UTILIZATION OF *PIPER BETLE* L. IN DAIRY GOAT DIETS

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาอิทธิพลของใบชะพลู (*Piper betle* L.) ต่อกระบวนการหมักในรูเมน ไบโอไฮโดรจิเนชัน และการเจริญเติบโตของสัตว์ และประเมินผลต่อกิจกรรมของเอนไซม์ ภายในเซลล์ และปฏิกิริยาออกซิเดชันที่เกิดจากความเครียด ที่เป็นตัวบ่งชี้ที่สังเกตได้จากการให้นมระยะแรกของแพะนม การรวบรวมข้อมูลจากปริมาณสารประกอบอินทรีย์ การรวบรวมด้วยระบบการวิเคราะห์แบบ meta-analysis การศึกษาในหลอดทดลอง และการศึกษาในสัตว์ทดลอง ผลการศึกษาได้แสดงให้เห็นว่าใบชะพลูมีกรดแอสคอร์บิค และโพลีฟีนอล gallic acid, caffeic acid, syringic acid, *p*-coumaric acid, sinapic acid, ferulic acid, catechin, rutin, myricetin, quercetin, apigenin, kaempferol, และ eugenol เป็นสารประกอบสำคัญ จากการรวบรวมข้อมูลด้วยระบบ meta-analysis พบว่าไม่มีความสัมพันธ์ระหว่างการทดลองในหลอดทดลอง และผลการทดลองในสัตว์ (R^2<0.10) ซึ่งเป็นตัวบ่งชี้ว่าจำเป็นต้องมีการดำเนินการศึกษาในหลอดทดลอง และการศึกษาในตัวสัตว์ การศึกษาใช้ใบชะพลูที่ 0 5 25 50 75 และ 100 มิลลิกรัม/การบ่ม เป็นวัตถุแห้ง (DM) เสริมในหลอดทดลอง ใบชะพลูปรับปรุงลักษณะการหมักกับการลดแก๊สมีเทน ไบโอไฮโดรจิเนชันในรูเมนของใบชะพลู ซึ่งมีการสะสมกรดสเตียริกผ่านการเปลี่ยนไบโอไฮโดรจิเนชันของกรดไขมัน (FAs) หลังจากนั้นจะผลิตกรดคอนจูเกตลิโนเลอิก (CLA) มากขึ้น ในการพิจารณาการกินได้ของสัตว์ที่มีผลเสียต่อโรคเมแทบอลิซึม ช่วงของการใช้ใบชะพลูที่ 5 กรัม สิ่งแห้ง (ประมาณ 1.25% ของอาหารทั้งหมด) ได้นำไปใช้ในการทดลองในตัวสัตว์ การศึกษาของแพะนมพันธุ์ซาแนน 12 ตัว มีน้ำหนัก (42±1.00 กิโลกรัม) ในช่วงให้นม 14 วัน ได้รับการสุ่มมาใช้ในการทดลองโดยแบ่งกลุ่มทดลองออกเป็นสองกลุ่มในการทดลองซึ่งเลี้ยงเป็นเวลา 6 สัปดาห์ หลังจากระยะเวลาในการปรับสัตว์ 2 สัปดาห์ เมื่อเทียบกับแพะที่ได้รับอาหารกลุ่มควบคุม (0 กรัม/กิโลกรัมของใบชะพลู) แพะที่ได้รับอาหารที่มีใบชะพลูที่ (12.8 กรัม/กิโลกรัมสิ่งแห้ง) แสดงให้เห็นว่ามีกรดไขมันที่ระเหยได้และปริมาณน้ำนมสูงขึ้น และพบว่ามีปริมาณของกรดลิโนเลอิกเพิ่มขึ้นสองเท่า (1.77 ถึง 2.62 กรัม/100 กรัมกรดไขมัน) ผลลัพธ์นี้อาจเกิดขึ้นเนื่องจากการเปลี่ยนแปลงของกระบวนการไบโอไฮโดรจิเนชันที่เกี่ยวข้องกับจุลินทรีย์ในกระเพาะรูเมน ยิ่งไปกว่านั้นใบชะพลูกำจัดลิพิดเปอร์ออกซิเดชัน ตลอดจนเพิ่มกิจกรรมของ superoxide dismutase (SOD) และลด thiobarbituric acid-reactive acid (TBARS) ในของเหลวในรูเมน เนื้อเยื่อเต้านม นม และเลือด ซึ่งสามารถแนะนำว่าการผลิต TBARS ที่ลดลงนั้นมีความสัมพันธ์กับการการแสดงออกของ mRNA ของการเพิ่มประสิทธิภาพของยีน κ light polypeptide ใน B-cells (NFKB) แต่การแสดงออกที่มากขึ้นของ SOD1 SOD2 SOD3 และ nuclear factor (erythroid-derived 2)-like 2 (NFE_2L_2) ในเนื้อเยื่อเต้านม ดังนั้น

ผลดังกล่าวแสดงให้เห็นว่าการใช้ใบชะพลูที่ปริมาณ 112.8 กรัม/กิโลกรัม ในอาหารของแพะนมซึ่งเป็นผลกระทบเชิงบวกต่อการเจริญเติบโตของสัตว์ สุขภาพสัตว์ และเป็นมิตรกับสิ่งแวดล้อม ความ สำเร็จนี้แนะนำให้มีการปรับใช้ประโยชน์ของใบชะพลู เพื่อให้มีคุณสมบัติในการต้านจุลินทรีย์ และคุณสมบัติในการต้านอนุมูลอิสระ ศึกษาเพิ่มเติมเกี่ยวกับการสะสมของไฟโตเคมิคอลในนม ที่เป็นผลิตภัณฑ์ที่ได้มาจากกระเพาะรูเมนอย่างรอบคอบ

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา_____

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

RAYUDIKA APRILIA PATINDRA PURBA : UTILIZATION OF *PIPER BETLE* L. IN DAIRY GOAT DIETS. THESIS ADVISOR : ASSOC. PROF. PRAMOTE PAENGKOUM, Ph.D., 277 PP.

ANTIOXIDANT/BIOHYDROGENATION/CONJUGATED LINOLEIC ACID/ LIPID PEROXIDATION/METABOLIC-RELATED DAIRY GOAT/*PIPER BETLE* LINN

This research was performed to investigate the influence of *Piper betle* L. on rumen fermentation, biohydrogenation and animal performance, and evaluate its effect on intracellular enzyme activity and oxidative stress indicator observed in early lactation of dairy goats. Data collection from organic compound quantity, systematic review with meta-analysis, *in vitro* and *in vivo* studies were determined. The obtained results demonstrate that *P. betle* L. contained ascorbic acid and polyphenol with gallic acid, caffeic acid, syringic acid, *p*-coumaric acid, sinapic acid, ferulic acid, catechin, rutin, myricetin, quercetin, apigenin, kaempferol, and eugenol as major compounds. According to this present systematic review with meta-analysis, there was no relationship between *in vitro* observations and *in vivo* results ($R^2 < 0.10$), indicating that it is required to conduct an *in vitro* study and *in vivo* study as well. Further studies, *P. betle* L. dosed at 0, 5, 25, 50, 75 and 100 mg/incubation as dry matter (DM) were supplemented into glass syringe containing substrate. *P. betle* L. improved fermentation characteristics, with promising methane mitigation. Ruminal biohydrogenation was affected by *P. betle* L. which possessed stearic acid accumulation by changing the biohydrogenation pathway of fatty acids (FAs), thereafter producing more conjugated linoleic acid (CLA) content. To consider animal intake with deleterious consequences on metabolic diseases, the selected

dose of *P. betle* L. at 5 g DM (ca. 1.25% of total feed) was proposed to undergo feeding trials (*in vivo*). The final observation, twelve multiparous Saanen goats (42±1.00 kg) in 14 days of day in milk were randomly assigned to two experimental groups in an experiment which lasted for 6 weeks after a 2-week adaptation period. Compared with goats receiving a control diet (0 g/kg of *P. betle* L.), goats receiving a diet with *P. betle* L. inclusion (12.8 g/kg DM) shown to have greater volatile fatty acid and milk yields, with CLA content found a double increasing (1.77 to 2.62 g/100 g FA). These outcomes may occur due to a change of ruminal microorganism-related biohydro-genation. Moreover, dietary *P. betle* L. limited lipid peroxidation through increasing superoxide dismutase (SOD) activity and decreasing thiobarbituric acid-reactive substances (TBARS) in ruminal fluid, mammary tissue, milk and blood. It is suggested that alleviated TBARS production was associated with dwindling a mRNA expression of κ light polypeptide gene enhancer in B-cells (NFKB) but a greater expression of SOD1, SOD2, SOD3 and nuclear factor (erythroid-derived 2)-like 2 (NFE_2L_2) in mammary tissue. Therefore, present results show that a provision of *P. betle* L. dosed of 12.8 g/kg in the diet of dairy goats addresses the positive impacts on animal performance, animal health and environmentally-friendly concerns. These achievements suggest a well-documented modulated benefit of *P. betle* L. to have antimicrobial and antioxidant properties. Further study of phytochemical's depositions in milk as rumen-derived product is wisely recommended.

School of Animal Technology and Innovation Student's Signature _____

Academic Year 2019 Advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

ALA	=	α-Linolenic Acid
BW	=	Body Weight
C17:0	=	Heptadecanoic Acid
C18:0	=	Stearic Acid
CAT	=	Catalase
CH_4	=	Methane
CHD	=	Coronary Heart Disease
CLA	=	Conjugated Linoleic Acid
CO_2	=	Carbon Dioxide
CT	=	Condensed Tannins
DI	=	Desaturation
DIM	=	Day in Milk
DM	=	Dry Matter
DMI	=	Dry Matter Intake
DPPH	=	1,1-Diphenyl-2-Picrylhydrazyl-Scavenging Activity
EC50	=	Half Maximal Effective Concentration
EO	=	Essential Oil
FA	=	Fatty Acid
FAME	=	Fatty Acid Methyl Ester
FCM	=	Fat-Corrected Milk
FO	=	Flavonoid

LIST OF ABBREVIATIONS (Continued)

GPx	=	Glutathione Peroxidase
H_2	=	Hydrogen
HDL	=	High-Density Lipoprotein
HR	=	Hydrogen Supply
HT	=	Hydrolysable Tannins
IVDMD	=	*In Vitro* Dry Matter Degradability
IVOMD	=	*In Vitro* Organic Matter Degradability
LA	=	Linoleic Acid
LDL	=	Low-Density Lipoprotein
LOD	=	Limit of Detection
LOQ	=	Limit of Quantitation
MT	=	Mixed Tannin
MUFA	=	Monounsaturated Fatty Acid
NFE_2L_2	=	Nuclear Factor (Erythroid-Derived 2)-Like 2
NFKB	=	κ Light Polypeptide Gene Enhancer In B-Cells
OA	=	Oleic Acid
PA	=	Phenolic Acid
PO	=	Piper Oil
PP	=	Piper Powder
PRISMA	=	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PRP	=	Piper Residue Powder

LIST OF ABBREVIATIONS (Continued)

PUFA	=	Polyunsaturated Fatty Acid
$\triangle^9$	=	Delta-9
RA	=	Rumenic Acid
RBB+C	=	Repeated Bead Beating Plus Column
RBH	=	Rumen Biohydrogenation
RF	=	Rumen Fluid
RT-qPCR	=	Real-Time Quantitative PCR
RUSITEC	=	Rumen Stimulation Technique
SA	=	Stearic Acid
SCD	=	Stearoyl Co-A Desaturase Enzyme
SEM	=	Standard Error of Mean.
SFA	=	Saturated Fatty Acid
SMD	=	Standardized Mean Difference
SOD	=	Superoxide Dismutase
TBARS	=	Thiobarbituric Acid-Reactive Substances
TMR	=	Total Mixed Ration
VA	=	Vaccenic Acid
VFA	=	Volatile Fatty Acid

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

During last decade, there is potent interest by consumers focussing on a well-behaved life, especially consumption of saturated fatty acid (SFA) from dairy products. Cancer and coronary heart disease (CHD) have been believed in advance as impact of consuming routinely SFA (Mensink et al., 2003; Shingfield et al., 2013), whereas the polyunsaturated fatty acid (PUFA) is assessed safer than SFA (Dewhurst et al., 2006; Elgersma et al., 2006). For this reason, it is reported that milk PUFA naturally seems to have a healthy FA by giving a conjugated linoleic acid (CLA) to improve human health properties in which concerning in prevention of CHDs, abating or eliminating cancer, enhancing immunity and treating obesity through the development of fixed lean body mass (Whigham et al., 2000; Derakhshande-Rishehri et al., 2015).

