± SD).

Fatty acid (g/100g of total fatty acid)	CON	5% OPEFB	10% OPEFB	SEM	P value
Capric acid, C10:0	1.12 ± 0.29	1.05 ± 0.29	0.99 ± 0.32	0.068	0.754
Lauric acid, C12:0	3.31 ± 0.66	3.11 ± 0.79	2.59 ± 0.54	0.166	0.194
Myristic acid, C14:0	3.55 ± 1.77	4.02 ± 1.26	3.50 ± 1.46	0.339	0.809
Myristioleic acid, C14:1	1.33 ± 0.81	1.31 ± 0.59	1.17 ± 0.67	0.155	0.908
Pentadecanoic acid, C15:0	1.73 ± 0.68	1.86 ± 0.36	1.47 ± 0.43	0.119	0.422
Palmitic acid, C16:0	31.20^{a} ± 5.07	35.71 ± 2.25^{b}	38.93 ± 1.61^{b}	1.066	0.004
Margaric acid, C17:0	9.13 ± 5.14	6.71 ± 3.11	7.84 ± 4.21	0.968	0.622
Stearic acid, C18:0	31.53 ± 3.52	31.79 ± 2.05	29.63 ± 2.80	0.674	0.384
Oleic acid, C18:1n-9	10.56 ± 1.19	10.60 ± 0.68	9.88 ± 0.93	0.227	0.366
Linoleic acid, C18:2n-6	6.53 ± 4.04	3.85 ± 1.18	4.00 ± 0.91	0.626	0.146
Total SFA	81.58 ± 4.25	84.25 ± 1.69	84.95 ± 1.40	0.706	0.166
Total UFA	16.81 ± 1.77	16.18 ± 1.47	15.45 ± 1.11	0.349	0.375
Total MUFA	11.90 ± 1.83	11.90 ± 0.94	11.04 ± 1.32	0.328	0.499
UFA : SFA	0.23 ± 0.07	0.19 ± 0.02	0.18 ± 0.02	0.011	0.125
PUFA : SFA	0.08 ± 0.06	0.05 ± 0.01	0.05 ± 0.01	0.009	0.153

CON (Control: 0 %, basal diet: concentrate + napier grass (40:60 %)), OPEFB extract diet (5% and 10% OPEFB extract per DM diet). SFA (saturated fatty acid): C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0). UFA (unsaturated fatty acid): C14:1, C18:1n-9, C18:2n-6). MUFA (Monounsaturated fatty acid): C14:1, C18:1n-9. PUFA (polyunsaturated fatty acid): C18:2n- 6. UFA: SFA (unsaturated fatty acid / saturated fatty acid). PUFA: SFA (polyunsaturated fatty acid / saturated fatty acid).

The effect of OPEFB extract supplementation on *in-vitro* rumen fatty acid profiles were presented in Table 4.5 above. The content of palmitic acid in the rumen was significantly affected ($p<0.05$) with the inclusion of OPEFB extract in the treatment diet. The highest content of palmitic acid (C16) in the rumen was found in the 10% OPEFB treatment diet and the lowest was recorded in the treatment diet without the extract. On the other hand, the inclusion of OPEFB extract in the diet did not affect other fatty acids such as capric acid (C10), lauric acid (C12), myristic acid(C14), myristoleic acid (C14:1), pentadecanoic acid (C15), stearic acid (C18), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), total saturated, total unsaturated fatty acid, total monoenes, unsaturated to saturated ratio and polyunsaturated to saturated ratio.

4.4 Discussion

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. 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, 1994). Furthermore, Nozière *et al*., (2011) reported that 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 our study. Therefore, both phytochemicals and lipids contained in the OPEFB extract decreased the molar proportion of acetate.

A trend showing increasing levels of iso-butyric acid and iso-valeric acid production in the rumen, parallel with the increasing levels of OPEFB extract in the diet. The concentration of isoacids in the rumen is the balance between the N produced from degradation and the N utilized by the bacteria to synthesize microbial protein in the rumen as stated by Hume (1970). Although insignificant, the NH_3N concentrations for all the treatment diets were above the level limiting in-vitro ruminal microbial growth (5 mg/100 mL) (Satter and Slyter, 1974). This suggested that ruminal microbial growth is not restricted especially cellulolytic bacteria which utilizes isoacid in the rumen, since the concentration of isoacid increased as well as the inclusion of OPEFB extract in the diet.

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 gas production rate (c) while in our study, the volume of gas production from insoluble fraction (b), and potential gas production (a+b) were decreased, and gas production rate (c) was increased which may be explained by the high roughage diet imposed to the animal. This is in line with the study done by Nathani *et al*. (2015), which reported that diet has an impact on microbial diversity further resulting in variability of the carbohydrate-active enzyme families present in the rumen. The differences were prominent in the solid phase rumen at a higher roughage fraction meanwhile in the liquid phase rumen the carbohydrate-enzyme families presence was reduced at 75:25 (roughage to concentrate ratio), and increased at 100% roughage diet. Furthermore, previous studies also observed that more gram- negative bacteria were found as 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).

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 our study where feed with the highest value of propionate concentration produce the lowest value of total gas production, is 5% OPEFB. It is worth noting that the amount of lignin and gross energy in 5% OPEFB is the highest among the diets. It has been highlighted by Casler (2001) that lignin and phenolic acid

were the limiting factor in the usage of energy in the rumen. Other than that, the presence of ferulic acid methyl ester (Item 39, Appendix C1) may also cause the decrease of gas production as ferulic acid can inhibit the growth of microbes (Zuhainis *et al.,* 2007; Saad *et al.,* 2008). In a review, Wang et al. (2022) stated that ferulic acid inhibits cellulose digestibility, negatively impact on rumen microbes but also reduces enzyme activity.

This study also highlights the effect of OPEFB extract on microbial population in *in-vitro* rumen fermentation. Inclusion of OPEFB extract decreased the population of total bacteria and *B. fibrisolvens* while total protozoa were thriving in the diet without OPEFB extract, decreased at 5% extract inclusion, and increased again at 10% extract inclusion diet. The reduced population of rumen protozoa in this study can be called partial rumen protozoa inhibition. Previous study suggested that supplemental phytochemicals and lipids can be effective in reducing methane production when rumen protozoa numbers are below 7 Log10 cells/mL, especially by supplemental saponins, tannins, and MCFA (Dai and Faciola, 2019) which is consistent with the result in our study where 5% of OPEFB extract diet shows rumen protozoa population less 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 with OPEFB inclusion 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 biohydrogenating bacteria population (Khiaosa-Ard *et al.,* 2009). It was in line with the study done by Zhu *et al*. (2014), where the *Butyrivibrio* group in the study was reduced as garlic oil was supplemented in 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. Other study also showed *B. fibrisolvens* was affected by tannin from sainfoin leaves by causing bacteria 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 plant extract on the animal such as antioxidant, anti-inflammatory, immune status, and antimicrobial have been shown against a wide variety of microorganisms (Gram-positive or Gram-negative bacteria, fungi, viruses, and protozoa), but are more effective against the Gram-positive. 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; Szumacher- Strabel *et al*., 2009). It was also found that some polyphenol may inducing cell apoptosis through breaking up the cell membranes of microorganisms (Lobiuc *et al.,* 2023).

It has been shown in the present study that rumen fatty acid profiles were altered by the inclusion of EFB extract in the diet. As the level of EFB inclusion increases, the fat content of the feed increases (Table 4.1). The present study also showed rumen palmitic acid increases as the dietary EFB increases. It was due to the fact that the extract from EFB 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 EFB after the stripping and threshing process of sterilized FFB where palm oil fruitlet removed from the branches (Ng *et al*., 2020). The temperature during EFB sterilization is 140°C which caused the leakage of palm oil from the fruitlet (Abdullah and Sulaiman, 2013) and thus formed a coating over EFB surfaces. Other than 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 by Vasta *et al*., (2010), where the content of palmitic acid in the rumen was increased when fed diet added with tannin, compared with 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 (2020) shows feeding palmitic acid-enriched supplement increase fiber digestibility while nitrogen metabolism remains unaffected, same with the findings by Wenner and St-Pierre, (2019). Conversely, other studies (Mosley *et al*., 2007 and Piantoni *et al*., 2013) found that supplementation of palmitic acid increase milk yield and milk fat concentration without lowering fiber digestibility and dry matter intake. Rumen UFA was decreased as the dietary EFB increase, although it was found insignificant in this study, which signify increase in rumen biohydrogenation. It was in line with the reduction in population of *B. fibrisolvens* in the current study as it was known that *B. fibrisolvens* participates in the biohydrogenation processes in the ruminant (Maia *et al*. 2010, Rodriguez Hernaez *et al*., 2018).

In general, bovine-sourced rumen inocula showed longer colonization compared to caprine-sourced rumen inocula. However, it has been reported by Noguera *et al*. (2011) that DM and OM degradation kinetics did not affect by different sources of rumen inoculants (Bovine and Caprine). Partition factor (PF), as suggested by Blummel *et al*., (1997) is defined as the quantity of degraded substrate (mg) with the total gas production during fermentation (ml). Feed with higher PF values suggests that the fraction of the degraded substrates used for microbial biomass synthesis is higher. Noguera *et al.,* (2011), in their study, showed caprine-sources rumen inocula have higher PF values compared with bovine-sources rumen inocula, which indicates caprine-sources rumen inocula has lower efficiency fermentation process as compared with rumen inocula from bovine. It was reported by Leng (1997), as organic matter truly digested in the rumen increases, a higher portion of digested organic matter is used to supply building block material for the cell polymer synthesis, and proportionally, fewer is directed into VFA, methane and heat production. Although Noguera *et al.,* (2011) reported Caprine-sources rumen inocula are fermentation processes are less efficient compared with bovines's, caprine ingestive behavior comprises of high selective activity, better masticatory activity, which leads to smaller feed particle sizes, shorter retention times of the feed particles, and high tolerance to acidic pH in the rumen (Rapetti and Bava, 2008) may compensate the inefficiency.

Therefore, it suggests that large ruminant is better in optimising forages with high cellulose content by increasing particle retention times in the anterior digestive tract meanwhile, goats are less efficient in utilizing high cellulose forages thus negate this inefficiency by chose to consume the digestible part of forages which has lesser particle retention time (Lechner-Doll *et al*., 1995).

4.5 Conclusion

The result of this experiment demonstrated that incubation with 5% and 10% dietary EFB extract added substrate did not affect rumen fermentation activity which is reflected by the insignificant gas production and total VFA production. However, differences were detected in rumen VFA whereby acetate concentration was notably decreased while the isoacid concentrations increased. Increased palmitic acid concentration in the rumen was observed in the incubation with EFB extract added substrate accompanied by the decrease in the population of total bacteria, protozoa, and *B. fibrisolvens*. 5% OPEFB extract was chosen for the *in-vivo* study as it is more practical due to the limitation of the low extract yield of OPEFB. These in-vitro results served as a basis for further in-vivo trials to test the effect of 5% OPEFB extract supplementation as a diet for the dairy goat.

CHAPTER 5

EFFECT OF OIL PALM EMPTY FRUIT BUNCH METHANOLIC EXTRACT ON *IN-VIVO* RUMEN FERMENTATION CHARACTERISTIC, BLOOD CHEMISTRY PROFILE, AND FATTY ACID PROFILE OF RUMEN, BLOOD, AND MILK IN SAANEN GOAT

5.1 Introduction

Milk contains a high level of saturated fat, which provides around 50% of the energy infants need (Koletzko *et al.,* 2011) but for adult consumers, it may raise the risk of heart disease and stroke. Medium-chained fatty acids (MCFA), which typically have a chain length of 8 to 14, are comprised of fatty acids in milk fat (Granot *et al*., 2016; Gardner *et al*., 2017). It has been well-documented that MCFA is more easily absorbed and oxidized for energy than long-chained fatty acid (LCFA) in humans (Bach and Babayan, 1982). Goat milk as compared with cow and human milk, has significantly higher levels of short-chained fatty acid (SCFA) and MCFAs (Park, 2010) which provides sufficient health benefits for the younger and elderly populations and according to some studies, it may be consumed by the majority of the population who are allergic to cow milk (Song *et al*., 2020). Thus, producers nowadays are expected to produce better milk products to meet consumer demands. As a result, there is a need to improve the current production and quality of milk in order to provide consumers with the option of healthier milk products. OPEFB extract has been deemed suited to be used at 5% supplementation (in Chapter 4) in improving the production of milk without detrimental effects on the rumen fermentation dynamics' of the animal. The extract was also enriched in long-chain fatty acids (LCFA) which is well suited to enhancing milk's nutritional profile especially the fatty acid composition of milk.