Regardless of valuable benefits, CLA is an intermediate product produced by shortening linoleic acid (LA; C18:2 cis-9, cis-12) and α-linolenic acid (ALA; C18:3 cis-9, cis-12, cis-15) through lipolysis, isomerisation and biohydrogenation. The dominant isomers of CLAs are C18:2 cis-9, trans-11 (RA; Rumenic acid) and C18:2 trans-7, cis-9, representing 75-90% and 3-16% of the total CLAs, respectively (Tanaka, 2005). CLA can be hydrolysed on rumen biohydrogenation (RBH) and it is present in limited ranges of 0.34-1.07% of total fat in milk (Dhiman et al., 2005). Literally, a

major CLA production produced by desaturation though deletion of two hydrogen atoms from vaccenic acid (VA; C18:1 trans-11) to form RA supported by the $\triangle^9$-desaturase enzyme (Dhiman et al., 2005; Vasta et al., 2009; Buccioni et al., 2017a). It is reviewed that the approach of synthesizing CLAs involves changing biohydrogenation pathways by modifying CLA precursors in the diets (Dhiman et al., 2000). LA and ALA including supplementation of feeds or supplied fats, forages, vegetable oils, marine oils and animal lipids suggested to increase CLA concentration in milk (Glasser et al., 2008). However, LA and ALA could not be synthesized by ruminant's body (Nakamura and Nara, 2003). There are two possible suggestions by Chilliard et al. (2007) to achieve PUFAs, including CLAs, in ruminant-derived products are as follows: a shifted biohydrogenation pathway with regard to microorganism reductase and the origin of the diet in the feeding regimen, especially lipid supplementations as CLA precursors. Although a strategy in improving CLA fraction of FA in milk through dietary lipids of PUFA in animal diets, this activity suggested an initially exacerbated animal health due to lipid peroxidation in which occurs in an unbalanced animal metabolism for maintenance and production (Scollan et al., 2006). In practical, it could be inevitably conditions where animals must face with these deals to perform their productivity without leading to other problems, particularly in animal oxidative stress. Therefore, a tactical approach to achieve these regards should be adhered to supplementary feed addictive with a high accessibility for farmer and a great impact on environment.

Early lactation of dairy animals could be noted as a critical phase to obtain more oxidative stress and an achieving antioxidant by dietary antioxidant agent in diets should be considered to improve animal health (Celi, 2010). Studies in goats

revived that administering 100 mg/kg ascorbic acid or vitamin C intramuscularly, maintained live weight and decreased oxidative stress (Nwunuji et al., 2014). In numerous references, dietary plant polyphenols supplemented alone or combined with vitamin C or E in dairy animal diets had been documented to have potential double benefits, namely antioxidant and antimicrobial (Gobert et al., 2009; Schogor et al., 2013a; Jafari et al., 2018). It may be accepted because of plant polyphenols able to be degraded during substrate fermentation in rumen, then mobilized to all animal body by blood stream. According to McSweeney et al. (2001), a high molecular weight of polyphenols could be characteristic as tannins and the tannins could be degraded by microbial rumen on intermediate scheme, then transferred to duodenum metabolism or directly absorbed on rumen wall and ended to mammary gland carried by blood stream. Also, well-absorbed polyphenols in rumen have a positive effect to induce microbial rumen to degrade the available fermentable carbohydrates, resulting in escalated readily organic matter. In previous systematic review and meta-analysis by Purba et al. (2020) who observed the links between supplementary tannin levels and CLA formation in ruminants based on numerical data from 38 selected publications consisting of 3712 treatments. Tannins could modulate CLA either in milk and/or meat with imposing RBH tending to lower concentration of (SA; C18:0). These occurs were as consequence of tannin performance to have a positive interaction towards endogenously $\triangle^9$ desaturation. It could be noticed that a strong motion of tannin is antimicrobial than antioxidant itself. References in studying combination of polyphenols exhibiting antimicrobial and antioxidant in animal diets are scarce. Thus, looking for plant polyphenols for further studies in modulation between altered rumen

biohydrogenation and attenuated oxidative stress of dairy goats is definitely recommended.

Piper betle L. (Family: *Piperaceae*) is tropical plant in which a perennial dioecious and semi-woody climber (Figure 1.1). This plant is easy to grow with stems located at papillose and node. *Piper betle* L. leaves are edible and eligible matter for human and animal. These leaves had been reported owning a nutrient value for protein, fat, carbohydrate and mineral about of 3-3.5%, 0.4-1.0%, 0.5-6.10% and 2.3-3.3% of total nutrient as dry matter (DM), respectively (Guha, 2006). In addition, this leaves were reported containing vitamin and polyphenol ranged in descending order: ascorbic acid < phenolic acids (including tannins) < essential oils < flavonoids (Purba and Paengkoum, 2019). In other studies, a minor chemical constituent of *Piper betle* is essential oils (eugenol, safrole, allyl diacetoxy benzene, and chavibitol acetate) following by solid substrates as major chemical constituent, e.g. phenolic, alkaloids, steroids, tannins and flavonoids (Mohottalage et al., 2007; Alam et al., 2013). In numerous earlier publications, *Piper betle* L. had a well-documented health benefit namely antimicrobial (Dorman and Deans, 2000; Datta et al., 2011), anti-inflammatory (Alam et al., 2013; Das et al., 2016), antifungal (Trakranrungsie et al., 2008; Prakash et al., 2010; Basak and Guha, 2015), antioxidant (Dasgupta and De, 2004; Arambewela et al., 2006; Prakash et al., 2010; Alam et al., 2013; Das et al., 2016), antidiabetic (Arambewela et al., 2005), analgesic (Alam et al., 2013) and anthelmintic (Sujon et al., 2008; Amin et al., 2010; Baloyi et al., 2012; Ahmed et al., 2014). Ultimately, holding *Piper betle* L. leaves as alternative supplementary plant as polyphenols source for dairy animal diets may accurately be suitable way. In contrast, information on the effect of *Piper betle* L. leaves in crude extraction, essential oils and

phenolic compound non-oils on perspectives of rumen fermentation related to biohydrogenation, microbial fermentation and oxidative stress related to lipid peroxidation as reflection of antimicrobial and antioxidant objectives in dairy goats is fairly limited.

Figure 1.1 *Piper betle* L. planted in SUT organic farm.

1.2 Research objectives

1.2.1 To evaluate the influences of varied threshold of *Piper betle* L. forms; Piper powder (PP), Piper oil (PO) and Piper residue powder (PRP) on feed intake, nutrient digestion, end-product of rumen fermentation, biohydrogenation, microbial population related to biohydrogenation, milk production and animal performance in lactating goats.

1.2.2 To study the effect of the best levels of *Piper betle* L. forms on intracellular enzyme activity related to lipid peroxidation in the rumen fluid, blood,

mammary tissue and milk, and the mRNA abundance of oxidative stress-related genes in mammary tissue of dairy goats in early lactation.

1.3 Research hypotheses

1.3.1 PP, PO and PRP of *Piper betle* L. have a major behaviour as antimicrobial on microbial rumen resulting in an altered nutrient digestibility, a shifted biohydrogenation pathway and modulated animal performance.

1.3.2 Either PP, PO or PRP of *Piper betle* L. with an initial level have a well-documented health benefits suggesting to improve antioxidant value through ameliorating oxidative stress on early lactation in dairy goats.

1.4 Scope and limitation of research

In current study, the observations have been focused on phytochemicals derived from selected plant for modulating animal performances, including productivity in term of fat biosynthesis and metabolic-related animal in animal health purpose. Animal as target population of present study is pointed to the healthy Saanen dairy goat under early lactation. Further study regard of phytochemical's depositions in milk as rumen-derived product is wisely recommended.

1.5 Expected benefits

1.5.1 To provide a potent interest in utilization of local plant richer in phytochemicals or polyphenols such as phenolic compounds, essential oils and flavonoids which have the positive impacts on animal performance, animal health and environmentally-friendly using feed additive.

1.5.2 To address a objectives-first framework of recent results as references in commercial perspectives.

1.5.3 To present a beneficial outcome of current study for further observation in exerting molecular weight of phenolic compounds, essential oils and flavonoids for evaluating antimicrobial and antioxidant profiles in mammalian.

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CHAPTER II

LITERATURE REVIEWS

2.1 *Piper betle* Linn

Piper betle Linn is tropical plant and evergreen herb with a stem swollen at papillose before moving on to nodes in mature session. This plant is characterized as *Piperaceae* family and has ability to act a semi-woody climber. To maintain its growth, *Piper betle* L. needs warm and moist growth conditions (Datta et al., 2011). *Piper betle* L. has a strong pungent aromatic flavour. Since it has been cultivated in tropical areas, *Piper betle* L. has common name, namely common names are betel (English), paan (Indian), phlu (Thai) and sirih (Bahasa Indonesian). *Piper betle* L. leaves are edible and eligible matter coming along with a varied component that is depend on growth location. Leaves contained protein 3-3.5%, carbohydrate 0.5-6.10%, minerals 2.3-3.3%, and tannins 0.1-1.3%. It contained vitamin B, vitamin C, vitamin A calcium, phosphorus, iron, iodine and potassium (Prakash et al., 2010). Other studies reported that these leaves had been reported owning a nutrient value for protein, fat, carbohydrate and mineral about of 3-3.5%, 0.4-1.0%, 0.5-6.10% and 2.3-3.3% of total nutrient as dry matter (DM), respectively (Guha, 2006).

In addition, this leaves were reported containing vitamin and polyphenols ranged in descending order: ascorbic acid < phenolic acids (including tannins) < essential oils < flavonoids (Purba and Paengkoum, 2019). Also, a minor chemical constituent of *Piper betle* is essential oils (safrole, allyl diacetoxy benzene, and

chavibitol acetate) following by solid substrates as major chemical such as phenolic, alkaloids, steroids, tannins and flavonoids (Mohottalage et al., 2007; Alam et al., 2013). In several previous references, *Piper betle* had a well-documented health benefit namely antimicrobial (Dorman and Deans, 2000; Datta et al., 2011), anti-inflammatory (Alam et al., 2013; Das et al., 2016), antifungal (Trakranrungsie et al., 2008; Prakash et al., 2010; Basak and Guha, 2015), antioxidant (Dasgupta and De, 2004; Arambewela et al., 2006; Prakash et al., 2010; Alam et al., 2013; Das et al., 2016), antidiabetic (Arambewela et al., 2005), analgesic (Alam et al., 2013) and anthelmintic (Sujon et al., 2008; Amin et al., 2010; Baloyi et al., 2012; Ahmed et al., 2014). Therefore, natural polyphenols derived from *Piper betle* L. may give a varied mode, where phenolics, essential oils and flavonoids working on their sole or shared way.

2.2 An overview of natural polyphenols: origin biosynthesis pathways, bioavailability and metabolism

Phenolic compounds, those are commonly mentioned as polyphenols, a complex natural micronutrient is broadly disseminated throughout the plant kingdom and is rife in vegetables, beverages and plant-based foods (Zhang et al., 2014; Purba and Paengkoum, 2019). Polyphenols is secondary metabolites that synthesized in plant by transforming their sugar carbon, especially pentose phosphate to specific compounds namely phenolic acids and essential oils via Shikimate and Phenylpropanoid pathways, and flavonoids through flavonoids pathway (Figure 2.1). In general, polyphenols have been classified into flavonoid and non-flavonoid forms. During biosynthesis, 3-carbon bridge of flavonoids usually forms an oxygenated

heterocycle, producing a 3-ring structure formed of 15 carbons. Regard of the degree of oxidation of the heterocycle structure, the type of sugar residue and the degree of polymerization, flavonoids can be divided into flavanones, dihydroflavonols, flavonols, leucoanthocyanins and proanthocyanidins (Kalinowska et al., 2014). The polymerization of trans or cis derived from flavan-3-ol leads to the formation of proanthocyanidins or commonly known as condensed tannins (CT). The degree of polymerization of CT is relatively big molecular weight which is approximately 30,000 Da and this CT favoured to poor soluble.

Likewise, non-flavonoid form are phenolic acids, vanillin, eugenols and gallic acids (HT: hydrolysable tannins), are synthesized through intermediates of shikimate and phenylpropanoid pathways. Gallic acid derivatives, place forming for HT, available pentose phosphate synthesized by DHD/SDS to dehydroshikimic acid and protocatechic acid during shikimate metabolic, whereas phenolic acids form eugenols by transformation of *P*-coumaroyl coA and/or feruloyl coA associated with phenylpropanoid enzymes into the coniferyl acetate (Amano et al., 2018). Each plant has a varied possibly intermediating product and these facts of synthesizing polyphenols in plant suggested a correspond of defence responses in plant (Lin et al., 2016). For example, the plants perform massively substances to perform an attractive pollination, inducing camouflage by colouring performance and resist attack against herbivores as predator

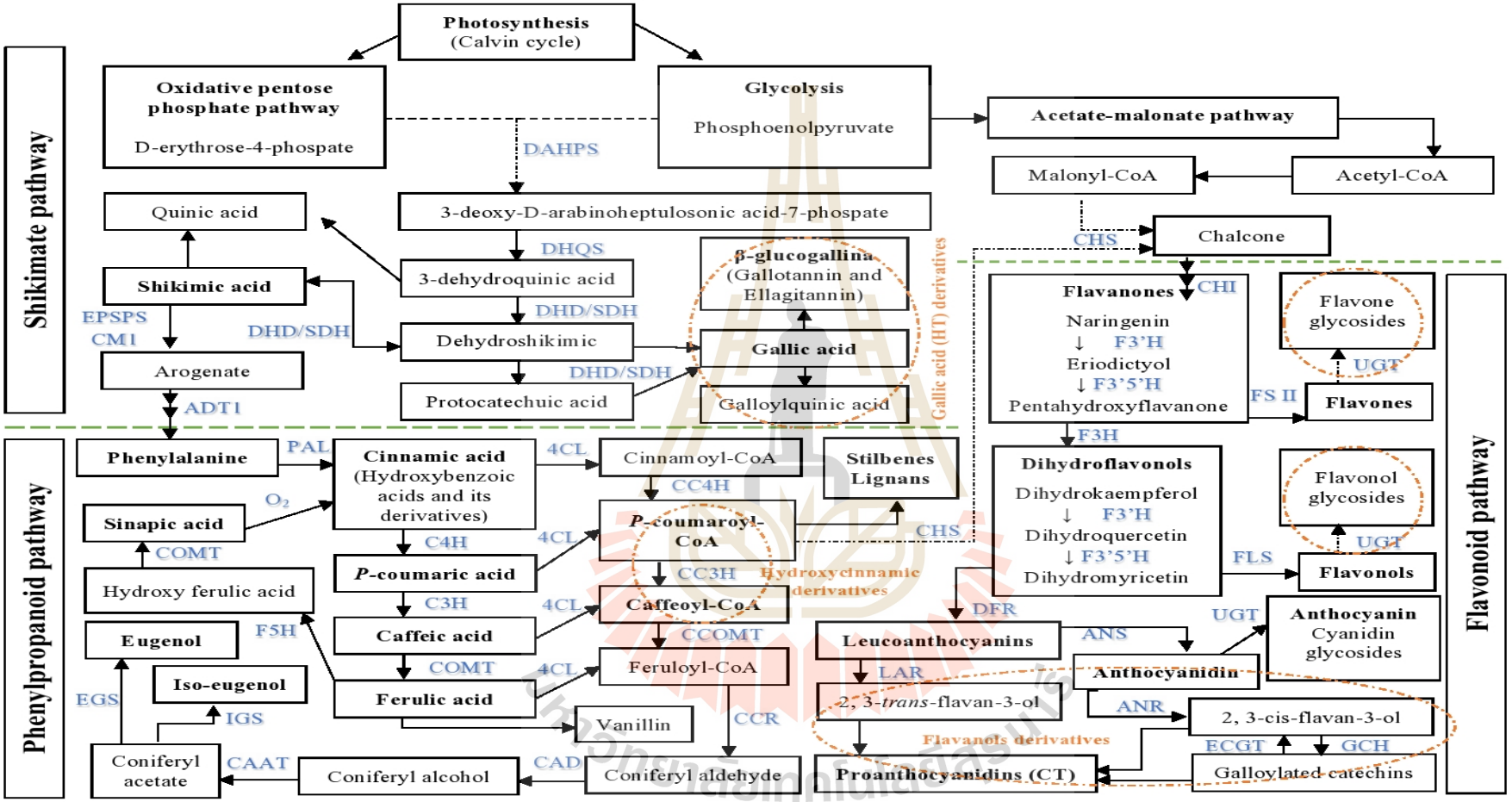

Figure 2.1 *Piper betle L.* biosynthesis pathways (Jiang et al., 2013; Rastogi et al., 2013; Kalinowska et al., 2014; Amano et al., 2018; Srinivasulu et al., 2018; Purba and Paengkoum, 2019). Biosynthesis enzymes, e.g. 3-deoxy-D-arabino-heptulosonate-

7-phosphate synthase (DAHPS), 3-dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHD), shikimate dehydrogenase (SDH), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate mutase 1 (CM1), arogenate dehydratase (ADT), phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), caffeoyl O-methyl transferase (COMT), coniferyl alcohol 5-hydroxylase (F5H), cinnamoyl-CoA 4-hydroxylase (CC4H), p-coumaroyl-CoA 3-hydroxylase (CC3H), caffeoyl-CoA O-methyl transferase (CCOMT), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), coniferyl alcohol acetyl transferase (CAAT), isoeugenol synthase (IGS), eugenol synthase (EGS), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3’H), flavonoid 3′, 5′-hydroxylase (F3’5’H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), uridine diphosphate Glycosyltransferase (UGT), galloylated catechins hydrolase (GCH) and epicatechins 1-O-galloyl-β-D-glucose O-galloyltransferase (ECGT).

Notably, there is potent interest to notice that polyphenols are inevitably content in animal diet in grazing system (Średnicka-Tober et al., 2016). However, polyphenols may be less active within the body due to polyphenols seeming to have a lower intrinsic activity, poorly absorbed from the intestine and highly metabolized or rapidly eliminated (Manach et al., 2004). The native substances in biological activity of polyphenols may differ animal metabolites because of chemical structure and sugar binding on their absorption and bioavailability (Lewandowska et al., 2013). It is reported that polyphenols could shift in simple phenolics (e.g., ellagic and gallic acids), oligomeric compounds (e.g., anthocyanins, eugenols, flavonoids and lignans), and polymeric compounds with high molecular weight (e.g., CT; Kalinowska et al. (2014)), and has ability to perform conjugation using available substrates of glycosides, organic acids and lipids.

In previous evidences, type of polyphenols is reported to have a slightly different mechanism whether in human or in ruminal putative pathways (Figure 2.2), as adapted from human (He and Giusti, 2010; Marín et al., 2015). After consumption of polyphenol-containing feeds, ruminants obtaining polyphenols by performing rumination includes saliva to break feed matrix. First polyphenol absorption could initiate in rumen after transformation and degradation by microorganisms and hydrolysation of the glycoside and cleavage of the heterocyclic ring (McSweeney et al., 2001). It is noteworthy that varied possibly bioavailability may occur during ruminal fermentation. Flavonoids and essential oils (especially eugenol as ferulic derivatives) had been suggested to favour more hydrophobic motion in rumen, leading to less bioavailability (Biasutto et al., 2010). It is clear that the small intestine is main place to absorb polyphenols for second times (He and Giusti, 2010). In this stage,

endogenic β-glucosidases are induced to release aglycones from extensively flavonoid-glu and to limit the extent of flavonoid -gal, -xyl, and -ara. The process is relatively slow and free aglycones could penetrate and osmosis into the epithelial layer passively. A possibly reason of this fact suggests to molecular weight of free aglycones that are smaller size than the glycosides. In contrast, intact glycosides are also absorbed by the small intestine (He and Giusti, 2010), either through passive diffusion or by the sodium-dependent glucose transporter (SGLT1).

Unabsorbed polyphenols during second stage forwarded to the colon, where microorganism colon supports reabsorption by providing catalytic and hydrolytic potential (Scheline, 1973). Colonic microflora then cleaved glycosidic and ester bonds (Bokkenheuser et al., 1987). The readily aglycones in this stage, thereby undergo spontaneous ring fission transferred into liver by portal vein. In liver, absorption derived from rumen, small intestine and colon is suggested to follow the liver II metabolism (methylation, glucuronidation and sulfation), where absorbed polyphenols are assigned to become conjugated and transported to the bloodstream again until they are secreted in urine. Some of the liver conjugates are then excreted as bile components back into the intestine (enterohepatic recirculation) and deconjugated compounds are regenerated by gut microbial enzymes before being reabsorbed again (Rechner et al., 2004; Aura, 2008; Cardona et al., 2013). In term of dairy ruminant, hydroxyl moieties are mobilized into mammary gland and their incorporated tissue and synthesised in milk (Tian et al., 2019). During intermediate of transit or movement of hydroxyl moieties diverse tissues and organs, the bioactive compounds of polyphenols seemed to perform their modulation-promoting effects, particularly antimicrobial, anti-inflammatory and antioxidant (He and Giusti, 2010).