The increasing use and importance of oil palm-based wastes in goat production further warrants investigation of the use of these materials as feedstuff in goats. While the limited information available seems to suggest that either rumen total VFA improved in a study on the usage of oil palm frond silage on Thai native-Anglo Nubian goat (Chanjula *et al*., 2021) or remained unchanged in a study of oil palm frond extract on Boer goats (Al-Jumaili, 2018). The result from both studies, however, could not provide a comparison of the LCFA identified since the studies mentioned did not present relevant further information on fatty acid profile in either rumen, blood or milk. Animal breeds, age, sex, fat depots and anatomical location, diet and feed supplementations are factors that influence the fatty acid profile in milk or meat (Banskalieva *et al*., 2000). Modifying fatty acid profile in milk through dietary supplementation represents an interesting approach to further improve milk product. To regulate the fatty acid profile in milk fat, feeding oil as feed supplement are among of the approaches previous studies have explored. Previous study by Bett *et al*., (2004) found that sunflower oil seed supplement in the diet altered the milk fatty acid profile by increasing the concentration of unsaturated fatty acids and decreasing saturated fatty acids.

Thanasak *et al*., (2010) in their study of fatty acid profile in dairy cow fed with soybean and sunflower oil-rich diet showed a decreased in C16 while increased in C18 content in the rumen and blood plasma while an increased in C18:2n-6 and C18:3n-3 content in milk. The changes in fatty acid profiles as a result of using oil palm-based wastes in animal feed are lacking, especially in milk and blood but not in rumen (AbuGhazaleh *et al*., 2002; Ebrahimi *et al*., 2015 ; Aiman-Zakaria *et al*., 2017; Al-Jumaili, 2018 ; Chanjula *et al*., 2021), and meat (Ebrahimi *et al*., 2012 ; Ebrahimi *et al*., 2013). Nutritional properties of milk can be further improved through the increased in essential milk fatty acids and an increase in milk anti-oxidant capacity. Incorporation of polyphenol-rich OPEFB extract in the diet may influence fatty acid and natural phenolic compound transfer from diet to milk, hence delivering value- added milk to consumers. Therefore, this study was conducted with the objective to determine the effect of oil palm empty fruit bunch methanolic extract on in-vivo rumen fermentation characteristics, rumen microbial populations, and fatty acid profile of rumen, blood and milk in Saanen goat. Milk composition, blood chemistry profile, lipid oxidation in rumen, blood and milk were also determined to further explained animal status physiologically due to dietary treatments.

5.2 Materials and methods

5.2.1 Animal management and experimental design

The experiment protocol was approved by the Animal Care and Ethics Committee of the Universiti Putra Malaysia (UPM/IACUC/AUP-R0048/2017). Four female Saanen goats with body weight ranges 54.3 ± 9 kg (mean body weight ± standard deviation) were randomly chosen to undergo a crossover experimental design in which each animal was assigned to their respective treatment sequence. Each of the treatment diets consisted of 4 animals where in each period 2 of the animals received the control diet and the other 2 animals received the 5% OPEFB diet likewise for the 2nd experimental period, the animals proceeded to receive the next treatment diet, respectively. Animals were housed in individual metabolic crates with free access to clean drinking water and mineral blocks. The feeding trial started at day 14 post-partum and consisted of 10 days of adaptation period followed by 7 days of the experimental period, with a washout period of 12 days to ensure no carry-over effect for the next trial period. Animal feed for both treatments was freshly prepared daily in the feed trough, for each animal to ensure the animals received the exact amount of extract for the period of feeding trials, minimising oxidation of the said extract, and avoiding feed spillage and wastage occurring. Animals were weighed before the start of feeding trials, and weekly. The amount of feed given was adjusted to the weight of each animal, weekly.

Milk was collected twice daily and the yield was recorded while samples of milk (50ml each) were kept for further evaluation of milk composition and fatty acid profile. Blood and rumen were collected on day 17, 2 hours post-feeding. Blood samples (10ml) were taken via jugular vein puncture on a standing animal

meanwhile rumen fluids were obtained via stomach tubing. The serum sample of the blood was further evaluated for serum biochemistry profile and stored for further analysis of lipid oxidation, meanwhile plasma part of the blood undergoes fatty acid profile evaluations. The fatty acid profile of the feeds is presented in Table 5.1 and the biochemical analysis of methanolic oil palm empty fruit bunch extract is presented in Table 3.1.

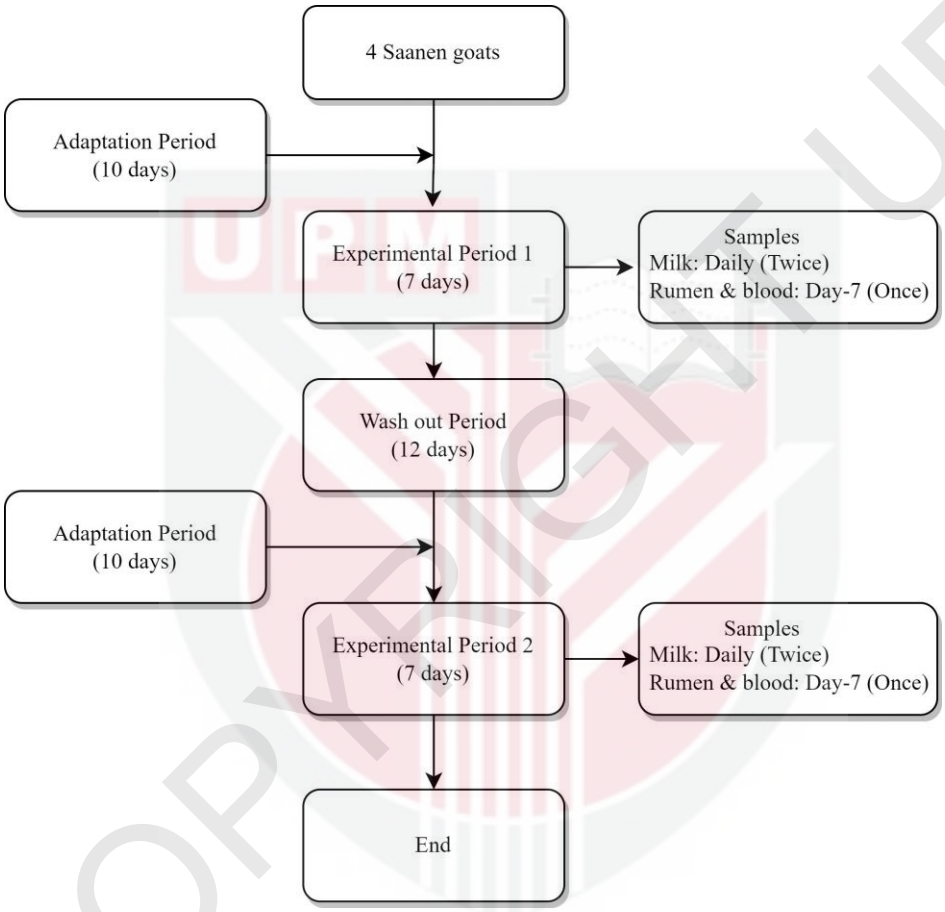

Figure 5.1: Experimental flowchart.

Table 5.1: Fatty acid (% of total identified fatty acids) of the experimental diets without (control) and with oil palm empty fruit bunch extract supplement (5% OPEFB extract supplement).

Fatty acids, g/100g of total fatty acid	CON	5% OPEFB
Capric acid, C10:0	0.65	0.60
Lauric acid, C12:0	6.90	7.33
Myristic acid, C14:0	2.25	2.39
Myristioleic acid, C14:1	0.77	n.d.
Pentadecanoic acid, C15:0	n.d.	0.68
Pentadecanoleic acid, C15:1	1.22	1.12
Palmitic acid, C16:0	14.65	15.94
Palmitoleic acid, C16:1	0.76	0.93
Stearic acid, C18:0	3.75	2.42
Oleic acid, C18:1n-9	12.55	12.66
Linoleic acid, C18:2n-6	37.14	36.41
α-linolenic acid, C18:3n-3	17.40	19.53
Total Saturated Fatty Acids	28.20	29.35
Total Unsaturated Fatty Acids	71.80	70.65
Total Monoenes	15.30	14.71
n-3:n-6 ratio	2.27	1.88
UFA: SFA ratio	2.59	2.44
PUFA: SFA ratio	2.05	1.95

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract). *n.d.: not detected

5.2.2 Determination of ruminal pH, NH_3-N, and volatile fatty acid

The rumen samples were collected through stomach tubing and were strained with 4 layers of muslin cloth to discard large feed particles. Rumen pH was determined by a pH electrode (Mettler-Toledo Ltd., England). Then, rumen samples were taken and kept for evaluation for ammonia N content and VFA content. As a preservation method, 5% of trichloro-acetic acid (TCA) was added to the samples for ammonia-N determination in a 1:1 ratio while for volatile fatty acid content, 10ml of rumen samples were acidified with 2ml of 25% meta-phosphoric acid. The determination of rumen ammonia nitrogen was done according to Parsons *et al*. (1984) as described previously in Chapter 4 (section 2.5). The determination of volatile fatty acid in the rumen was done according to Filipek and Dvorak (2009) as ascribed in Chapter 4 (section 2.4).

5.2.3 Rumen microbial population determination by qPCR

The determination of total bacteria, total protozoa, total fungi, total methanogens, methanobacteriales, *Ruminococcus albus, Ruminococcus flavefacien, Fibrobacter succinogenes,* and *Butyrivibrio fibrisolvens* population were done

through qPCR. The microbial population study was done according to the procedure described in detail in previous Chapter 4 (section 2.6).

5.2.4 Serum biochemistry profile

The serum biochemistry analysis was conducted at the Veterinary Haematology and Clinical Biochemistry Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, using respective kits on Hitachi 902 Automatic Analyser (Roche Diagnostics, Basel, Switzerland). Serum samples were analysed for the total protein, total glucose, blood urea nitrogen, total cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL).

5.2.5 Milk composition

Milk samples from individual goat was used to determine major component of the milk which is protein, fat, lactose, total solid and solid non-fat content at the dairy laboratory, Animal Science Department, Universiti Putra Malaysia. Approximately, 20 ml of milk sample was incubated in water bath (Techne Tempette Junior TE-8J Water Bath) at 40°C for 5 min. Then, the evaluation of milk composition was done by using a milk analyser (Laporte and Paquin, 1999), Milkoscan (134 A/B series equipment, Foss Electric, Denmark).

5.2.6 Determination of fatty acid profile by gas chromatography

The fatty acid of feed, rumen, plasma and milk were extracted according to the method of Folch *et al*., (1957) with minor modifications describe by Ebrahimi *et al*., (2012) by using chloroform: methanol (2:1) (v/v) containing butylated hydroxytoluene to prevent oxidation during sample preparations. The transmethylation was 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) equipped with a flame ionization detector (FID). The split ratio was 10:1 after injection of 1ul of the FAME. The injector and detector temperature was programmed at 250°C. The column temperature program initiated runs at 140°C for 5 minutes, warmed to 200°C at 3°C/min, held for 7 minutes, warmed to 220°C at 2.3°C /min, and then held for 3 minutes for facilitate optimal separation. The peak identified through comparison of equivalent chain lengths with a reference standard (Supelco 37 Component FAME Mix; Sigma- Aldrich, Inc., St. Louis, MO, USA). The fatty acid concentrations are expressed as g/100 g of the identified fatty acid.

5.2.7 Lipid oxidation in rumen, serum blood and milk

Thiobarbituric acid reactive substances (TBARS) in the rumen, serum and milk were measured with the method described by Lynch and Frei (1993). About 1 ml of rumen or serum or milk sample was homogenised in 4 mL 0.15 M KCl + 0.1 mM BHT for 1 min at 6000 rpm with Ultraturrax homogenizer. Two hundred microliters of the homogenised sample were mixed with a solution (TBARS solution), heated in a water bath set at 95 ◦C for 1 h, for a pink colour to develop. The formation of a pink-coloured sample mixture is the result of the reaction of TBARS end products (malondialdehyde) with thiobarbituric acid (TBA). After samples were cooled under the tap water for about 5 minutes, three millilitres of n-butyl alcohol was then added to the extracts and homogenised. Then, the mixtures were centrifuged for 10 min at 5000 rpm. The absorbance of the supernatant was read with a spectrophotometer (Secomam, Domont, France) at 532 nm wavelengths against n-butyl alcohol as a blank. TBARS concentration of samples was determined from a standard curve of 1, 1, 3, 3-tetraethoxypropane and calculated as µml malondialdehyde (MDA)/ml samples.

5.2.8 Statistical analyses

All statistical analyses were carried out using SPSS (IBM version 22) by using an independent t-test to examine the effect of 5% OPEFB feed on in-vivo rumen fermentation characteristics, rumen microbial populations, serum biochemistry, milk composition, lipid oxidation and fatty acids profile in rumen, blood plasma and milk. Differences between treatment means were deemed significant when P values ≤ 0.05 were observed.