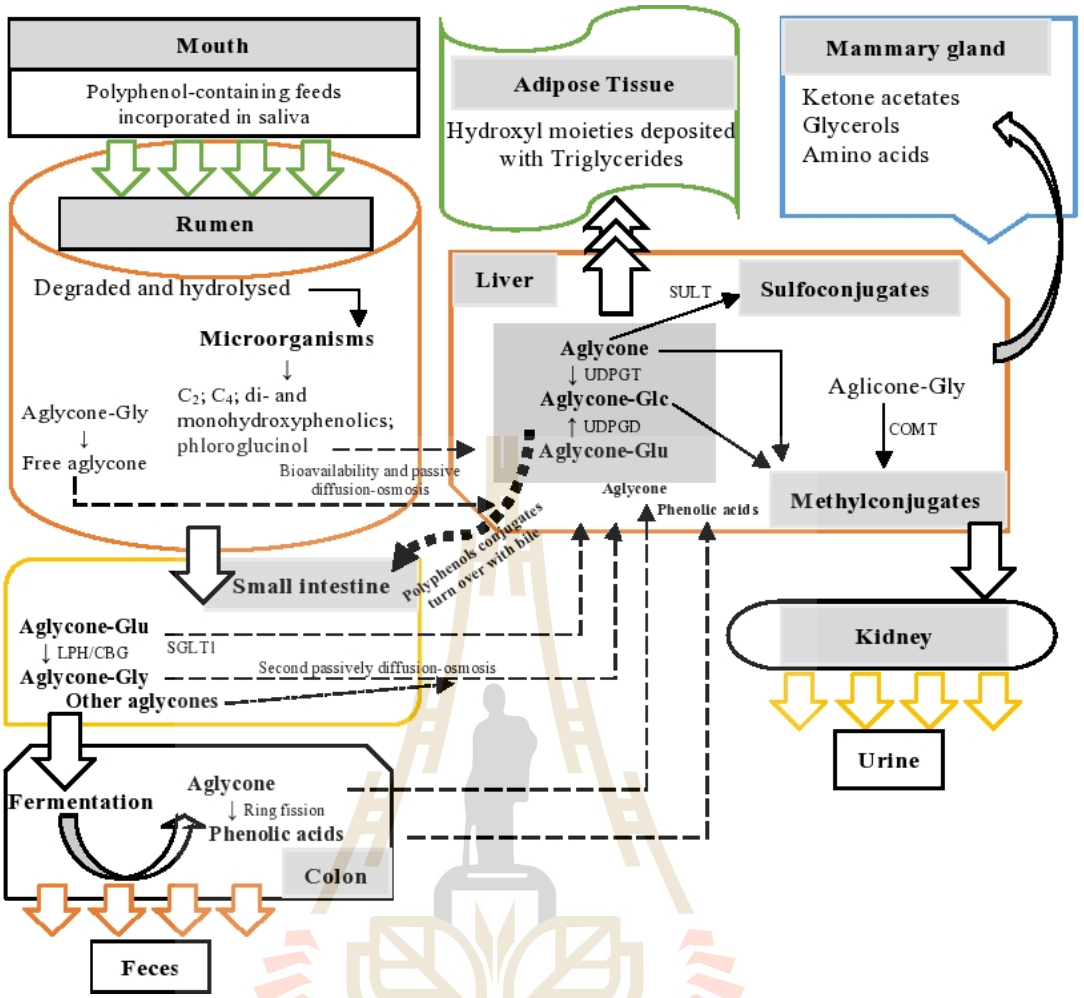

Figure 2.2 Putative pathway of dietary polyphenols on absorption, metabolism, distribution, and excretion in ruminant, adapted from human pathway (He and Giusti, 2010; Marín et al., 2015).

2.3 Effects of dietary polyphenols incorporated into dairy ruminant diets

Numerous studies are available in the literature regarding the effects of polyphenols on ruminant nutrition. In particular, tannins have anti-bloat properties. In particular, polyphenols have antimicrobial properties (Patra and Saxena, 2009), inhibit

protozoal mobilisation (Patra, 2010), attenuate methane production (Calsamiglia et al., 2007; Benchaar et al., 2008; Jayanegara et al., 2012b), enhance nitrogen utilization (Wina et al., 2005), alter intermediate of ruminal biohydrogenation relate to fatty acid (FA) component (Cabiddu et al., 2010; Jayanegara et al., 2012a; Rana et al., 2012; Vasta et al., 2019), and consequently, contribute to improve the antioxidant profile in body requirement and rumen-derived product, especially in milk content (Schogor et al., 2013b; Tian et al., 2018; Tian et al., 2019). Polyphenols are one of secondary compounds with variable phenolic structures and promote good flavour. However, preserving polyphenols in which richer in essential oils in diet might provide a slightly sour-bitter taste leading to an affinity in eating rate and feed intake (Patra et al., 2019). Although, it is reported that dietary feed intake of dairy animals was inconsistent (Frutos et al., 2004b; De Nardi et al., 2014; Ma et al., 2017). It seemed likely that animals could adapt in manipulated rations due to formulation of basal feed, e.g. dietary lipids (Dewhurst et al., 2006)

Moreover, in term of milk production, contrasting results are reported in the literature. In most cases, unvaried effects have been reported, irrespective of the kind of polyphenols and the use of pure extracts or ago-industry by-products in both dairy animals (Elgersma et al., 2006; Dschaak et al., 2011). In fact, dietary polyphenols with abundance of nutrient in protein and fat inclusion may shift a milk composition. For instance, Winkler et al. (2015) reported a higher content of lactose in milk from dairy cows received a plant product consisting of green tea and curcuma extract (GTCEx) compared with initial control diet on early lactation stage. However, the content of fat, protein and casein was not affected by dietary GTCEx. In addition, a greater content of fat and protein may find in general studies of polyphenols inclusion

in the diets tending to express a higher efficiency of digestibility rate in the rumen (Berger et al., 2015), then modulating various hydrolysing and conjugating enzymes in small intestine as primary substates (Abbas et al., 2017)

2.4 Microbial rumen during substrate fermentation

Ruminants accept a raw natural resource to face their nutrient requirement. They need feedstuff with high fibre and their rumen cultivates slowly to breakdown feedstuff through their gut especially passage of fibre, which involves enable microbial enzyme time to digest the constituent digestion resistant polymers, mainly cellulose and xylene (Hungate, 1969; Bodas et al., 2008; Nkosi et al., 2016). According to van de Vossenberg and Joblin (2003), mammalian enzymes cannot break down cellulose or xylene. The predominantly reason could explain the rumen microbes having responsibility to ferment the released sugars transforming to release ATP in which as energy to their growth. This occurs need required a suitable condition where rumen condition preferring to more anaerobic, therefore supporting to substrate fermentation. In this anaerobic fermentation, the major result of generating ATP on metabolic pathways (Figure 2.3). During fermentation, rumen inhabitant degrades a substrate through anaerobic stage accompanied by hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) production as result of cellulose and hexose hydrolysis (Wolin, 1979). CH_4 is commonly now well recognized and detected as a greenhouse gas, a significant contributor to global warming.

Moreover, other essential substance of this side for microbial growth is nitrogen that mainly in the form of protein derives from bulk or crude of the diet (Patra and Saxena, 2011). Hence, proteolysis and amino acid transport are invited into

these schematics supporting significantly to microbial growth. In fact, microbial proteolysis impacts to lesser energy absorption because its generally exceeds the capability of the microbes to utilize the hydrolysis products (Walker et al., 2005; Wina et al., 2005). Consequently, the metabolism involving the excessive catabolism should be balance, where energy requirement should be fully supported to avoid metabolism misleading. This occur could lead to one of the major nutritional losses like N excretion from animal agriculture to unfriendly environment (Hristov et al., 2015).

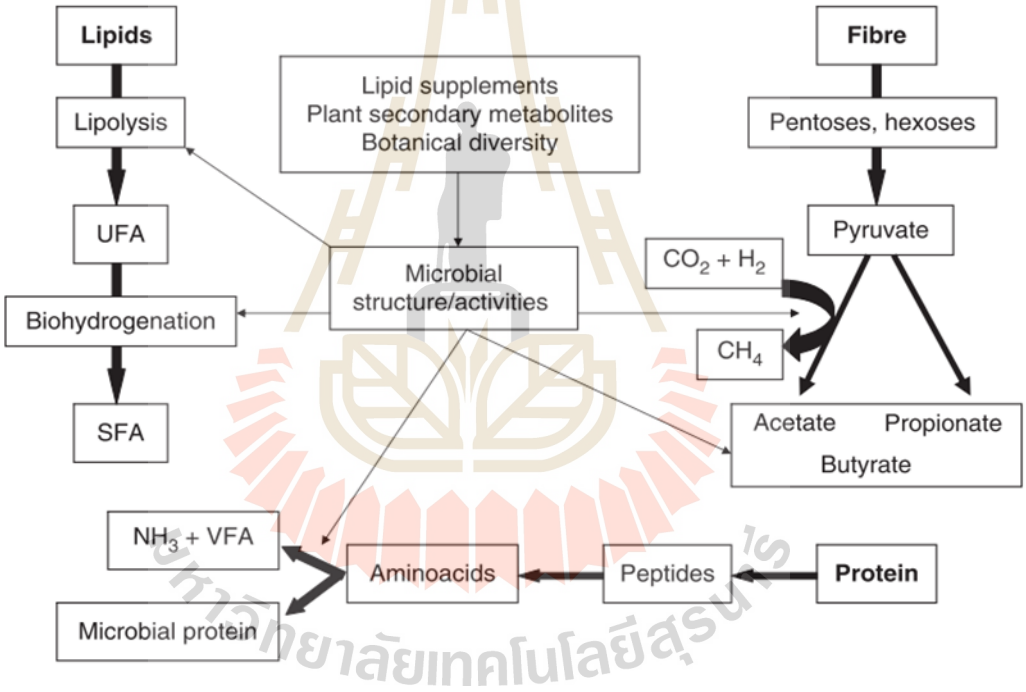

Figure 2.3 Interventions to manipulate lipid metabolism in the rumen inevitably lead to effects on other processes. Sometimes the target organisms have several functions, in other cases the metabolic pathways are linked, for example by the availability of H_2. UFA=unsaturated fatty acid; SFA=saturated fatty acid; VFA=volatile Fatty Acids (Lourenço et al., 2010).

In contrast, the metabolism of dietary lipid is opposite assessment. Dietary fat intake is considered as prior requirement to build fat metabolism. Also, it seems to have an important role as nutrient source to ruminal microorganisms. The microorganisms can synthesize their own FA and indeed do so extensively in the mixed community (Boguhn et al., 2008). They also cannot get energy from β-oxidation, which does not occur anaerobically. Hence, lipid metabolism scheme may be lesser important of microbial rumen for growth-generating activities of rumen microbial community issue. However, it is extremely valuable for 'microorganisms' aspect to survive against a toxic challenge (Macheboeuf et al., 2008), where it is basic in influencing the nutritional quality of ruminant products (Chilliard et al., 2007).

Literally, the main members of the microbial community are bacteria, archaea, protozoa and fungi. Bacteria are the most abundant, followed by archaea (the CH_4 producers) (Buddle et al., 2011; Patra, 2012; Patra and Yu, 2013), ciliate protozoa (Kamra et al., 2006; Lourenço et al., 2010; Santra et al., 2012) and in lower numbers the anaerobic fungi (Zhang et al., 2008; Mao et al., 2010; Zhou et al., 2011). Precisely, different species have different roles which associate with rumen microbial ecosystem and its collective activity. For example, the gram-negative bacterium, *Butyrivibrio fibrisolvens* is a key player in fibre digestion but many strains are also highly proteolytic (Wallace et al., 2006; Yang et al., 2009). It also presents and leads dominating its peer of the biohydrogenation of FA. Thus, targeting one microbial activity for manipulation always has consequences for others (Lourenço et al., 2010; Song et al., 2010). Likewise, cellulolytic is particularly vulnerable to disruption, for illustration in previous references (Maia et al., 2007; Weimer et al., 2010). Utterly, an attempt to block one aspect of ruminal activity should be guided by wisely objective

in monitoring other effects. The secondary effect may not necessarily be detrimental (Makkar, 2003).

2.5 Ruminal biohydrogenation and fundamental transport of milk

The major FA substrate for biohydrogenation in grazing animals is linolenic acid (LNA=cis-9, cis-12, cis-15-18:3), because it is the most abundant FA present in glycolipids and phospholipids of grass and other forages, whereas for animals accepting dietary lipid supplements, linoleic acid (LA=cis-9, cis-12-18:2) in the form of triacylglycerols will usually be the main substrate for biohydrogenation (Lourenço et al., 2010). LA metabolism in the rumen involves the transient formation of CLA, mainly cis-9, trans-11-18:2 or rumenic acid, which is then converted to VA, and finally stearic acid (18:0**;** Figure 2.4). LNA is metabolized in a similar way**,** though as there are two double bonds to be reduced the pathway is slightly more complicated (Jenkins et al., 2008). Coming along with the main 18:3 up intermediate from isomerization of LNA in combining ruminal digesta has described by Wąsowska et al. (2006) and Boeckaert et al. (2007), one of the possible considerably conformation suggested by Lourenço et al. (2008). Other intermediates identified included trans-9, trans-11, cis-15-18:3 and trans-11, cis-15-18:2. Conjugated trienes may have just as important health implications as CLA (Tsuzuki et al., 2004) although much less work has been done on the trienoic than the dienoic fatty C-18 FA

Rumenic acid or cis-9, trans-11 18:2 could be assessed as the predominant CLA isomer. Its form is determined in rumen (Shingfield and Griinari, 2007) and milk component (Shingfield et al., 2006; Chilliard et al., 2007; Jenkins et al., 2008). However, the trans-10, cis-12 isomer may become a major intermediate, when

Rumenic acid content exceedingly in rumen and milk cause to detrimental effect for animal (Lourenço et al., 2010). Numerous studies have conducted in supplementation of highly starch and fish/vegetable oil containing feedstuff (Glasser et al., 2008; Song et al., 2010; Schmidely and Andrade, 2011; Ferreira et al., 2014; Szczechowiak et al., 2016). Also, high trans-10 18:1 concentrations presenting in digesta and consequently in the FA flowing to animal tissues (Daniel et al., 2004; Shingfield and Griinari, 2007) lead to milk fat depression occurs coming along with other consequences to the animal such as lower intake and decreased fibre digestion (Bauman and Griinari, 2001). For illustrating, post-ruminal infusion experiments first indicated that trans-10, cis-12 18:2 exerts anti-lipogenic effects in the lactating cow (Baumgard et al., 2000). Recent studies suggest that it may actually be trans-10 18:1 rather than the trans-10, cis-12 CLA that decreases mammary lipogenesis (Shingfield et al., 2006).

On FA biohydrogenation, bacteria play the main role (Jenkins et al., 2008; Boeckaert et al., 2009). In early microbiological studies (Polan et al., 1964), was identified *Butyrivibrio fibrisolvens* as undertaker for FA biohydrogenation and was creating CLA and VA form as intermediates during the biohydrogenation of LA. An old observation addressed that stearic acid was not formed from LA. Bacteria carrying out stearate formation were identified as *Fusocillus spp* (Wallace et al., 2006). Subsequently, a species named *Clostridium proteoclasticum* was also identified as a stearate producer with morphological and metabolic properties that were indistinguishable from those reported for *Fusocillus* (Wallace et al., 2006; Wallace et al., 2007). van de Vossenberg and Joblin (2003) and Moon et al. (2008) had isolated, identified and renamed prior stearic producer (*Clostridium proteoclasticum*) to be *Butyrivibrio proteoclasticus*, as consideration using its 16S rRNA gene sequence.

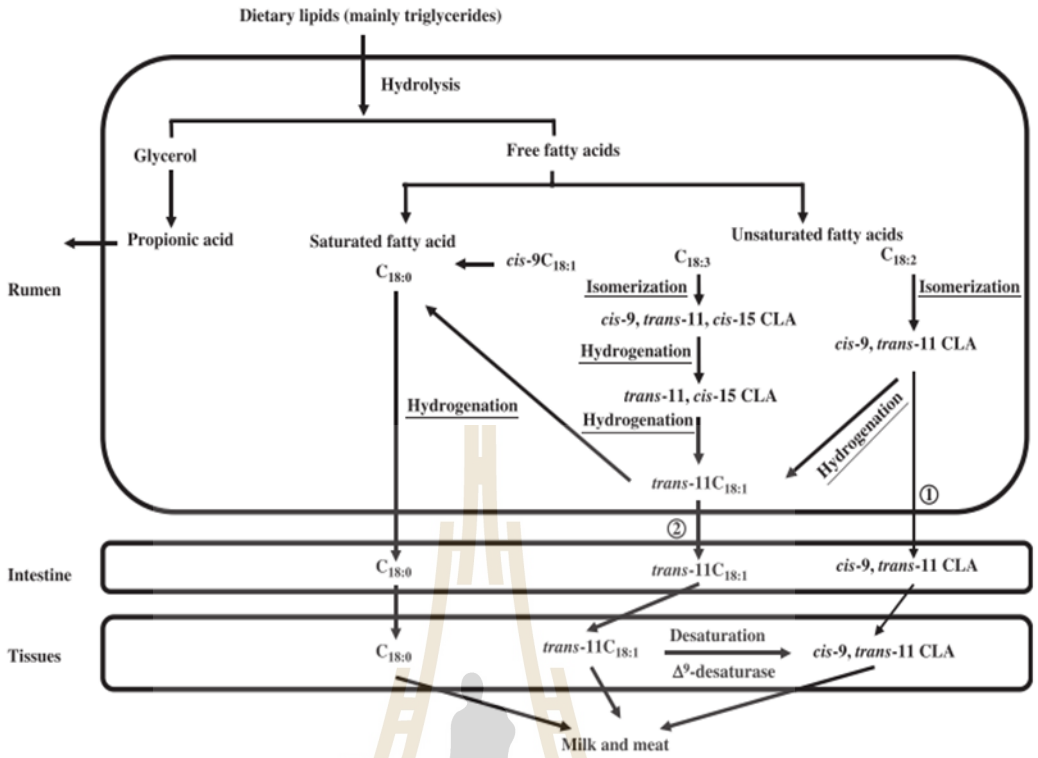

Figure 2.4 Lipid metabolism in the rumen and the origins of conjugated linoleic acid in ruminant products (Tanaka, 2005).

In stand point, the bacteria could vary steps of biohydrogenation process and those could classified as group A (*Butirivibrio fibrisolvens*) and group B (*Butiryvibrio proteoclasticus*) (Lourenço et al., 2008; Lourenço et al., 2010). Group A bacteria hydrogenated LA and LNA to VA, whereas group B bacteria convert the same FA to stearic acid. To straightforward regard of CLA production and milk depression, metabolism of LA by Butyrivibrio group declares in the formation of cis-9, trans-11 CLA and VA but no trans-10, cis-12 CLA or trans10-18:1 is formed (McKain et al., 2010). This group has responsible for milk depression occur, therefore, other aspects likely different enzymic mechanism of cis-9, trans11 associating to produce this deterioration (Wallace et al., 2007). Besides, rich starch fraction organizing large cocci

identified as *Megasphaera elsdenii* had observed by Kim et al. (2002) tending to milk stress depression accumulation to associate with firstly inflammation. Thus, considering milk depression occurs while CLA production is paid more attention on shifting CLA in rumen-derived products.

In general, most metabolic processes in the rumen are affected by others because of common metabolic pathways or common microbial species. The common factor of H_2 metabolism is an important feature of these interdependencies. H_2 is produced by the fermentation of sugars and used in a number of processes as well as biohydrogenation (Lourenço et al., 2010). Interspecies H_2 transfer is vital to maintain ruminal fermentation of dietary nutrients because an accumulation of H_2 can inhibit the activity of cellulolytic bacteria (Latham and Wolin, 1977). Methane and propionate are the two largest sinks for ruminal H_2. When methanogenesis falls, propionate proportions increase, as bacteria such as *Selenomonas ruminantium* alter their metabolism to dispose of H_2 (Latham and Wolin, 1977). Biohydrogenation was originally proposed to be an alternative H_2 sink to methanogenesis or propionigenesis. However, Greening et al. (2019) concluded that H_2 sinker metabolism is complex. Low H_2 in fermentation may be due to other bacteria, such *Blautia*. Amino acid deamination is another aspect of ruminal metabolism that depends on H_2 metabolism, because the disposal of reducing equivalents and the NADH/NAD ratio are important effectors of branched-chain amino acid fermentation (Hino and Russell, 1985). Thus, methanogenesis, propionigenesis, amino acid metabolism and biohydrogenation are all linked metabolically.

FA metabolism in animal tissues, particularly the mammary gland, has also been targeted as a means of altering FA composition in ruminant products. While

ruminal production of cis-9, trans-11 CLA contributes to milk fat CLA that endogenous synthesis of cis-9, trans-11 CLA also makes a significant contribution (Griinari et al., 2000). Endogenous synthesis of cis-9, trans-11 CLA involves the enzyme $\triangle^9$-desaturase and the substrate are trans-11 C18:1, another intermediate formed in the ruminal biohydrogenation of polyunsaturated C18-fatty acids. In order to quantify the relative contribution of endogenous synthesis, the present study supplied trans-11 C18:1 as exogenous substrate for desaturation to cis-9, trans-11 CLA as well as cyclopropane fatty acids to inhibit $\triangle^9$-desaturase. It seems likely that CLA is produced mainly in endogenously $\triangle^9$-desaturasion and this step could be included on shifting CLA formation in rumen-derived product, such as in milk.