5.3 Results

Table 5.2: Effect of OPEFB supplementation on *in-vivo* rumen fermentation parameters of goats (mean ± SD).

Parameters	CON	5% OPEFB	SEM	P value
pH	6.28 ± 0.12	6.35 ± 0.12	0.037	0.394
VFA (mol/100mol)				
Acetic	42.24 ± 5.03	39.52 ± 5.16	1.406	0.358
Propionic	27.38 ± 3.87	30.09 ± 3.29	1.038	0.205
Isobutyric	2.08 ± 0.50	2.72 ± 0.61	0.164	0.045
Butyric	21.96 ± 3.15	23.02 ± 2.57	0.734	0.491
Isovaleric	2.92 ± 0.86	4.04 ± 0.95	0.271	0.033
Valeric	2.29 ± 0.40	2.67 ± 1.16	0.215	0.410
Total VFA (mM/ml)	73.34 ± 3.21	70.94 ± 3.10	0.906	0.199
A/P	1.52 ± 0.41	1.34 ± 0.27	0.090	0.364
NH_3N, mg/dL	16.76 ± 4.75	21.36 ± 4.56	1.236	0.060

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract).

The effect of supplementation 5% OPEFB feed on the rumen fermentation profile is presented in Table 5.2. Significant differences were found in the iso-butyric and iso-valeric concentrations, where it increased significantly ($P<0.05$) with the supplementation of 5% OPEFB feed. Although insignificant, acetic acid content was decreased while propionic, butyric and valeric acid were increased in the 5% OPEFB feed. Thus, leading to a decrease in total VFA and A to P ratio. Meanwhile, the ammonia-nitrogen content of the rumen was observed to have increased in concentration, albeit not significantly different.

Table 5.3 : Effect of OPEFB supplementation on *in-vivo* rumen microbial populations of goat (mean ± SD).

Microbial population, Log_{10} copy no. per ml of DNA extract	CON	5% OPEFB	SEM	P value
Total bacteria	10.96 ± 0.43	10.63 ± 0.13	0.088	0.073
Total protozoa	5.90 ± 0.48	6.93 ± 1.05	0.212	0.043
Total methanogens	5.21 ± 0.32	4.84 ± 0.32	0.090	0.036
Total fungi	6.52 ± 0.44	6.34 ± 0.27	0.082	0.342
Methanobacteriales	5.37 ± 0.32	4.98 ± 0.28	0.089	0.022
R. albus	8.32 ± 0.34	7.78 ± 0.41	0.109	0.009
R. flavefaciens	7.37 ± 0.16	6.99 ± 0.16	0.056	<.0001
F. succinogenes	5.26 ± 0.41	4.84 ± 0.12	0.099	0.016
B. fibrisolvens	5.29 ± 0.39	4.80 ± 0.36	0.105	0.014

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract).

Table 5.3 shows that 5% OPEFB feed significantly affects the rumen microbial population selected except for the total bacteria and total fungi populations. 5% OPEFB feed increased the population of protozoa ($P<0.05$) but in contrast, the population of other microbial populations measured were reduced ($P<0.05$).

Table 5.4 : Effect of OPEFB supplementation on serum biochemistry profile of goats (mean ± SD).

Parameters	Reference intervals	CON	5% OPEFB	SEM	P value
Total protein (g/L)	62-85	113.2 ± 6.89	117.0 ± 11.04	3.10	0.578
Glucose (mmol/L)	2.1-3.7	2.60 ± 0.29	2.45 ± 0.64	0.16	0.683
Urea (mmol/L)	4.1-13.4	6.98 ± 2.29	6.45 ± 2.28	0.75	0.756
Cholesterol (mmol/L)	1.0-5.0	1.65 ± 0.93	1.28 ± 0.75	0.29	0.554
Triglyceride (mmol/L)	-	0.04 ± 0.03	0.01 ± 0.02	0.17	0.177
HDL (mmol/L)	-	1.30 ± 0.72	1.06 ± 0.60	0.22	0.637
LDL (mmol/L)	-	0.52 ± 0.20	0.42 ± 0.13	0.06	0.421

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract).
[a] University of Guelph (2023).

No significant differences were found in both, total protein and glucose content of the blood serum of goats fed with or without 5% OPEFB feed. The study also found that the 5% OPEFB feed has no effect on urea content in the serum. The result of cholesterol, triglyceride, HDL and LDL content in the blood serum was also found in a similar manner where there is no significant differences when animals treated with 5% OPEFB feed.

Table 5.5 : Effect of OPEFB supplementation on milk composition of goats (mean ± SD).

Parameters	Control	5% OPEFB	SEM	P value
Milk yield (kg/day)	1.20 ± 0.23	1.12 ± 0.44	0.05	0.407
Milk composition				
Fat (%)	4.11 ± 0.65	4.03 ± 0.98	0.07	0.612
Protein (%)	3.15 ± 0.27	3.28 ± 0.44	0.03	0.039
Lactose (%)	5.01 ± 0.20	4.87 ± 0.26	0.02	0.001
Total solid (%)	12.62 ± 0.87	12.47 ± 1.24	0.09	0.420
Solid non-fat (%)	8.70 ± 0.41	8.64 ± 0.61	0.04	0.505

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract).

The results of this study on the milk composition of goats supplemented with and without 5% OPEFB diet are shown in Table 5.5. Current study showed no significant difference in the milk yield of the goats. Significant differences ($P<0.05$) were found in the protein content and lactose content of the milk. Contrariwise, 5% OPEFB diet has no significant effect on in milk fat content, total solid content and solid non-fat content of the milk.

Table 5.6 : Effect of OPEFB supplementation on *in-vivo* rumen fatty acid profile of goat (mean ± SD).

Fatty acid, g/100g total fatty acid	CON	5% OPEFB	SEM	P value
Capric acid, C10:0	0.41 ± 0.26	0.81 ± 0.60	0.107	0.087
Lauric acid, C12:0	5.16 ± 1.37	4.90 ± 1.34	0.244	0.601
Myristic acid, C14:0	4.83 ± 0.90	5.96 ± 1.44	0.257	0.026
Myristioleic acid, C14:1	1.51 ± 0.46	1.35 ± 0.44	0.092	0.392
Palmitic acid, C16:0	27.19 ± 3.75	30.94 ± 3.52	0.824	0.019
Stearic acid, C18:0	35.53 ± 4.33	41.11 ± 6.14	1.227	0.019
Oleic acid, C18:1n-9	15.74 ± 1.72	13.66 ± 2.34	0.476	0.024
Linoleic acid, C18:2n-6	4.13 ± 1.27	5.31 ± 1.38	0.341	0.029
Total SFA	79.65 ± 2.44	77.35 ± 2.64	0.572	0.042
Total UFA	21.04 ± 2.86	22.65 ± 2.64	0.565	0.163
Total MUFA	18.10 ± 2.73	15.39 ± 3.00	0.604	0.022
UFA : SFA	0.27 ± 0.04	0.29 ± 0.04	0.009	0.132
PUFA : SFA	0.05 ± 0.01	0.07 ± 0.02	0.003	0.018

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract). SFA (saturated fatty acid): C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0). UFA (unsaturated fatty acid): C14:1, C18:1n-9, C18:2n-6). MUFA (Monounsaturated fatty acid): C14:1, C18:1n-9. PUFA (polyunsaturated fatty acid): C18:2n-6. UFA: SFA (unsaturated fatty acid / saturated fatty acid). PUFA: SFA (polyunsaturated fatty acid / saturated fatty acid).

Present study results of rumen fatty acid content as affected by 5% OPEFB feed were presented in Table 5.6. Table showed a significant increase ($P<0.05$) in the amount of myristic, palmitic, stearic, linoleic and polyunsaturated fatty acid to saturated fatty acid ratio. Meanwhile, significant decreases ($P<0.05$) were observed in the concentration of oleic acid, total saturated fatty acid and total monounsaturated fatty acid in the rumen. However, no significant differences in the concentration of capric, lauric, myristoleic, total unsaturated fatty acid and the ratio of unsaturated to saturated in rumen were also observed.

Table 5.7 : Fatty acid composition of blood plasma from goats fed OPEFB supplementation (mean ± SD).

Fatty acid, g/100g total fatty acid	CON	5% OPEFB	SEM	P value
Capric acid, C10:0	6.70 ± 0.69	7.87 ± 1.12	0.264	0.022
Lauric acid, C12:0	5.98 ± 1.24	7.04 ± 1.85	0.462	0.271
Myristic acid, C14:0	5.63 ± 1.21	6.34 ± 0.52	0.243	0.148
Palmitic acid, C16:0	20.32 ± 1.37	19.80 ± 0.91	0.289	0.385
Margaric acid, C17:0	6.93 ± 2.00	6.52 ± 2.06	0.468	0.677
Stearic acid, C18:0	11.19 ± 1.73	12.65 ± 1.17	0.445	0.090
Oleic acid, C18:1n-9	13.42 ± 4.37	18.14 ± 2.41	1.175	0.039
Linoleic acid, C18:2n-6	29.17 ± 4.12	22.66 ± 4.02	1.255	0.005
Total SFA	54.71 ± 2.01	61.29 ± 6.44	1.433	0.024
Total UFA	44.70 ± 1.20	42.73 ± 4.84	0.946	0.333
UFA : SFA	0.81 ± 0.04	0.65 ± 0.17	0.038	0.030
PUFA : SFA	0.53 ± 0.09	0.38 ± 0.09	0.029	0.006

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract). SFA (saturated fatty acid): C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0). UFA (unsaturated fatty acid): C14:1, C18:1n-9, C18:2n-6). MUFA (Monounsaturated fatty acid): C14:1, C18:1n-9. PUFA (polyunsaturated fatty acid): C18:2n-6. UFA: SFA (unsaturated fatty acid / saturated fatty acid). PUFA: SFA (polyunsaturated fatty acid / saturated fatty acid).

In this study, 5% OPEFB feed does not have any effects on lauric, myristic, palmitic, margaric, stearic and total unsaturated fatty acid (UFA) content of the blood plasma. Contrastingly, capric, oleic, linoleic, total saturated fatty acid (SFA), unsaturated to saturated fatty acid (UFA: SFA) ratio and poly-unsaturated fatty acid (PUFA: SFA) ratio was affected by the 5% OPEFB feed. The major fatty acid in the blood plasma is palmitic, stearic, oleic, and linoleic acid which accounted for approximately 72% of the total identified acid (Table 5.7). 5% OPEFB feed increased the content of capric, oleic and total SFA, at the same time, decreased the content of linoleic, UFA: SFA ratio and PUFA: SFA ratio.

Table 5.8 : Fatty acid composition of milk from goats fed OPEFB supplementation (mean ± SD).

Fatty acid, g/100g total fatty acid	CON	5% OPEFB	SEM	P value
Capric acid, C10:0	9.64 ± 3.20	13.01 ± 3.43	0.709	0.014
Lauric acid, C12:0	7.35 ± 2.35	9.50 ± 2.76	0.550	0.048
Myristic acid, C14:0	8.91 ± 3.81	12.29 ± 3.24	0.759	0.022
Myristioleic acid, C14:1	7.53 ± 3.84	6.62 ± 2.51	0.655	0.495
Palmitic acid, C16:0	26.09 ± 3.15	28.67 ± 3.85	0.724	0.075
Stearic acid, C18:0	9.26 ± 2.19	7.40 ± 2.15	0.433	0.029
Oleic acid, C18:1n-9	26.06 ± 6.91	20.47 ± 5.38	1.258	0.024
Linoleic acid, C18:2n-6	8.31 ± 3.11	8.03 ± 2.34	0.582	0.818
Total SFA	61.17 ± 10.57	70.28 ± 10.6	1.990	0.019
Total UFA	38.83 ± 10.57	30.88 ± 8.44	1.960	0.039
Total MUFA	31.60 ± 8.96	25.22 ± 7.32	1.636	0.049
UFA : SFA	0.69 ± 0.32	0.47 ± 0.197	0.054	0.043
PUFA : SFA	0.15 ± 0.07	0.13 ± 0.05	0.013	0.381

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract). SFA (saturated fatty acid): C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0). UFA (unsaturated fatty acid): C14:1, C18:1n-9, C18:2n-6). MUFA (Monounsaturated fatty acid): C14:1, C18:1n-9. PUFA (polyunsaturated fatty acid): C18:2n-6. UFA: SFA (unsaturated fatty acid / saturated fatty acid). PUFA: SFA (polyunsaturated fatty acid / saturated fatty acid).