According to Shennan and Peaker (2000), five routes of milk secretion across the mammary secretory epithelium from the blood side to milk, four transcellular and one paracellular (Figure 2.5). The figure describes sites, as 1) membrane route, 2) Golgi route, 3) milk fat route, 4) transcytosis and 5) paracellular route. In the membrane route, substances may traverse the apical cell membrane (and for those directly derived from blood, the basolateral membrane). Water, urea, glucose, Na^+,K^+, and Cl^- is traversed in this step. In the Golgi route, secretory products are transported to or sequestered by the Golgi apparatus and secreted into the milk space by exocytosis. Milk constituent includes casein, whey proteins, lactose, citrate, and calcium. In the milk fat route, milk fat globules are extruded from the apex of the secretory cell surrounded by membrane (milk-fat-globule membrane). Among cytoplasm are sometimes involved in this route. Examples are milk fat, lipid soluble material and leptin explained to be involved in this route. In transcytosis, vesicular transport included several organelles. This route may connect to some cases with route

2. Examples are immunoglobulins during colostrum formation, transferrin, prolactin and material which be produced during peripartum. Lastly, in the paracellular route, there is direct passage from interstitial fluid to milk. In full lactation, routes 1, 2, 3, and, possibly, 4 predominantly work.

2.6 Ruminal oxidation

As a crucial time during which dairy animal such dairy cows are highly susceptible to oxidative stress (Sharma et al., 2011). The period is characterized by high metabolic demand and physiological adjustments to the onset of lactation. Abuelo et al. (2015) recently provided a review of the *in vivo* benefits of dietary antioxidants on udder health, uterine health and reproductive performance, and incidence of production diseases of periparturient cows. Insufficient dietary antioxidants during this period were suggested to possibly increase oxidative stress and occurrence of retained placenta in dairy cows (Brzezinska-Slebodzinska et al., 1994). However, supplementing transition cows and periparturient heifers with vitamin E resulted in improved signs of oxidative status with regards to higher serum α-tocopherol level, decreased lipid peroxidation and reduced oxidative damage in liver (Brzezinska-Slebodzinska et al., 1994; Bouwstra et al., 2008; Bouwstra, 2010). In addition, a meta-analysis of 19 experiments suggested that dietary addition of vitamin E and Se could decrease the average relative risk of mastitis by 34% (Zeiler et al., 2010). However, individual supplementation of Se was more potent in reducing the risk of mastitis compared with the individual supplementation of vitamin E (40% v. 30%). Moreover, dietary addition of vitamin E and Se apparently increased milk yield with mean of 1 kg milk/animal per day and this effect was greater for vitamin E than

Se (Zeiler et al., 2010). Consequently, activity in supplementing dairy animal in peripartum period by offering antioxidant source is remarkable strategic.

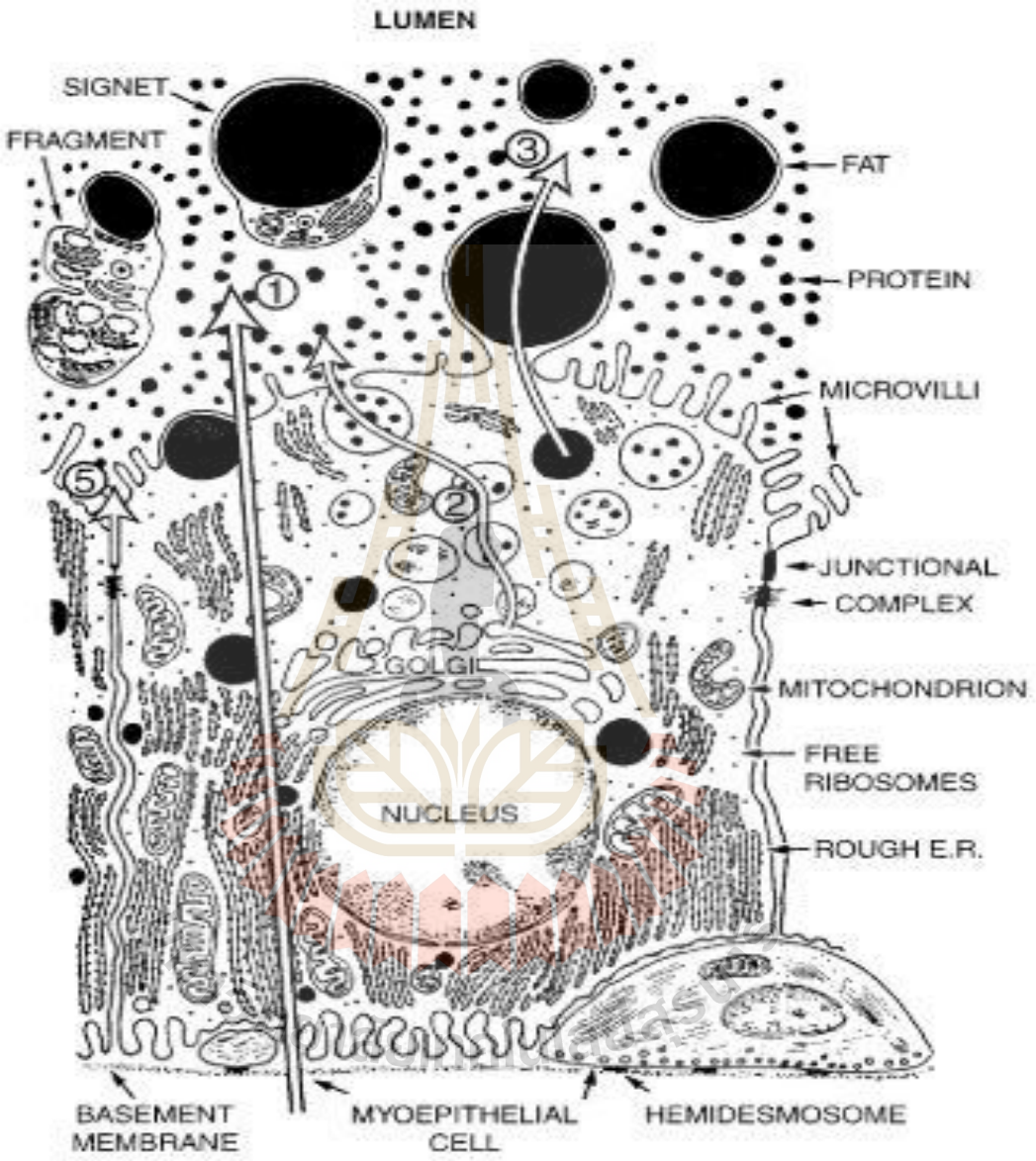

Figure 2.5 Five major routes of secretion across the mammary epithelium.

A succinct detail is provided in this review. The mechanisms of antioxidant protection in the biological system of animals have been extensively reviewed by several authors (Brand-Williams et al., 1997; Heim et al., 2002; Lykkesfeldt and

Svendsen, 2007; Sordillo and Aitken, 2009; Zhong and Zhou, 2013). In reality, the animal is naturally endowed with an overwhelming biological antioxidant system to combat the free radicals that are continuously produced as a result of several metabolic activities in the body. Free radicals include reactive oxygen species and reactive nitrogen species such as superoxide anion, hydroxyl radical and hydrogen peroxide (Kalam et al., 2012). However, there is a certain limit to the protection that could be offered by the endogenous antioxidant barrier. This limit is further compromised by the presence of factors that could trigger excessive production of free radicals and/or weaken the efficiency of the biological antioxidant system, thereby causing oxidative stress. Such factors include consumption of high-PUFA (first objective) or rancid diet, nutritional deficiency, pathogenic infections, stress-related practices.

Free radicals are unstable and highly reactive chemical species with an unpaired electron which induces them to trap electron from biological macromolecules such as DNA, lipids and proteins, in order to neutralize themselves. The reaction of free radicals with biological molecules results in oxidative damage of such macromolecules and potential cellular damage. In a counter protective response, antioxidants act by either directly scavenging the free radicals or stabilizing the free radicals by donating the electron required (Figure 2.6). The biological antioxidant system consists of both the enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), etc.) and non-enzymatic (selenium, vitamins E, C and A, etc.) components (Table 2.1). In essence, oxidative stress is the deteriorative condition, which results from the imbalance between the endogenous generation of free radicals and the biological antioxidant defence systems in the body (Rathee et al.,

2009). In situations of excess free radical production, there is a keen need for exogenous intake of antioxidants to prevent potential cellular damage.

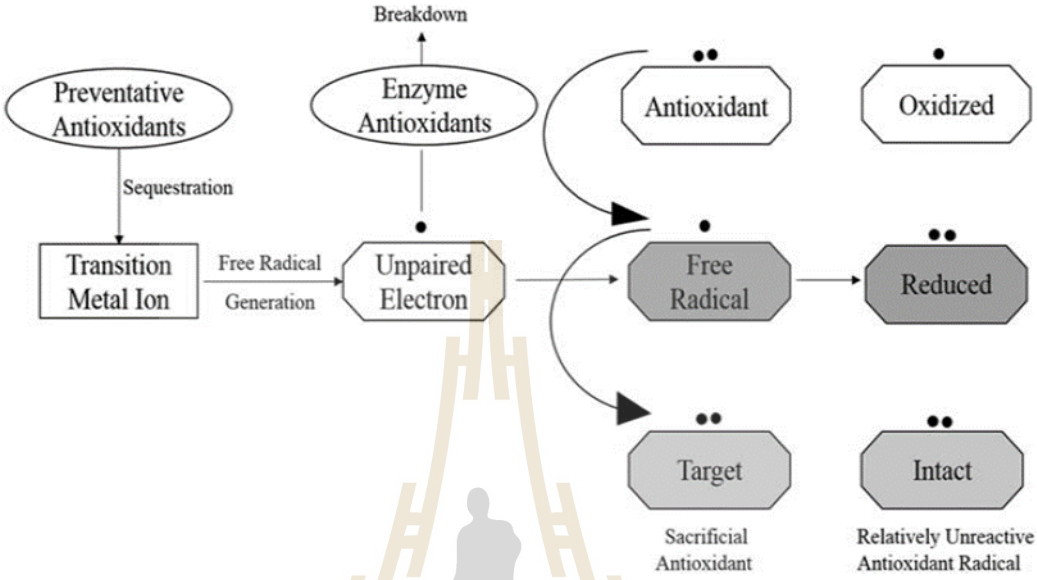

Figure 2.6 Mechanism of action of antioxidants adapted from (Kalam et al., 2012).

Based on the nature of antioxidants, they can be grouped into water-soluble (e.g. ascorbic acid) and lipid-soluble (e.g. vitamin E and carotenoids) antioxidants. The former and the latter are located in the hydrophilic and lipophilic compartments of the cell, respectively (Yeum et al., 2004). There are emerging indications that redox cooperation exists between these two groups of cellular antioxidants, which accumulate to antioxidant synergism. Example of such redox cooperation is the ability of terminal hydrophilic ascorbic acid to repair oxidized tocopheroxyl radical of vitamin E in order to allow vitamin E perform its antioxidant function again (Buettner, 1993). Similarly, Iglesias et al. (2009) demonstrated that exogenous phenolic compound, grape procyanidins, had the ability to repair oxidized α-tocopherol and delay the depletion of ascorbic acid in the muscle tissues of fish. Thus, this highlights

the importance of supplementing livestock with both groups of antioxidants to enhance duality of action which has proven to have synergistic effects.

Table 2.1 Antioxidants in the biological system of animals (Salami et al., 2016).