The composition of milk fatty acids in the present study is shown in Table 5.8. It was found that major fatty acids in the milk were palmitic, oleic, capric, and myristic, respectively which account for 74% of the total identified fatty acid in the milk. There were significant differences in the content of capric, lauric, myristic, stearic, oleic, total SFA, total UFA, total monoenes, and UFA: SFA ratio in the milk. On the contrary, no significant changes was seen among myristoleic, palmitic, and linoleic content of the milk. 5% OPEFB feed showed increased content of capric, lauric myristic and total SFA but decreased content of stearic, oleic, total UFA, total monoenes, and UFA: SFA ratio in the milk.

Table 5.9 : Lipid oxidation of rumen, serum and milk from goats fed with and without OPEFB supplementation (mean ± SD).

Lipid oxidation (μM MDA/ml)	CON	5% OPEFB	SEM	P value
Rumen	25.28 ± 7.13	19.24 ± 3.24	1.46	0.035
Serum	5.01 ± 0.78	7.13 ± 2.09	0.49	0.025
Milk	11.76 ± 3.60	8.59 ± 1.65	0.52	0.002

CON (0 %, basal diet: concentrate + napier grass (40:60 %)), 5% OPEFB (basal diet+5% of OPEFB methanolic extract).

In this study, lipid oxidation of rumen, blood serum and milk was investigated and presented in Table 5.9. Major lipid oxidation did occur in the rumen, followed by milk and blood serum respectively. Results of this study showed that 5% OPEFB feed decreased lipid oxidation in rumen and milk, significantly ($P<0.05$), but resulting in a significant increase in blood serum ($P<0.05$).

5.4 Discussion

The 5% OPEFB supplementation in animal feed improves the energy density of the feed since it is high in fat content. High content of palmitic acid is also another characteristic of oil palm agricultural waste. Incorporation of 5% OPEFB feed in the animal diet increases milk protein and lactose content as well as de novo fatty acid. Not to mention, the value added to the milk as the result of feeding 5% OPEFB to the animal, considering the polyphenol contained in OPEFB may be transferred from the diet to the milk.

In this study, the rumen pH remains unaffected by the 5% OPEFB supplementation in the diet. The optimum range of ruminal pH for the activity of cellulolytic microbes is between the pH of 6.0 to 7.0 which is the same as for the absorption of VFA (Erdman, 1988). The present study observed pH value of both diets was between the pH of 6.28 to 6.34, similar to the findings of Bannink *et al*. (2008) in which fibre degradation was not inhibited when the rumen pH was around pH 6.2. This is in line with Erdman (1988) and Mourino *et al*. (2001) report saying that the pH of 6.0 to 6.3 is the critical pH below which fibre degradation is impaired.

Significant differences were shown in the concentration of iso-acid (iso-butyric and iso-valeric acid) content of the rumen. These differences were probably due to the fermentation of amino acids as well as branched amino acid by the microbes. Iso-butyric and iso-valeric acids are branched-chain volatile fatty acids (BCVFA) that are a conversion product of oxidatively deaminated branched-chain amino acid (BCAA), valine, iso-leucine and leucine (Allison, 1978); of which are among the metabolite produced alongside NH_3N (Apajalahti, 2019). The concentration of rumen NH_3N in the present study was similar to a study on oil palm leaf extract in-vitro, conducted by Aiman-Zakaria *et al*. (2017) where NH_3N concentrations were between 18-23 mM/dL, albeit insignificant. It is worth

noting that the rumen NH_3N concentration required for optimum digestion ranges between 10-20mg/L (Kraidees, 2005). Since the present study found that NH_3N concentration falls higher than the optimum range, it would suggest that the NH_3N was more than adequate for microbial growth. Meanwhile, the molar proportion of other volatile fatty acids is similar in both treatments at 2 hours post-feeding although, the fermentation pattern changed subtly towards an increase in propionate production (Table 5.3) in the 5% OPEFB diet group. In times when acetate production was reduced, lesser hydrogen ions were produced and as the methanogenesis and propionate production used hydrogen, therefore, it created competition. This is in agreement with Janssen (2010) who stated that inhibiting methanogenesis can shift rumen fermentation towards propionate production. Methanogen formation by rumen fermentation is closely associated with the VFA profile formed (Ungerfeld, 2020). Present study showed total methanogens and methanobacteriales reduced in the 5% OPEFB diet. These findings corroborated with the findings of Aiman-Zakaria *et al*. (2017) in a study of oil palm leaf extract and Jayanegara (2015), in a study with different forms of tannins in ruminants. While most studies reported a reduction in methanogens with a reduction in the total protozoa population, current study reported a significant increase in total protozoa with EFB diet. The above may suggest that OPEFB extract may have a direct effect on the methanogenic archea population which is not associated with the protozoa. This is in agreement with a study by Jafari *et al*. (2016), where methanogens were reduced while protozoa population remained unchanged. It was favourable that protozoa remains unaffected as it plays a significant contribution in the breakdown of carbohydrate as well as producing enzyme (pectinesterases, cathepsins and some glycosyl hydrolase families) that are uncommonly found produced by protozoa (Williams *et al*., 2020). The reduction of methanobacteriales in the 5% OPEFB diet may due to the anti-microbial effect of tannin towards Gram-positive archea. Methanobacteriales is an archea that is stained as Gram-positive bacteria which has thin cell walls composed of pseudomurien (Sowers, 2009). The antibacterial effectiveness of tannin may be related to its action on the bacteria membrane and/or their ability to complex metal ions as suggested by Kaczmarek (2020) and Engels *et al*. (2011). Notably, a recent study by Su *et al*. (2021), reported that the rumen microbial community shifted towards methane inhibition and propionate enhanced by utilising cashew nut shell liquid diet in Holstein cows. Such favourable changes in fermentation were also reported in the rumen of cattle (Shinkai *et al*., 2012; Watanabe *et al*., 2010), dairy cows (Coutinho *et al*., 2014), and sheep (Kang *et al*., 2018).

In the present study, *R. albus* was the most dominant across cellulolytic bacteria observed. This is in agreement with earlier studies with Aiman-Zakaria *et al*. (2017). Contrary to OPLE, the current study showed a decreased in cellulolytic bacteria with OPEFB extract diet. A possible explanation for this might be that of physiochemical content of OPEFB extract is in a different trend than that of OPLE, one of the differences is the high content of tannin. It is possible through a selective anti-microbial action against rumen microbes by the phenolics of 5% OPEFB extract, especially. As stated by Ungerfeld (2015), antimicrobial activities may induce a rumen microbial community shift, resulting in decreased methane synthesis and a higher propionate production, given that these pathways competitively consume metabolic hydrogen in the rumen. Many studies indicated

that tannin have significant inhibitory effect on rumen fibrolytic bacteria. Tannins also are known for their antimicrobial activity either on cellulolytic or proteolytic bacteria (Goel *et al*, 2005). Reduction in *R. albus, F. succinogenes* and *R. flavefacien* may be due to the tannin binding to the substrate cell wall leading to lesser availability of the binding site in the cell wall for rumen microbes to attach to (Ghasemi *et al*, 2012).

Butyrivibrio fibrisolvens are rumen microbes that are important for the digestion of fibre (Hungate, 1966). Many years ago, *B. fibrisolvens* were recognised to undertake biohydrogenation of fatty acid (Polan *et al*. 1964) and to form conjugated linoleic acid (CLA) as an intermediate in the process (Kepler *et al*. 1966) based on the landmark studies published in 1960's. Rumen biohydrogenation increases as unsaturation increases (Marin *et al*., 2018). Current study showed B. fibrisolvens population were reduced by the 5% OPEFB diet. It was suggested that the presence of polyunsaturated fatty acid may cause a bacteriostatic effect towards *B. fibrisolvens* during biohydrogenation (Maia *et al*., 2010). Thus, *B. fibrisolvens* reduction could be due to the increased content of linoleic acid in the 5% OPEFB diet compared to CON diet (Table 2). Kim *et al*., (2000) stated that linoleic acid inhibited the growth of B. fibrisolvens A38 and in a different study discovered that *Butyrivibrio/Pseudobutyvibrio* phylogenetic grouping had different sensitivities to growth inhibition by linoleic acid. In addition, Henderson (1973) reported that lauric acid (C12) has an inhibitory effect on *B. fibrisolvens* which provides another explanation to the decrement in *B. fibrisolvens* population in the 5% OPEFB diet since it contains higher C14 content than the control diet.

The current study also looks into the effect of 5% OPEFB feed on the serum blood chemistry of goats. Although there are no significant effects among serum blood profiles, total serum protein in 5% OPEFB feed shows a higher value than CON feed while having a lower value of other serum blood parameters such as serum glucose, serum urea nitrogen, serum total cholesterol, serum triglyceride, serum HDL cholesterol and serum LDL cholesterol. Total serum protein content in the present study (113.18 g/L in the CON feed and 117 g/L in the 5% OPEFB feed) was slightly higher than 72.25 g/L and 80.12 g/L, respectively, for the control diet and 8% of canola with palm oil blend diet as reported by Adeyemi *et al*., (2016). Meanwhile, serum glucose content in the current study obtained (2.6 mmol/L in the CON feed and 2.45 mmol/ L in the 5% OPEFB feed) was near 2.79 mmol/L and 2.51 mmol/L respectively for the control diet and C16 supplemented diet in cows during early lactation as reported by De Souza *et al*., (2019). De Souza *et al*. (2019) in the same study reported total serum cholesterol of 2.06 mmol/L and 2.07 mmol/L for the control diet and C16 supplemented diet, respectively, slightly higher values than the current study obtained which is 1.65 mmol/L and 1.275 mmol/L, for CON feed and 5% OPEFB feed respectively. Results in the Table 5.5 showed that serum urea nitrogen values correspond to 6.975 mmol/L for the CON feed and 6.450 mmol/L for the 5% OPEFB feed which fall in the optimal BUN ranges (4.00-9.89 mmol/L) for goats (Lewis, 1957) and also within normal ranges (3.57-7.14 mmol/L) proposed by Kaneko *et al.,*(2008). It has been reported by Wang *et al*., (2008), that the lack of variation in serum urea nitrogen was due to the fact that ammonia produced in the rumen has met

the requirement of microbial protein and the excess N ingested from the diet resulted in non-equilibrium of amino acids in ruminant (Wang and Lu, 1999). As for serum triglyceride content in our study, it would appear to remain unchanged, and its concentration in both groups was within the normal range (< 1.13 mmol/L) according to Van Saun (2000).

Table 5.6 shows that the 5% OPEFB feed increased milk protein content and decreased milk lactose content while milk yield, milk fat, milk total solid and milk solid non-fat remained unchanged. It is widely known that the fat supplementation in the diet of dairy cows and ewes leads to lowered milk protein content and modifying coagulated properties, which this negative effect was not seen in goats (Chilliard and Bocquier, 1993) since most fat supplementation in goat results in increasing milk protein content, which concurred with the current study. It has also been observed by Chilliard *et al*., (2003) that the addition of fat to the goat diet not only causes no reduction in milk protein content, but an increase in 14 out of 20 reviewed trials. The lactose content is the only source of carbohydrates in milk (Chandan *et al*., 1992). Milk lactose content in the present study was in a similar range to the findings of Simos *et al*., (1991); Chandan *et al*., (1992) and Greyling *et al*., (2004). Milk lactose content is positively correlated with milk yield (r=0.10) (Mioc *et al*., 2008) which is in line with the result of present study where milk yield is decreased, albeit insignificant.

The evident increase in C18:0 is the result of the extensive biohydrogenation in the rumen (Cremonensi *et al*., 2018). Current study showed that 5% OPEFB dietary group increase the concentration of myristic (C14), palmitic (C16), stearic (C18) and linoleic acid (C18:2n6), and decrease in oleic acid (C18:1n9), total saturated fatty acid and total mono-unsaturated fatty acid, in the rumen. The reduction of oleic acid (C18:1n9) in the rumen may be caused by most of it being biohydrogenated to stearic acid and thus leading to a substantial increase in the concentration of stearic acid (C18:0) in 5% OPEFB diet. The increases in C18:0 in the rumen may also be due to the high content of linolenic acid in 5% OPEFB diet as it is suggested by Vargas *et al*., (2018) that linolenic acid was biohydrogenated faster than linoleic acid. This suggests that 5% OPEFB was unable to hinder fatty acid biohydrogenation in the rumen. Lourenco *et al*. (2010) reported that oils containing PUFA such as linolenic acid would be expected to have a greater effect on rumen biohydrogenation and fermentation processes than those rich in linoleic acid or oleic acid. Additionally, linoleic acid increases in 5% OPEFB diet may be due to its accumulation in the rumen as it is reported by Vargas *et al*., (2018) that when the concentration of linoleic acid in the diet is greater than linolenic acid, linoleic acid tends to accumulates in the rumen. The accumulation of linoleic acid in the rumen may also be due to the complex biohydrogenation of linolenic acid in the rumen since diets contain high in linolenic acid (Shingfield and Wallace, 2014). The disappearance of linoleic acid also rather, lower than linolenic acid as observed by Juoany *et al*., (2007). Earlier studies by Maia *et al*., (2007 and 2010) also agreed that PUFA is more disruptive against biohydrogenation bacteria than di- or monoenoic fatty acid. Tannin presence in the diet may be able to reduce the extent of PUFA biohydrogenation by linoleic acid and CLA isomers accumulation in the first and second step of biohydrogenation (Jayanegara *et al*., 2011). Another study reported by Or-Rashid *et al*., (2007), myristic acid may also inhibited methanogenesis in dairy

cow without altering the conjugated linoleic acid or vaccenic acid profile in milk. In addition, recent studies reported that C16 and C18 may have acted as pivotal regulators of metabolism and gene transcription in ruminants than unsaturated fatty acid, which a different from the non-ruminant model (Bionaz *et al*., 2012) through the activation of PPARα and PPARγ target genes in bovine and goat cells, as it was increased by saturated long- chain fatty acid (LCFA), as observed by Bionaz *et al*., (2015). On the other hand, the present study was unable to determine the CLA content of the rumen due to the separation and structural identification of all peaks in the CLA range of the GC chromatogram, including the extremely small peak, which was not attempted or even possible. Total saturated fatty acid in the present study were decreased in the 5% OPEFB diet. It is in agreement with a study done by Vasta *et al*., (2008) where they found that reduced saturated fatty acid, while increased CLA isomer and PUFA while using tannin in their diets.

In this study, a decrease in C18:2n6 of blood plasma was observed in the 5% OPEFB diet when compared to the control diet. Present results are consistent with that of Pesqueira (2016), who indicated a partial inhibition of the biohydrogenation process of C18:2n6 due to the content of plasma C18:2n6 trans decreased significantly in animals consuming algae. Supplementation of 5% OPEFB in the diet modified plasma fatty acid profile where C10 and C18:1n9 content was observed higher than control diet with decreasing amount of C18:2n 6. It was observed by Prom *et al*., (2021) that the content of cis 9 C18:1 in plasma triglyceride increases linearly as the amount of oleic acid abomasum- infused was increased, indicating the increase in the absorption of cis-9 C18:1. The increased in the absorption of oleic acid may be due to its emulsifying properties (Freeman, 1969) that improve micellar formation thus allowing improvement in absorption of all FA not only cis-9 C18:1. These changes in plasma fatty acid profile reflect the fatty acid composition of the dietary treatment (Tsiplakou and Zervas, 2013) especially C18:1n9 and C18:2n6 as they are among the predominant fatty acid in the diet.

Alteration in milk fatty acid profile has been shown to accompany the supplementation of the 5% OPEFB diet. Among the fatty acids identified in Table 5.8, only myristoleic, palmitic, linoleic and PUFA: SFA ratio was unaffected by the supplementation. Result from current study was similar to a previous study by Chamberlain and DePeters (2017) where high content of palmitic acid in milk was found and it was due to the high content of palmitic acid in the diet. The current study observed a significant increment of milk fatty acid occurs at medium-chained fatty acid which is capric acid, lauric acid, and myristic acid that originated from de novo synthesis in the mammary gland where acetate and β-hydroxybutyrate are the major substrate sources. While acetate is the end-product of carbohydrate fermentation in the rumen, β-hydroxybutyrate is produced by the rumen epithelium from absorbed butyrate (Bernard, Leroux and Chilliard, 2008). This increment may be explained by both of the concentrations of rumen acetate and butyrate in this study, although statistically insignificant, showing an increase in amount when 5% OPEFB feed is given to the animal. This results in the total SFA increases in milk of 5% OPEFB feed. As for LCFA in the milk, they are absorbed from blood plasma where the resources are from

dietary lipid absorption and from body reserves mobilisation. In the current study, supplementation of the 5% OPEFB feed has led to decreased concentration of stearic and oleic acid in the milk which suggests that the 5% OPEFB extract diet improved energy density availability to animals. This is because when the energy balance is negative, animals mobilise lipids stored in adipose tissue, mainly in the form of NEFA. Since ruminant adipose tissues are in high content of palmitic, stearic and oleic acid as described by Bas *et al*., (1987) in goat tissues, 59% of the variability in milk content of C18 and C18:1 is related to energy-balanced changes, in goats with differences in milk yield during the first four months of lactation, fed with a classic diet of hay and concentrate diet (Chilliard *et al*., 2003). A lower UFA: SFA ratio was observed in milk of 5% OPEFB feed due to a higher proportion of SFA content in the milk. The ratio of UFA: SFA is used to reflect the hardness of butter which varies throughout lactation (Soyeurt *et al*., 2007), as the values increase rapidly to 2 up to 100 days in milk (DIM) and decrease slowly until 365 DIM, in cows. The changes appear due to the seasonal effect which is mainly due to changes in feeding especially in summer as stated by Soyeurt *et al*., (2007). The value of milk UFA: SFA ratio in this study (0.468-0.685) was similar to the findings of Coreddu *et al*., (2016) in the study of dietary supplementation of grape seed and linseed on milk fatty acid of ewes. Additionally, PUFA: SFA ratio is another frequently used indices that was used to evaluate fatty acid profile related to cardiovascular health (Chen and Liu, 2020). Current study shows the ratio of PUFA: SFA was similar to the study by Mierliţă (2018) of dietary hemp supplementation on ewe's milk where its PUFA: SFA ratio is in the range of 0.106-0.175, while PUFA: SFA ratio of this study was in the range of 0.126-0.150. Nevertheless, the factors that change the milk fatty acid which stems from rumen fatty acids are rumen biohydrogenation and fatty acid esterification (Lanier and Corl, 2015). Among the challenges that prevent easy incorporation of dietary fatty acids into milk are the fatty acids cellular uptake and the differences in fatty acids incorporation into milk as stated by Lanier and Corl (2015), although these received lesser research attentions.

The effect of supplementation 5% OPEFB diet on the oxidative stress of lactating dairy goats was also investigated using the level of malondialdehyde (MDA) production as an indicator. Lipid oxidation analyses were done to assess the extent of oxidative stress occurring in animals. Due to it being the end product of lipid peroxidation, MDA formation is expedited by oxidative stress (Horie *et al*., 1997). Hence, analysing MDA content in the sample may indicates the the levels of oxyden free radicals and further reveal the extent of lipid peroxidation. MDA was used due to its simple interaction with thiobarbituric acid, MDA generation has often been considered to be a carcinogenic by-product of lipid peroxidation by omega-3 and omega-6 fatty acids (Ayala *et al*., 2014). The current study showed the 5% OPEFB feed significantly decreased the TBARS concentration in the ruminal fluid, and milk in goats but increased in serum blood. The increment of MDA concentrations suggests that the amount of anti-oxidant available in the blood was inadequate to scavenge ROS accumulation which may lead to an increased risk of oxidative stress (Castillo *et al*., 2004). These findings corroborate with the result of Emami *et al*. (2015) who reported that there is no significant effect on MDA in plasma and liver at 5% or 10% levels of pomegranate seed pulp meal, but only at 15% milk and plasma MDA decreased significantly. In another study by Sharifi *et al*., (2017), they found that increasing

levels of date palm seed diet did not affect lipid peroxidation in blood serum and milk, which is in contrast with a study by Purba *et al*., (2022) where a decrease TBARS production was found in the ruminal fluid, mammary tissue, milk, and plasma in early lactating dairy goats when supplied with piper meal diet in which contains bio-actives polyphenols. It is suggested that dietary plant polyphenols were expected to protect against oxidative stress (Schogor *et al*., 2013) by lowering TBARS concentrations in rumen fluids due to improved ruminal epithelial cells and defence systems (Ma *et al*., 2018). Due to its promising result, 5% OPEFB can be used as a natural-antioxidant rich sources in animal feed as it may benefits the dairy animal in terms of alleviating stress due to metabolic demand and milk production as well as increases its milk value.

5.5 Conclusion

Overall, 5% OPEFB supplemented feed increases rumen iso-butyric and iso-valeric acid concentrations and total protozoa population while decreasing the population of total methanogens, methanobacteriales and rumen cellulolytic bacteria. 5% OPEFB feed also increases SFA accompanied by a decrease in UFA and MUFA in the blood plasma and milk. At the level and method of extract inclusion used in the present study, 5% OPEFB feed increases milk protein content but decreases milk lactose content while decreases lipid oxidation in the rumen and milk but not in blood plasma. Therefore, the use of 5% OPEFB in animal feed shows promising results as it improves milk composition and antioxidant capacity and thus adds value to milk products.

CHAPTER 6

GENERAL DISCUSSION

Oil palm plant components have proven their usefulness to animals in various ways. Studies have shown that the usage of oil palm leaves, fronds, and empty fruit bunch has impacted animal production due to their high fiber content, further impedes their usage as a potential roughage resource in Malaysia (Dahlan, 2000). Nonetheless, previous studies reported various interventions done in order to improve their palatability, digestibility, and voluntary intake; which includes physical (Goh, 2002; Ebrahimi *et al.*, 2012; Wu *et al.*, 2020), biological (Nur Nazratul *et al.*, 2019) and chemical pre-treatment (Chanjula *et al.*, 2021). In lieu of these challenges, acquiring plant extract from plant material offers a feasible approach to the incorporation of any oil palm fibrous material in animal feed. It is considered an attractive alternative method as it provides practicality with the added benefits of natural compounds. In accordance with natural compound content, it is stated by Al-Al-Goranya *et al.*, (2020) that ethanolic extract of oil palm empty fruit bunch contained natural compounds that have antioxidant, anti-inflammatory, antimicrobial, antifungal, antitumor, antiviral, anti-convulsant, analgesic and as well as antidiabetic properties. It was also reported that oil palm empty fruit bunch 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).

This study focused on the effect of oil palm empty fruit bunch methanolic extract on dairy production performances. The composition of OPEFB extract was addressed in Chapter 3 where the phytochemical content of OPEFB extract was determined, and hexadecanoic acid, methyl ester was the main compound of interest contained in the OPEFB extract. Next, chapter 4 was done in order to evaluate the OPEFB extract on the *in-vitro* rumen fermentation profile, which results in a decrease in acetate production, gas production kinetics, the population of total bacteria, protozoa, and *B. fibrisolvens* but an increase in the production of palmitate in the rumen. Further into Chapter 5, the study evaluates on the *in-vivo* rumen fermentation profiles, blood biochemical parameters, milk production parameters (yield, compositions, and fatty acid profiles), and lipid peroxidation (rumen, blood, and milk). From the results obtained, it was clear that 5% EFB extract feed reduces rumen acetate production, methanogens, methanobacteriales, cellulolytic bacteria, as well as *B. fibrisolvens*. It also increased protozoa populations, milk protein content, and antioxidant capacity in rumen and milk.

The current study showed that OPEFB extract contained a total phenolic content of 3.98%, a total tannin content of 2.3%, and a total flavonoid content of 1.3%. The total phenolic content of the OPEFB extract found in this study was 75.9% higher than the phenolic content of the methanolic extract of EFB as reported by Tsouko *et al.*, (2019), but 12.48% lower than in leaves as reported by Aiman-Zakaria *et al.*, (2017). Meanwhile, the flavonoid content of the OPEFB extract in

the current study was 81% higher than the OPEFB extract recorded by Han and May (2012). Current results indicated that the OPEFB was a great source of flavonoid while a moderate source of phenolic content when compared to the other oil palm by-products. On the other hand, 70 compounds were identified through a GC-MS characterization study with 12 major compounds discovered. It was recorded in the current work that the compounds with the highest content in OPEFB extract are hexadecanoic acid, methyl ester, (41.3%) and the second highest content is 11-Octadecenoic acid, methyl ester (32.3%). Previous studies by Usha and Nazarine (2003) indicate that hexadecanoic acid, methyl ester can be used safely in animal feeds as a supplementary source of fat. It was recorded that 0.2% of hexadecanoic acid, methyl ester in feedlot lamb feed increases 70% of its content in intramuscular fat (Borghi *et al.,* 2016). Meanwhile, previous study reported an increase milk yield and UFA along with a decrease in SFA with 3.34% hexadecanoic acid, methyl ester in buffalo feed (Shelke *et al.,* 2012). Meanwhile, the usage of up to 1% of 11-Octadecenoic acid, methyl ester in rabbit feed increases milk yield, improved blood lipid profile as well as antioxidant status (El-Gindy *et al.,* 2022) Both of the compounds mentioned earlier, have antioxidant and antimicrobial properties, beneficial to the host animal when added into the feed as they can modify rumen fermentation dynamics along with mediate oxidative stress experienced by the host animal.