Component (Cell location)	**Nutrient involved**	**Function**
Superoxide dismutase (cytosol)	Cu and Zn	An enzyme that converts superoxide to hydrogen peroxide
Superoxide dismutase (mitochondria)	Mn and Zn	An enzyme that converts superoxide to hydrogen peroxide
Ceruloplasmin	Cu	An antioxidant protein, may prevent copper from participating in oxidation reactions
Glutathione peroxidase (cytosol)	Se	An enzyme that converts hydrogen peroxide to water
Catalase (cytosol)	Fe	An enzyme (primarily in liver) that converts hydrogen peroxide to water
α-Tocopherol (membranes)	Vitamin E	Breaks fatty acid peroxidation chain reactions
β-Carotene (membranes)	β-Carotene	Prevents initiation of fatty acid peroxidation chain reactions

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CHAPTER III

A HPLC METHOD OF *PIPER BETLE* L. FOR QUANTIFYING PHENOLIC COMPOUND, WATER-SOLUBLE VITAMIN AND ESSENTIAL OIL IN FIVE DIFFERENT SOLVENT EXTRACTS

3.1 Abstract

A reversed-phase HPLC-DAD was developed and validated to estimate the phenolic acids (gallic acids, caffeic acids, syringic acids, *p*-coumaric acids, sinapic acids, and ferulic acids), flavonoids (catechins, rutins, myricetins, quercetins, apigenins, and kaempferols), ascorbic acids, and eugenols. The chromatogram condition was set in suitable wavelengths 272 nm and run flow rate 0.7 µl/min using HPLC Agilent Technologies 1260 Infinity, a reversed-phase Zorbax SB-C18 column (3.5 µm particle size, i.d. 4.6 x 250 mm) with mobile phase solution (1:9, HPLC-grade acetonitrile:1% acetic acid). The linearity, precision, LOD, LOQ, and accuracy were R^2>0.9907, RSD <1%, 0.005 µg/ml, 0.015 µg/ml, and 96-102%, respectively. As a result, all selective compounds were successfully separated, identified, and quantified. The enormous contents were found in quercetin and eugenol, expressing crude content (mean, 5.989 mg/g) and residue content (mean, 1.934 mg/g) for quercetin, while crude content (mean, 3.209 mg/g) and residue content (mean, 0.184 mg/g) for eugenol.

Consequently, this method could be applied, repeated, and developed for later observation, especially in commercially inclination of *Piper betle* analysis.

Keywords: Phenolic compounds, flavonoids, essential oil, *Piper betle* L., gradient HPLC, different solvent extracts

3.2 Introduction

Phenolic compounds or commonly as polyphenols had been considered as rife organic compound characteristic, ubiquitously in plants, fruits, and vegetables. These are secondary metabolites that contain simple phenolic molecules and benzene rings through shikimic and pentose phosphate pathway. Lin et al. (2016) reviewed polyphenols have a host of medicine values such as anti-inflammatory, antioxidant, antidiabetic, and anticarcinogenic, representing action by its members: simple flavonoid, phenolic acid, complex flavonoid, and hydroxycinnamic acid. Interaction of polyphenols with neighbourhood substances and its derivative had been linked to specifically other functions. For instance, polyphenols persuading essential oil as eugenol had been used revealing antimicrobial mode and stress-linked phytochemical corresponding to water-soluble vitamin profoundly as ascorbic acid. In earlier study, Begam et al. (2018) reported ethanol *Piper betle* L. extraction bring up ten major fractions, leaded by eugenol using GC-MS procedure. Moreover, Syahidah et al. (2017) confirmed the *Piper betle* L. has an abundant of flavonoids, tannins, and alkaloids where the methanolic assay was subjected under HPLC-DAD machine. Hence, elaborating study to obtain, extract, and modulate a comprehensive knowledge whether (about) using single polyphenols or combining with other essential compounds should be more investigated.

Piper betle Linn. (Family: *Piperaceae*) is herb plant (Figures 3.1) whose has a perennial dioecious, semi-woody climber, and swollen stems. Its common names are betel (English), paan (Indian), phlu (Thai), and sirih (Bahasa Indonesian). *Piper betle* L. is cultivated in tropical Asian area and has a strong pungent aromatic flavour. The plant is credited for conventional treatments covering antiseptic, antimicrobial, and medicinally useful for pulmonary affection by locals. In addition, nutrient value of this plant was categorised low protein (3.5-3.94%), fat (0.25-0.33%), and energy 21.8 MJ/kg (unpublished data). Dasgupta and De (2004) conducted *in vitro* study used *Piper betle* extracts exhibiting antioxidant role and Datta et al. (2011) reported ethanol extract of *Piper betle* had confirmed diminishing significantly towards to human pathogenic bacteria (gram-positive and gram-negative). As a result, we hypothesized that natural organic compound of *Piper betle* L. and corroboration method for compelling its interaction were suspected necessary for further study.

Therefore, quantification of natural present contents and validation method including separation and identification of active constituents were a prerequisite. Hopefully, the actual sentence of these results could be clear-cut and useful for pharmaceutical biotechnology community, human nutrition, and animal welfare group. Thus, the aim was to identify and measure phenolic compound, water-soluble vitamin, and essential oil of *Piper betle* L. leaf as potential antioxidant, antimicrobial, and anticarcinogenic using different polarity of solvents by reverse phase High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD).

Figure 3.1 *Piper betle* L. for experiment.

3.3 Materials and methods

3.3.1 Plant material and extract processing

Piper betle L. leaves were purchased from local market, Prachinburi area, east Thailand. Fresh biomass of leafy material plant was pooled, rinsed and kept overnight at 4°C. Residue content of *Piper betle* L. leaves was made by after steam-distillation using Clevenger's apparatus to collect an essential oil (separated report) and crude content of *Piper betle* L. leaves was as untreated steam-distillation. The crude and residue content were air-dried using oven set 40°C for 2 days, made a powder, and kept in sealed plastic until extraction time.

Five grams of crude or residue content were extracted with 20 ml solvents on the basis of their polarity. Water, methanol, ethanol, chloroform, and hexane were used in this section. The extraction was run with Soxhlet apparatus for

3-4 hours. The extract was filter and kept. Remaining solid residue on the filter paper was reextracted for 3 times with volume of fresh solvent, Soxhlet and filtered. All filtrates were pooled and combined based on solvent followed by evaporation using Rotavapor (Buchi R300, USA). The extracts were finally filtered through 0.45µm PVDF syringe paper and the volume was made up to 10 ml using the same solvent and stored at -20°C. Moreover, regard of collecting essential oils, leaves were collected, dusted and subsequently subjected into the Clevenger apparatus using ratio *Piper betle* L. leaves and deionized water (1:4) for 2 h. Steam distillation product were rinsed, separated and collected using hexane. Hexane was completely removed using Rotavapor (Buchi R300), as described above.

3.3.2 Chemical reagent, standard chemical preparation and HPLC equipment

The HPLC-grade solvents were purchased from Anapure (Bangkok, Thailand) for acetic acid, Avantor (USA) for ethanol and methanol, Labscan (Bangkok, Thailand) for hexane and chloroform, and Duksan (Gyunggido, Korea) for Acetonitrile. Standard chemicals, phenolic acids (gallic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acids), flavonoids (catechins, rutins, myricetins, quercetins, apigenins and kaempferols), essential oils (eugenols) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). However, ascorbic acid was purchased from Carlo erba (Strada Rivoltana, France). All water used in all preparations was of the double distillate water grade (Millipore, Illkirch-Graffenstaden, France).

A standard stock solution (1 mg/ml) was made by diluted 1 mg selecting standard with 0.5 ml HPLC-grade methanol followed by sonication for 15 minutes in ice and vortex. The standard stock solution was then adjusted to 1 ml with

mobile phase solution (1:9, HPLC-grade acetonitrile:1% acetic acid). Furthermore, HPLC analyses were performed with HPLC Agilent Technologies 1260 Infinity (USA and Canada) with four solvent delivery system quaternary pumps (61311B) including a diode array detector (DAD 61315D) with 10 mm flow cell, an automatic sample injection valve equipped with a 100 loop and Agilent OpenLAB CDS 1.8.1 system manager as data processor. The separation was achieved by a reversed-phase Zorbax SB-C18 column (3.5 µm particle size, i.d. 4.6 x 250 mm).

3.3.3 Chromatographic condition and method validation

A method for chromatographic analysis followed to Seal (2016) with modification. The gradient elution, flow rate, temperature setting is presented in Table 3.1. Injected volume was maintained at 20 µl. A photo diode array UV detector run wavelengths at 272 nm according to absorption maxima of analysed compounds was set to detect HPLC chromatogram with analysis total time per sample 65 mins. Identification of each compound was decided with identical condition by its retention time inviting spiked standard and reference mode was turned on critically for identification of integration data analysis. Quantification of each injected sample was completely success by measuring integrated area. As a result, content number of samples was calculated based on calibration curve by plotting peak area, respective standard sample was also considered. The data was subjected in quadruplicate.

The analytical method was validated according to ICH guideline namely the effectiveness, the limit of detection (LOD), the limit of quantitation (LOQ), the linearity, the precision and the accuracy (Table 3.2). The effectiveness of HPLC method was confirmed by recognizing peak of standard solvent randomly after it was diluted with diverse solvent. Acetonitrile and 1% acetic acid resolved well in this study,

however, other solvents such as methanol and formic acid might be considered. Theoretically, LOD and LOQ were calculated from the calibration curve according to the following equations:

$$LOD=3.3\times\frac{\sigma}{S} \quad (1)$$

$$LOQ=10\times\frac{\sigma}{S} \quad (2)$$

where σ is the standard deviation of *y*-axis interception values and *S* is the angular coefficient.

To obtain linearity, a calibration curve was created with a stock standard solution (1 µg/ml) diluted to six concentrations (5, 10, 20, 30, 40, and 60 µg/ml) randomly injected in triplicate. The calibration curve was accepted to get $R^2 > 0.99$ indicating the significantly abundant linearity measurement. Precision evaluation was run repeatability and intermediate precision using the degree of proximity expressing as % relative standard deviation (RSD) of retention time and peak area. Two diluted concentration was selected (20 and 40 µg/ml) by injecting in 10 times to evaluate % RSD determination. Accuracy was evaluated based on recovery percentage of crude and residue extract spiking to 20 µg/ml and 40 µg/ml. Results of accuracy were calculated in terms of recovery (%R) according to:

$$R\ (\%)=\frac{a-b}{c}\times 100 \quad (3)$$

where *a* is amount found, *b* is amount contained, and *c* is amount added.

Table 3.1 Chromatographic design for gradient eluent, flow rate, and temperature.

Time (min)	Flow rate (µl/min)	Temperature (°C)	Mobile phase (%)	
			Acetonitrile	1% Acetic acid
0	0.7	28	10	90
28	0.7	28	40	60
39	0.7	28	60	40
50	0.7	28	90	10
55	0.7	28	10	90
65	0.7	28	10	90

3.4 Results and discussion

3.4.1 Optimization of chromatographic condition

The UV-Vis spectrum of all standard compounds showed successfully in wavelengths 272 nm. This wavelength provided a clearest separation of single peak area towards to retention time. Regardless of time observation, running at 43 minutes onwards was found slightly sensitive identification. All peak area is depicted in Figures 3.2.