The current study showed ruminal acetate decreased as dietary supplementation of OPEFB increased, *in-vitro*. This is probably due to the decrease in the population of total bacteria as the dietary supplementation of OPEFB increased since acetate is the product of structural carbohydrate fermentation by cellulolytic bacteria while propionate is the product of non-structural carbohydrate fermentation by amylolytic bacteria (Enjalbert *et al.*, 1999). It was reported the presence of polyphenols can reduce the degradation of fiber in the rumen by declining the attachment of microbes to fiber particles (Makkar, 2003) which explains the decrease in potential gas production fraction although the effect of polyphenol is better interpreted when the determination of microbial growth and/or feed degradability was included with gas measurement. Meanwhile, increased iso-butyric and iso-valeric content in the study indicates optimal rumen degradable protein ingested as microbial production of isoacid is related to the prospect of amino acid release and protein deamination in the rumen (Armentano *et al.*, 1993; Yang *et al.*, 2004). It is likely that the decrease in the population of total bacteria, total protozoa, and *B. fibrisolvens* is due to the increase in the content of total flavonoids of the OPEFB extract since it has been put forth that flavonoid may affect rumen microbial activity directly (Patra and Saxena, 2010) or through the production of new derivatives via biotransformation (Simon *et al.*, 2005). Flavonoids generally negate microorganisms through suppression of cytoplasmic function, bacterial cell wall synthesis, or nucleic acid synthesis (Cushnie and Lamb, 2005). The cellulolytic bacteria (*R. albus, R. flavefaciens,* and *F. succinogenes*) population in this study was unaffected by the OPEFB dietary supplement which is consistent with the findings of Aiman-Zakaria *et al.*, (2017) and Al-Jumaili (2018) in the *in-vitro* studies of oil palm leaves extract. The reduction of *B. fibrisolvens* populations may be due to the increment of palmitic acid in the rumen as Maczulak *et al.*, (1981) in their study showed some strains of *B. fibrisolvens* were affected by palmitic and stearic acid. It was also observed by Henderson (1973) in earlier studies where myristic,

palmitic, and stearic acid shows inhibitory effects towards *B. fibrisolvens* were recorded. Hence, this *in-vitro* study shows that 5% of OPEFB extract could be supplemented in the animal diet with the impact of polyphenol in the OPEFB extract being modest on the fermentation in the rumen. Current *in-vivo* study showed that feeding 5% of the OPEFB diet increases iso-acid contents (iso-butyrate and iso-valeric), and total protozoa populations, while reducing the population of total methanogens, methanobacteriales, and all four major fibrolytic bacteria (*R. albus, R. flavefaciens, F. succinogenes, B. fibrisolvens*). On the contrary, the protozoa population in the current *in-vivo* study increased, which was different than in the previous *in-vitro* result. The differences may be due to the different time of rumen samples taken as in the *in-vivo* study, rumen samples were obtained 2 hours post feeding while in *in-vitro* studies, the rumen samples were taken 12 hours post incubation. However, the difference between the current study apart of the study by Al-Jumaili (2018) and Aiman-Zakaria *et al.*, (2017) is the forage-to-concentrate ratio used in the studies. The ratio used in our study is a 60:40 forage-to-concentrate ratio meanwhile both of those studies used an equal ratio of forage-to-concentrate ratio which explains the increase in protozoa population in our study while the protozoa population in their studies is not affected. As pointed out by Huws *et al.*, (2009), rumen protozoa are rich in poly-unsaturated fatty acid due to the ingestion of chloroplast from forages, potentially protecting lipids in the rumen from biohydrogenation which is in line with the suggestion by Williams *et al.*, (2020) who suggests that protozoa in the rumen do not directly contribute to ruminal lipid metabolism since only two lipases of GDSL family were identified but not highly expressed in the meta 'omic studies. The increment of total protozoa population in the rumen with decreasing amount of hydrogen-competing microbes may suggest that biohydrogenation might be reduced, albeit not. It was found recently by Martel *et al.* (2011) and Devillard and Wallace (2006) that protozoa play a vital role in rumen biohydrogenation by keeping a ruminal pool of fatty acid in their protozoal membranes that are inaccessible to bio-hydrogenating bacteria, thus help to slow down fatty acid biohydrogenation. The 17.5% increase in the population of protozoa in rumen-fed OPEFB extract also explains the increase of rumen linoleic acid in the study since Devillard *et al.*, (2006) stated that protozoa may represent a vital source of CLA since according to Keeney (1970), approximately 75% of the microbial fatty acid in the rumen might originate from protozoa. The reduction of all cellulolytic bacteria in our study is likely due to the content of condensed tannin (CT) in the extract as it was suggested by Costa *et al.*, (2018) where some gram-positive specialized fibrolytic bacteria particularly *R. albus, R. flavefaciens,* and *F. succinogenes* are more susceptible to CT than gram-negative rumen bacteria. This further suggests the OPEFB extract in the diet reduces fiber degradation. A decrease by 7.1% in the total methanogens population and a 7.3% decrease in the methanobacteriales population of the OPEFB extract dietary group indicate the reduction of methane production as both microbes compete for the available hydrogen for biohydrogenation instead of methane production. According to Lan and Yang (2019), in order to improve accessibility to hydrogen, rumen methanogens can exist in the rumen through free-living, associated with protozoa or fungi. Hence, the decreased population of methanogens and methanobacteriales accompanied by an increased protozoa population suggests the portion of methanogens that exists lay towards free-living and fungi-associated protozoa. Newbold *et al.*, (1996) suggested that protozoa-associated methanogens in the rumen formed 9-25% of rumen

methanogens, approximately. The result for rumen fermentation concurs with the result of the rumen microbial population. Milk protein and lactose content showed inverted responses towards supplementation of dietary OPEFB extract. Although milk lactose content decreased with the addition of OPEFB extract, the content of oleic acid supplied was still within the content needed to maintain milk fluidity as suggested by Gama *et al.* (2008) where oleic acid concentrations need to exceed 8 g/100 g of fatty acids. The increase in milk protein content in our study may as a result of the presence of phenolic content in OPEFB diets since the extract in this study revealed a substantial amount of hexadecanoic acid, methyl ester which has anti-oxidant properties that may ameliorate oxidative stress as well as any inflammation especially during early lactation, thus causing an improvement in milk quality (Bonanno *et al.* 2019). It is in line with the result in our study which shows supressed lipid oxidation in milk which suggests that either the presence of polyphenol in milk helps with the lipid oxidation or the content of unsaturated fatty acid in milk is lower hence the result. Wang *et al.*, (1996) also observed an increased in milk protein content when supplementing ewes with *Lotus corniculatus* that contain high polyphenols. Additionally, Formato *et al.* (2022) stated that the effects on the variation of milk proteins probably vary on the ability of tannins to form protein–tannin complexes through the hydrogen bond formations which are stable in rumen pH (5–7) and capable of breaking down at the acid or alkaline pH of the abomasum and duodenum. Dietary OPEFB extract increases capric, lauric, myristic, and total saturated fatty acid in the rumen. Palmitic, oleic, UFA, MUFA, and UFA: SFA in the rumen were reduced when supplemented with dietary OPEFB extract. According to Lourenço *et al.*, (2010), both long-chain UFA and medium-chain SFA have an inhibitory effect on biohydrogenation (Jenkins *et al.*, 2008; Goel *et al.*, 2009) and methanogenesis (Martin *et al.*, 2009). The current study showed a 23.9% decrease in the MDA content of EFB-fed rumen while a 27% decrease in the MDA content of EFB-fed milk which indicates an improved antioxidant capacity in EFB-fed host animals. It was confirmed by Kapusta *et al.*, (2018) in their study that negative correlation between MDA content and the degree of antioxidant protection in milk. In this study, UFA content in the rumen was high, concurrently rumen MDA content was low, which suggest that degree of antioxidant protection was high in the rumen. Whereas, the current study shows a low content of UFA along with a high content of MDA in blood suggesting a lower degree of antioxidant protection by OPEFB extract in blood. Meanwhile, dietary OPEFB shows a low content of UFA with a low level of MDA in milk indicating a high degree of antioxidant protection in the milk of the OPEFB diet group. This study outcome was in agreement with Gross *et al.* (2011) as they discovered that C4 to C16 milk fatty acids rose with decreasing negative energy balance, but C18:0 and cis-9 C18:1 milk fatty acid reduced as adipose tissue mobilization decreased in early lactation cows. It would mean that in this study, the animals are not in negative energy balance, despite being conducted in early lactation.

Therefore, the overall outcome of the study showed that 5% of the OPEFB feed is useful to be fed when the animals experience stressful conditions for instance, during early lactation. As both of the major compounds of interest in the OPEFB extract, hexadecanoic acid, methyl ester, and 11-Octadecenoic acid, methyl ester may contained antioxidant properties, which may help the animal better manage current metabolic demand during peak milk production. Another

biological property of hexadecanoic acid, methyl ester, worth mentioning is anti-inflammatory (Othman *et al.*, 2015; Aparna *et al.*, 2012; Elwekeel *et al.,* 2023) which is most beneficial if it is utilized in the feed after kidding. The increase in milk SFA and protein content would help in faster increasing the weight of the kids as more energy and protein is ingested hence, leading to reduced weaning age of the kids.

CHAPTER 7

SUMMARY, GENERAL CONCLUSION AND FUTURE RESEARCH RECOMMENDATIONS

7.1 Summary and general conclusion

Briefly, the current work has identified hexadecanoic acid, methyl ester as the main bioactive phytochemical constituents of interest that are found in abundance in the OPEFB extract. Rumen acetate production was decreased, iso-butyric, iso-valeric, and palmitic acid productions were increased, and gas production kinetics were reduced with the population of total bacteria, protozoa, and *B. fibrisolvens* was decreased with an increased level of the OPEFB extract in the *in-vitro* rumen fermentation. While in the *in-vivo* study, rumen iso-butyric and iso-valeric productions were increased as well as the total protozoa population in the rumen, the population of methanogens, methanobacteriales, and rumen cellulolytic bacteria was decreased with the 5% of OPEFB methanolic extract supplementation in the animal's diet. The fatty acid profile in the rumen, blood, and milk was altered with the incorporation of the 5% OPEFB methanolic extract in the feed, with an increase in the milk SFA content along with the reduction of UFA and MUFA content in milk, especially. The 5% OPEFB methanolic extract diet also increases the milk protein content and milk antioxidant capacity while reducing the lactose content of the milk. Whilst, the serum biochemistry profile of the animal fed with the 5% OPEFB methanolic extract diet was unaffected. In conclusion, the OPEFB extract supplemented at 5% can be incorporated as a supplement into the animal feed especially when the animal experiences stressful conditions such as early lactation to alleviate oxidative stress of the animal and further improve the milk quality of the animal.

7.2 Future research recommendations

The outcome of the current study has managed to contribute new knowledge as mentioned briefly above. However, several improvements could be made for useful future research. There are:

- Duplicating the above research approach but using a high-concentrate feed as a basal diet.
- In-depth study on the production of long-chain fatty acids in the milk.
- In-depth study on the milk protein profile for example the total protein fraction, casein content, and whey content of the milk for the development of specialized dairy products.
- Increase the number of animals in the feeding trial to assess further physiological changes in the animals.

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APPENDICES

Appendix A : Institute Animal Care and Use Committee Approval for Animal Feeding Trials

UPM
UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	23th October 2017
AUP No.:	UPM/IACUC/AUP-R058/2017
Project Title:	Effects of Feeding Empty Fruit Bunches (EFB)-Based Diet On Milk Production In Dairy Goats.
Principal Investigator:	Assoc. Prof. Dr. Goh Yong Meng
Members:	Assoc. Prof. Dr. Awis Qurni Sazili; Assoc. Prof. Dr. Anjas Asmara @Abd. Hadi Samsuddin; Nur Liyana Akmal Harun; Elham Khazaie.
Attending Veterinarian:	Assoc. Prof. Dr. Goh Yong Meng
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Chronic
Category of Invasiveness:	B
Source of Animals:	Genetic Improvement Farm Technologies (GIFT) Sdn Bhd, Penang, No 20-1, Jalan PJS 10/2, Subang Indah. 46000 Petaling Jaya.
Number of Animals Approved:	27 Dairy Goats
Housing:	1. Experimental Facility, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia. 2. Farm 15, Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia
Duration:	23 October 2017 – 23 October 2018

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.

PROF. DR. MOHD HAIR BEJO
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1673,
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP)
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

Appendix B: Instrument used for *in-vitro* rumen fermentation study

Appendix C1 : GC-MS Identified Components of OPEFB extract

PK	Library/ID	R.T. (min)	Ref	Area Pct (%)	Qual (%)	Molecular formula	Molecular weight
1	1,2,3,4-Butanetetrol, [S-(R*,R*)]-	7.5756	9700	0.073	42	$C_4H_{10}O_4$	122.1198
2	Phenol, 2-methoxy- (Guiacol)	8.3582	10417	0.1526	90	$C_7H_8O_2$	124.1372
3	2-Propanone, 1-(2,5-dimethoxy-4-methylphenyl)-	9.7196	67573	0.0426	35	$C_{12}H_{17}NO_3$	223.272
4	3,5-Di(2-thienyl)pyridine	10.1586	96480	0.1244	14	$C_{13}H_9NS_2$	243.342
5	Carbamic acid, acetylthio-, O-methyl ester	10.2795	14587	0.0458	18	$C_4H_7NO_2S$	133.165
6	2-Methoxy-4-vinylphenol	12.4489	24419	0.4321	94	$C_9H_{10}O_2$	150.1745
7	4-Hydroxy-2-methylacetophenone	12.6779	24471	0.0378	70	$C_9H_{10}O_2$	150.1745
8	Phenol, 2,6-dimethoxy-	13.1296	27407	0.2006	95	$C_8H_{10}O_3$	154.1632
9	Phenol, 2-methoxy-4-(1-propenyl)-	14.7519	33412	0.1084	81	$C_{10}H_{12}O_2$	164.2011
10	Ethoxy(methyl)chlorosilane	14.8601	10262	0.072	43	C_3H_8ClOSi	123.631
11	Cyclotridecane	15.0382	47268	0.0693	86	$C_{13}H_{26}$	182.3455
12	2,11-Dodecanedione	15.3436	59505	0.1137	43	$C_{12}H_{22}O_2$	198.3019
13	Butylated Hydroxytoluene	15.4835	77555	0.1308	98	$C_{15}H_{24}O$	220.3505
14	Phenol, 2,6-bis(1,1-dimethylethyl)-	15.5472	66108	0.0618	81	$C_{14}H_{22}O$	206.3239

15	Undecanoic acid, 10-methyl-, methyl ester	15.7571	72721	0.0828	90	$C_{13}H_{26}O_2$	214.3443
16	1,5-Naphthyridin-4-amine	16.0434	21474	0.2519	32	$C_8H_7N_3$	145.165
17	1,2,4-Cyclopentanetrione, 3-(2-pentenyl)-	16.4442	45245	0.2749	46	$C_{10}H_{12}O_3$	180.203
18	1H-Purine-2,6-dione, 3,7-dihydro-1,3-dimethyl-	16.6987	46065	0.0203	30	$C_7H_8N_4O_2$	180.1640
19	2H-Cyclopentacycloocten-2-one, decahydro-3a-methyl-, trans-	17.1377	45663	0.0044	22	$C_{12}H_{20}O$	180.291
20	Pyrimidine-2,4(1H,3H)-dione, 6-hydroxy-5-methyliminomethyl-	17.2204	37655	0.1517	53	$C_6H_7N_3O_3$	169.14
21	1,4-Dimethyl-1,2,4-triazol-3-thione-5-one	17.4812	21298	0.055	37	$C_4H_7N_3OS$	145.18
22	Methyl (Z)-5,11,14,17-eicosatetraenoate	17.8184	159968	0.1002	38	$C_{21}H_{34}O_2$	318.501
23	1,5-Dodecadiene	17.952	35146	0.0952	64	$C_{12}H_{22}$	166.308
24	1,11-Tridecadiene	18.0602	45716	0.1054	38	$C_{13}H_{24}$	180.335
25	Methyl tetradecanoate	18.1556	95859	0.2394	86	$C_{15}H_{30}O_2$	242.3975
26	2-Pentadecanone	18.3273	82960	0.0974	90	$C_{15}H_{30}O$	226.404
27	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	18.4419	55967	0.1336	78	$C_{11}H_{14}O_3$	194.23
28	2(1H)-Benzocyclooctenone, decahydro-4a-methyl-, trans-(-)-	18.5755	56433	0.0542	74	$C_{13}H_{22}O$	194.318
29	Methyl tetradecanoate	18.6709	95859	0.8521	98	$C_{15}H_{30}O_2$	242.3975
30	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	18.9318	57688	0.1157	76	$C_{10}H_{12}O_4$	196.1999
31	5-Amino-1-pentanol, N,O-diacetyl-	19.218	51238	0.0966	22	$C_9H_{17}NO_3$	187.239

32	2-Pentanone, 1-(2,4,6-trihydroxyphenyl)	19.3453	69035	0.1938	64	$C_{11}H_{14}O_4$	210.229
33	Pentadecanoic acid, methyl ester	19.5298	107593	0.3164	97	$C_{16}H_{32}O_2$	256.4241
34	Tetradecanoic acid, 12-methyl-, methyl ester	19.6379	107611	0.277	90	$C_{16}H_{32}O_2$	256.4241
35	Oxirane, tridecyl-	19.9624	82972	0.0259	89	$C_{15}H_{30}O$	226.404
36	Pentadecanoic acid, methyl ester	20.026	107595	0.139	97	$C_{16}H_{32}O_2$	256.4241
37	Ethanone, 1-[2-(5-hydroxy-1,1-dimethylhexyl)-3-methyl-2-cyclopropen-1-yl]-	20.1214	81075	0.0663	47	$C_{14}H_{24}O_2$	224.344
38	E-8-Methyl-9-tetradecen-1-ol acetate	20.2487	117492	0.0577	68	$C_{17}H_{32}O_2$	268.441
39	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	20.3696	67357	0.3404	94	$C_{11}H_{12}O_4$	208.2106
40	Acetic acid, 7-chloro-3-methyl-6-oxo-hept-2-enyl ester	20.5541	75227	0.0855	16	$C_{10}H_{15}ClO_3$	218.677
41	Cyclooctene, 3-ethenyl-	20.6113	15736	0.0667	70	$C_{10}H_{16}$	136.238
42	Octacosyl acetate	20.7131	227661	0.116	38	$C_{30}H_{60}O_2$	452.7962
43	Pentadecanoic acid, 14-methyl-, methyl ester	20.8467	119425	0.3351	96	$C_{17}H_{34}O_2$	270.4507
44	7-Hexadecenoic acid, methyl ester, (Z)-	21.0503	117506	0.4767	99	$C_{17}H_{32}O_2$	268.4348
45	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis-	21.1712	154818	0.0819	58	$C_{19}H_{36}O_3$	312.4873
46	Hexadecanoic acid, methyl ester	21.3875	119400	41.2894	99	$C_{17}H_{34}O_2$	270.4507
47	n-Hexadecanoic acid	21.8392	107549	1.9438	99	$C_{16}H_{32}O_2$	256.4241
48	Hexadecanoic acid, 15-methyl-, methyl ester	22.1	131321	0.1052	95	$C_{18}H_{36}O_2$	284.484

49	Hexadecanoic acid, 15-methyl-, methyl ester	22.2082	131321	0.4422	97	$C_{18}H_{36}O_2$	284.4772
50	7-Hexadecenoic acid, methyl ester, (Z)-	22.3799	117506	0.1701	81	$C_{17}H_{32}O_2$	268.4348
51	Heptadecanoic acid, methyl ester	22.5772	131301	0.235	98	$C_{18}H_{36}O_2$	284.4772
52	1,9-Tetradecadiene	23.1052	56479	0.0602	93	$C_{14}H_{26}$	194.362
53	Z-3-Methyl-2-hexenoic acid	23.3151	12221	0.1	43	$C_7H_{12}O_2$	128.171
54	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	23.6014	139726	9.5539	99	$C_{19}H_{34}O_2$	294.4721
55	11-Octadecenoic acid, methyl ester	23.7605	141291	32.2902	99	$C_{19}H_{36}O_2$	296.4879
56	9,12-Octadecadienoic acid (Z,Z)-	24.2376	127649	0.1998	97	$C_{18}H_{32}O_2$	280.4455
57	trans-13-Octadecenoic acid	24.3394	129357	0.9587	99	$C_{18}H_{34}O_2$	282.4614
58	9,17-Octadecadienal, (Z)-	24.683	114272	0.0869	96	$C_{18}H_{32}O$	264.4461
59	cis-13-Octadecenoic acid	24.7975	129347	0.2745	86	$C_{18}H_{34}O_2$	282.4614
60	2,2-Dimethyl-1-oxa-spiro[2.4]heptane	25.1347	11478	0.0619	47	$C_8H_{14}O$	126.199
61	Methyl 9-cis,11-trans-octadecadienoate	25.2301	139701	0.077	51	$C_{19}H_{34}O_2$	294.479
62	7-Hexadecenoic acid, methyl ester, (Z)-	25.7009	117506	0.1748	58	$C_{17}H_{32}O_2$	268.4348
63	Silanol, triphenyl-	25.9045	124487	0.0253	86	$C_{18}H_{16}OSi$	276.4045
64	Oxacyclohexadecan-2-one	26.5279	94128	0.0824	62	$C_{15}H_{28}O_2$	240.3816
65	3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	27.425	127141	0.0857	38	$C_{14}H_{16}O_2S_2$	280.4

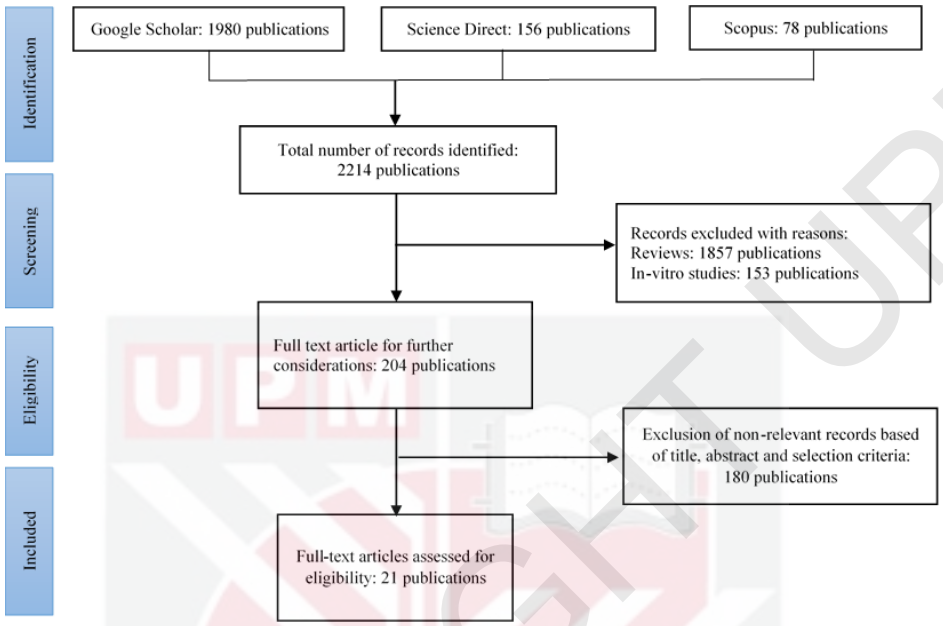
66	Myristoleic acid	27.654	82760	0.0409	38	$C_{14}H_{26}O_2$	226.3550
67	9-Cycloheptadecen-1-one, (Z)-	27.8131	102574	0.1217	38	$C_{17}H_{30}O$	250.4195
68	7-Oxabicyclo[4.1.0]heptane, 1,5-dimethyl-	27.8958	11486	0.2126	38	$C_8H_{14}O$	126.199
69	Methyl 18-methylnonadecanoate	28.5574	166215	0.3311	98	$C_{21}H_{42}O_2$	326.565
70	2-Cyclohexen-1-one, 3-(3-hydroxybutyl)-2,4,4-trimethyl-	29.4035	69372	0.0704	27	$C_{13}H_{22}O_2$	210.3126

Appendix C2: Materials and methods for systematic review on the effect of polyphenol supplementation on milk composition and fatty acid of dairy animal

A database of previous studies involving polyphenol supplementation and milk fatty acid profile was created based on the guideline requirements of the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) (Liberati et al., 2009). The literature search was performed in several databases, including Google Scholar, Scopus, and Science Direct. The keywords used to search the databases were "feed" "milk fatty acid", "extract", "polyphenol", and "rumen. The keywords were chosen to identify publications with experiments that were appropriate for further exploration. The response of interest included total phenolic content, milk fat content, milk protein content, milk lactose content, milk saturated fatty acid (SFA) content and milk medium-chain fatty acid (MCFA) content. A total of 2214 studies were identified through database searches: Google Scholar (1980), Science Direct (156), and Scopus (78). The first round of exclusion excluded review articles, books and encyclopedias, leaving 297 studies suitable for further screening. In the second round of screening, irrelevant articles were excluded which includes conference proceedings, *in-vitro* studies and reviews, which resulted in 81 articles remaining. In these two stages, the title and, if necessary, the abstracts were reviewed. The final screening was performed to select eligible studies according to the following criteria: (1) the study was conducted between 2008 and 2023, (2) the study used a control group without polyphenol supplementation, (3) the study reported at least one of the outcome variables listed, and (4) the study reported the mean values and associated error. Only those manuscripts from which the authors of this review could extract the required data were included. Finally, 21 articles met the required criteria and were included in this systematic review and the list of the chosen articles were tabulated in a table in appendix C4. Appendix C3 below showed the search flow and data extraction process of the current study.

The analysis was performed using Review Manager (RevMan version 5.3.5; The Cochrane Collaboration, The Nordic Cochrane Centre, Copenhagen, Denmark). The standard mean difference (SMD) approach RevMan implemented in the software was used as the basis of the comparison between the controls and treatments. Individual study weights were calculated as the inverse of the variance. Weighted averages and 95 % confidence intervals (CI) were pooled and a random effect model was used to compute a summary of the effect across studies (Cochrane, 2011). Given the different study locations covered by the review and the nature of the studies, assuming a single common fixed effect model was untenable. Heterogeneity among studies was evaluated using the Inconsistency index (Higgins and Thompson, 2002). The I^2 value describes the percentage of total variation across studies that is due to heterogeneity rather than chance (Higgins and Thompson, 2002). A classification of the I^2 values was used to interpret the heterogeneity magnitude: values around 25 %, 50 %, and 75 % were considered as low, medium, and high heterogeneity respectively (Higgins and Thompson, 2002). The asymmetry of the funnel plot, which consists of a scatterplot of the mean variable value against its standard error, was used to assess publication bias (Cochrane, 2011).

Appendix C3: Literature retrieval flowchart



Appendix C4: Data tabulation of the chosen *in-vivo* experiments for the systematic review analysis

No. Exp	References	Species	Parity status	Basal feed	Polyphenol sources	Type of polyphenol sources	Total phenolic content, % of DM	Adaptation period/ long treatment (day)	Milking (time/ day)
1	Yao *et al.* (2021)	Cow (Holstein)	Multiparous	TMR[2]; 60: 40 of forage to concentrate ratio (F: C)[1]	18% Soybean Meal	Agro-industrial byproduct	0.202	15/5	2
					11% Unfermented yellow wine less	Agro-industrial byproduct	0.582		
					11% Fermented yellow wine less	Agro-industrial byproduct	0.676		
2	Kholif *et al.* (2018)	Goat (Nubian)	Multiparous	40: 60 (F: C)[1] Egyptian berseem clover and concentrates mixture.	Control	Forages	-	15/7	2
					10ml *M. oleifera* leaves extract	Forages	0.6		
					20ml *M. oleifera* leaves extract	Forages	1.2		
					40ml *M. oleifera* leaves extract	Forages	2.4		
3	Khosravi *et al.* (2018)	Cow (Holstein)	Multiparous	TMR[1]	Corn silage	Forages	0.053	21/7	3
					Sorghum silage	Forages	1.698		
4	Mitsiopoulou *et al.* (2021)	Goat (Alpine x Local breed)	n/a	Component feeding; 50: 50 (F: C)[1]	Control	Forages	6.312	7/100	2
					5% of whole sesame seed	Seed/grain	9.43		
					10% of whole sesame seed	Seed/grain	12.603		
5	Cabiddu *et al.* (2009)	Sheep (Sardinian)		Grazing Sulla-based pasture	Sulla pasture on 4th May	Forages	3.21	14/14	2

					Sulla pasture on 17th May	Forages	3.55		
					Sulla pasture on 1st Jun	Forages	3.42		
6	Abbeddou *et al.* (2011)	Sheep (Awassi)	Multiparous	30: 70 (F: C)[1] except Olive cake diet 20:80 (F: C)[1]	Control	Forages	0.67	10/50	2
					Lentil straw	Forages	1.32		
					Atriplex leaves	Forages	0.57		
					Olive leaves	Forages	2.25		
					Olive cake	Agro-industrial byproduct	0.53		
					Tomato pomace	Agro-industrial byproduct	0.60		
7	Campione *et al.* (2021)	Sheep (Comisana)	Multiparous	Concentrates with *ad libitum* hay	Conventional concentrate as control concentrate	Agro-industrial byproduct	0.194	14/21	2
					Experimental concentrate: with Cocoa bean shell	Seed/grain	0.52		
8	Coreddu *et al.* (2016)	Sheep (Sarda)	Multiparous	TMR[2]	Control (corn, soybean, pea)	Seed/grain	-	14/56	weekly
					Grape seed	Seed/grain	0.037		
					Extruded linseed	Seed/grain	-		
					Mixed of grape seed and extruded linseed	Seed/grain	0.038		
9	Akhlaghi *et al.* (2022)	Cow (Holstein)	Primiparous	TMR[2]; 32: 68 (F: C)[1]	1.5% of palmitic acid-enriched fat with 8.7% pomegranate peel	Agro-industrial byproduct	0.288	21/7	3

					1.5% Ca salts of fish oil with 8.7% pomegranate peel	Agro-industrial byproduct	0.357		
					1.5% of palmitic acid-enriched fat without pomegranate peel	Agro-industrial byproduct	0.23		
					1.5% Ca salts of fish oil without pomegranate peel	Agro-industrial byproduct	0.22		
10	Kotsampasi *et al.* (2017)	Cow (Holstein)	Multiparous	TMR[2]	0% of Pomegranate pulp silage (PPS) as control	Agro-industrial byproduct	0.078	20/5	1
					7.5% of PPS	Agro-industrial byproduct	0.226		
					15% of PPS	Agro-industrial byproduct	0.364		
11	Todaro *et al.* (2017)	Sheep (Valle del Belice)	Multiparous	Component feeding; partial mixed ration (PMR)[3] of *ad libitum* hay with 600g of concentrates daily with 1 kg of FLP replaced 200 g of concentrates	0% of Fresh lemon pulp (FLP) as control	Forages	0.84	14/7	1
					9.01% of FLP	Agro-industrial byproduct	1.132		
					15.7% of FLP	Agro-industrial byproduct	1.322		
12	Cappucci *et al.* (2018)	Sheep (Comisana)	Multiparous	Component feeding; PMR[3] of *ad libitum* chopped lucerne hay with 100 g of rolled	0% of olive crude phenolic concentrates (OCPC)	-	-	21/14	2

				barley, and 800 g/animal daily	0.6% of OCPC	Agro-industrial byproduct	0.064		
					0.8% of OCPC	Agro-industrial byproduct	0.083		
					0.12% of OCPC	Agro-industrial byproduct	0.118		
13	Grosse Brinkhaus *et al.* (2016)	Cows (Holstein-Friesian)	Multiparous	Component feeding; PMR of grass hay, corn silage, and Extrulin 135 (contain 60% of extruded linseed oil and 40% of wheat bran) Experimental dietary variation generated by offering AL/SF/BT in amounts representing approximately 20% of the basal diet	Pelleted alfalfa (AL) (*Medicago sativa L.*'*Sanditi*')	Forages	0.97	21/7	2
					Pelleted sainfoin (SF) (*Onobrychis viciifolia L. 'Perly'*)	Forages	3.241		
					Pelleted birdsfoot trefoil (BT) (*Lotus corniculatus L. 'Polom'*)	Forages	1.633		
14	Heidarian Miri *et al.* (2013)	Goat (Alpine × Beetal)	n/a	TMR[2]; 50: 50 (F: C)[1] berseem hay and concentrate	0% of cumin seed extract (CSE)	-	-	28/2	2
					1.27% CSE	Seed/grain	3.89		
					2.53% CSE	Seed/grain	7.77		
15	Karimi *et al.* (2022)	Cow (Holstein)	n/a	TMR[2]	diet without soybean oil (SO0) and dried citrus pulp (DCP)	-	0.25	21/7	2
					SO0 + 8.65% of DCP	Agro-industrial byproduct	0.71		

					SO0 + 17.3% of DCP	Agro-industrial byproduct	1.17		
					17.3g of soybean oil + 8.65% of DCP	Agro-industrial byproduct	0.71		
					17.3g of soybean oil + 17.3% of DCP	Agro-industrial byproduct	1.17		
16	Claudio *et al.* (2022)	Cow (Holstein)	Multiparous	PMR[3] *Acacia mearnsii* bark (694g per kg DM of total tannin) as tannin source	Morning grazing + afternoon PMR meal with 9.0 g of tannins/kg of PMR DM	-	-	14/8	2
					Morning PMR with 9.0 g of tannins/kg of PMR DM + afternoon grazing meal	-	-		
					Morning grazing + afternoon PMR with 15.0 g of tannins/kg of PMR DM	Agro-industrial byproduct	6.885		
17	Santos *et al.* (2016)	Cannulated cow (Holstein)	n/a	60:40 (F: C)[1] corn silage as forage and concentrate of soybean meal, ground corn grain, wheat bran, urea and mineral supplements	Control (basal diet)	Forages	0.345	14/7	2
					Diet with flaxseed oil (FO)	Seed/grain	0.392		
					Diet with FO + propolis-based Product (PBP)	Agro-industrial byproduct	2.827		
					Diet with FO + PBP + vit E	Agro-industrial byproduct	2.923		
18			Multiparous	PMR of 76:24 (F: C)[1]	Control	Forages	-	21/5	2

	Huang *et al.* (2022)	Cows (Holstein-Friesian)			6% of paulownia leaves silage diet replacing alfalfa silage	Forages	0.36		
19	Kälber *et al.* (2012)	Cows (Holstein-Friesian)	n/a	Component feeding; partial mixed ration (PMR)[3] of *ad libitum* forage with 2kg of concentrates daily	Buckwheat diet	Forages	0.85	9/6	2
					Chicory diet	Forages	0.71		
					Ryegrass diet	Forages	0.82		
20	Silva *et al.* (2016)	Goats (crossbred Saanen)	n/a	TMR[2]; 60:40 (F: C)[1]	0% whole mango meal (WMM) as control	-	-	14/5	2
					33% WMM	Agro-industrial byproduct	3.96		
					66% WMM	Agro-industrial byproduct	7.91		
					100% WMM	Agro-industrial byproduct	11.9		
21	Tsiplakou *et al.* (2017)	Goat (cross-bred)	n/a	Component feeding; 50: 50 (F: C)[1]	Control	-	-	14/30	2
					1% of *Chlorella pyrenoidosa*	Marine microalgae	0.5		

Appendix C5: Preparation of buffer in *in-vitro* rumen fermentation analysis (Fievez *et al.,* 2005)

Phosphate buffer

The phosphate buffer used in the *in-vitro* rumen fermentation analysis contained 28.8 g $Na_2HPO_4 \cdot 12H_2O$ in a liter of distilled water, and the buffer was adjusted to the pH of 7 and were gassed with carbon dioxide for 1.5 hours.

Bicarbonate buffer

The bicarbonate buffer used in the *in-vitro* rumen fermentation analysis contained 39.2 g $NaHCO_3$ in a litre of distilled water, and the buffer was adjusted to the pH of 7 and were gassed with carbon dioxide for 1.5 hours.

Appendix C6 : Gas chromatography setting for fatty acid analysis

Oven programme

- Equilibration time : 0.5 min
- Max oven temperature : 240°C

	Rate °C/min	Value °C	Hold up time	Run time
Initial	-	140	5	5
Ramp 1	3	200	7	32
Ramp 2	2.3	220	3	43.696

- Post run : 240°C
- Post run time : 0 min

Column : J&W 112-88A7:250°C:100mm×250µm×0.25µm

Initial : 0 min
H_2 @ 140°C oven
Out : Ambient pressure

Flow : 2.4273 ml/min
Pressure : 2.7549 bar
Average velocity : 43.754 cm/sec
Hold up time : 3.8091 min

Inlet

- Heater : 250°C
- Pressure : 2.7549 bar
- Total flow : 29.7 ml.min
- Septum purge flow : 3ml/min
- Mode : split
- Split ratio : 10:1
- 24.273 ml/min

Detector

- Heater : 250°C
- H_2 flow : 35 ml/min
- Air flow : 350 ml/min
- Makeup flow (N_2) : 25ml/min

BIODATA OF STUDENT

Nur Liyana Akmal Harun, was born in Muar, Johor on 1st October 1989. She is the eldest of three of Dr. Harun Ongah and Mrs. Ruhaidah Yahya. She started her primary education in Dungun, Terengganu, and continues her primary and secondary education in Ampang, Selangor, due to her father's work relocation. She pursued her matriculation study in Negeri Sembilan for a year before enrolling in Universiti Putra Malaysia for the degree of Bachelor of Agriculture (Animal Science) completed in 2012.

She continued her learning journey and obtained a Master of Science in Animal Nutrition as her field of study in 2016 from the same university. For the next leg of her learning journey, she continued her Ph.D. study in Animal Production at the Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, under the supervision of Prof. Ts. Dr. Goh Yong Meng.

LIST OF PUBLICATION

Liyana, N.A.H., Goh, Y.M., Samsudin, A.A., & Sazili, A.Q. (2018). The effect of oil palm empty fruit bunch extract on rumen fermentation of goats. 4th Asian-Australasian Dairy Goat Conference, Tra Vinh, Vietnam. 17th-19th October 2018.

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