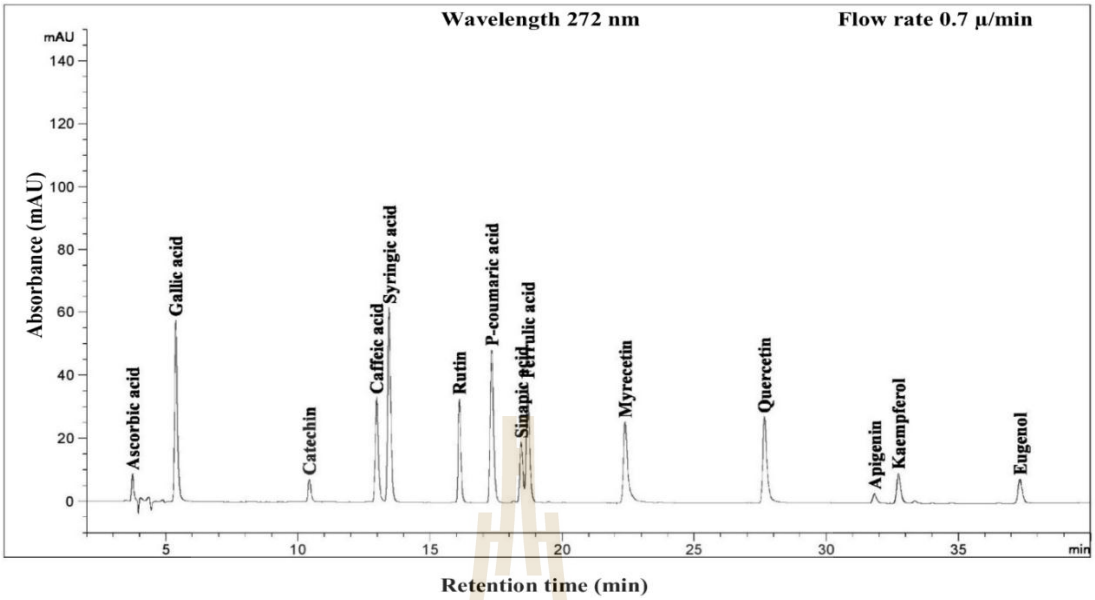

Figure 3.2 Standard HPLC chromatogram of phenolic compound, water-soluble vitamin, and essential oil.

3.4.2 Validation method of phenolic compound, water-soluble vitamin, and essential oil

In response of validation method, including linearity, precision, and accuracy is presented in Table 3.2. A compulsory-mixed standard chemical was found over R^2>0.9907 indicating all variables standing on linearity. According to precision, RSD in retention time and spiking peak area at two selectively standards, 20 µg/ml and 40 µg/ml were lesser 1% exhibiting repeatability running a rigid way. An automatically injection valve set maintaining evaporation off solvent and mixing genuinely vial were acceptable strategies for further recommending investigations. Furthermore, the LOD and LOQ were calculated as mean 0.005 and 0.015 µg/ml, respectively. The decreased values of these limits revealed high sensitivity of the method. In this study, the separation detector was set a strictly shorter than previous

study (Syahidah et al., 2017), In addition, the percentage of recovery was range 96-102. A breakthrough in number was particularly interesting in this stage, considering the method formula hinged on linearity, precision, and accuracy involving meticulousness could be applicated for qualitative and quantitative analysis of phenolic compound, water-soluble vitamin, and essential oil of this study.

3.4.3 Identifying compounds of phenolic compound, water-soluble vitamin, and essential oil

The phenolic compound, water-soluble vitamin, and essential oil chromatogram of crude content is captured in Figures 3.3-3.7, residue content in Figures 3.8-3.12 and oil content (only formed in water: Figure 3.13) following by water, methanol, ethanol, chloroform, and hexane, respectively. Clearly, polar solvents were easier to bond the organic compound due to uneven distribution of electron density, while non-polar solvents were opposite sentence. A relationship of O-H bonding and repeating extraction might be a hand of effecting factors stimulating compound sensitivity. As a result, flavonoid was most edible dissolved among essential oil and water-soluble vitamin (Rafiq et al., 2018). Overall, quercetin and eugenol were duo foremost in crude-residue contents of plant under investigation.

Table 3.2 Retention time and parameters of calibration curve, precision and repeatability, LOD, LOQ and percent recovery study of standard ascorbic acid, phenolic acids, flavonoids, and eugenol for HPLC method validation using wavelength at 272 nm.

Standard name	Retention time	RSD (%) of the retention time	RSD (%) of the peak area at conc 20 µg/ml	RSD (%) of the peak area at conc 40 µg/ml	Regression Coefficient R^2	LOD µg/ml	LOQ µg/ml	Percentage of recovery (%)
Ascorbic acid	3.74	0.277	0.602	0.074	0.9951	0.003	0.008	99.69
Gallic acid	5.42	0.318	0.842	0.518	0.9981	0.001	0.002	100.47
Catechin	10.55	0.502	0.291	0.220	0.9986	0.013	0.038	101.09
Caffeic acid	13.12	0.430	0.132	0.196	0.9990	0.003	0.008	101.94
Syringic acid	13.60	0.436	0.171	0.222	0.9988	0.001	0.004	101.66
Rutin	16.30	0.579	0.175	0.262	0.9990	0.006	0.018	97.52
P-coumaric acid	17.49	0.395	0.184	0.221	0.9984	0.002	0.007	100.80
Sinapic acid	18.62	0.373	0.467	0.973	0.9990	0.007	0.020	98.59
Ferulic acid	18.85	0.573	0.165	0.386	0.9996	0.008	0.023	102.62
Myricetin	22.53	0.283	0.299	0.562	0.9955	0.001	0.004	102.78
Quercetin	27.82	0.242	0.367	0.938	0.9910	0.001	0.003	99.86
Apigenin	32.00	0.211	0.522	0.293	0.9915	0.012	0.035	96.02
Kaempferol	32.90	0.205	0.957	0.276	0.9907	0.009	0.027	100.05
Eugenol	37.49	0.221	0.148	0.171	0.9908	0.004	0.011	100.26

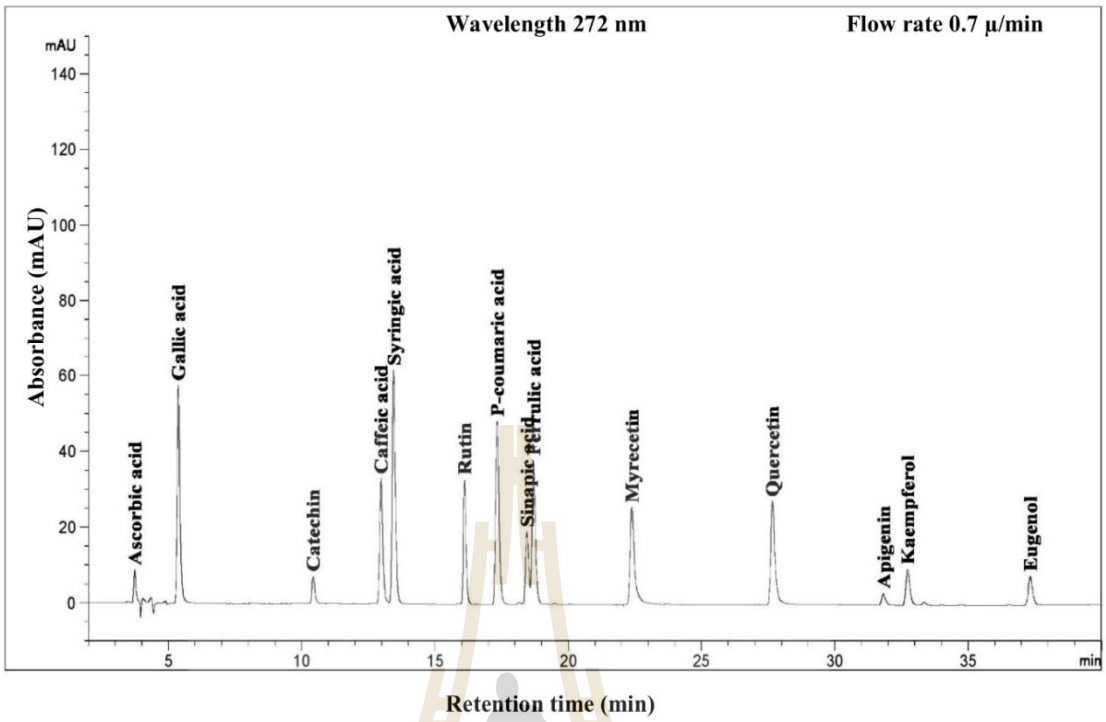

Figure 3.3 HPLC chromatogram of *Piper betle* L. crude content in water extract.

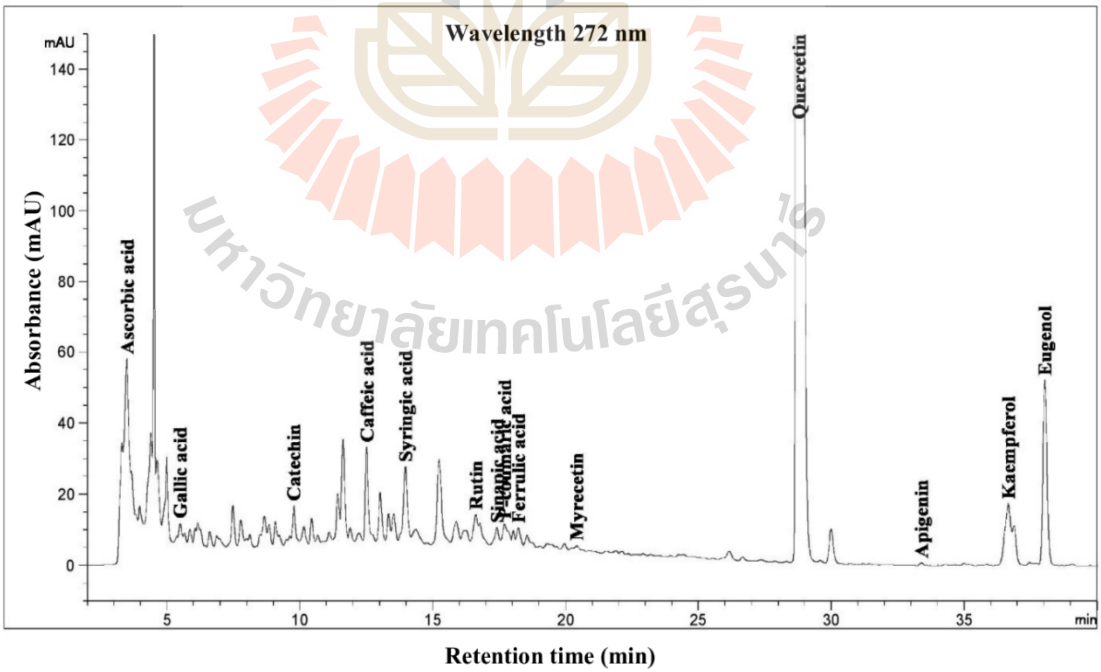

Figure 3.4 HPLC chromatogram of *Piper betle* L. crude content in methanol extract.

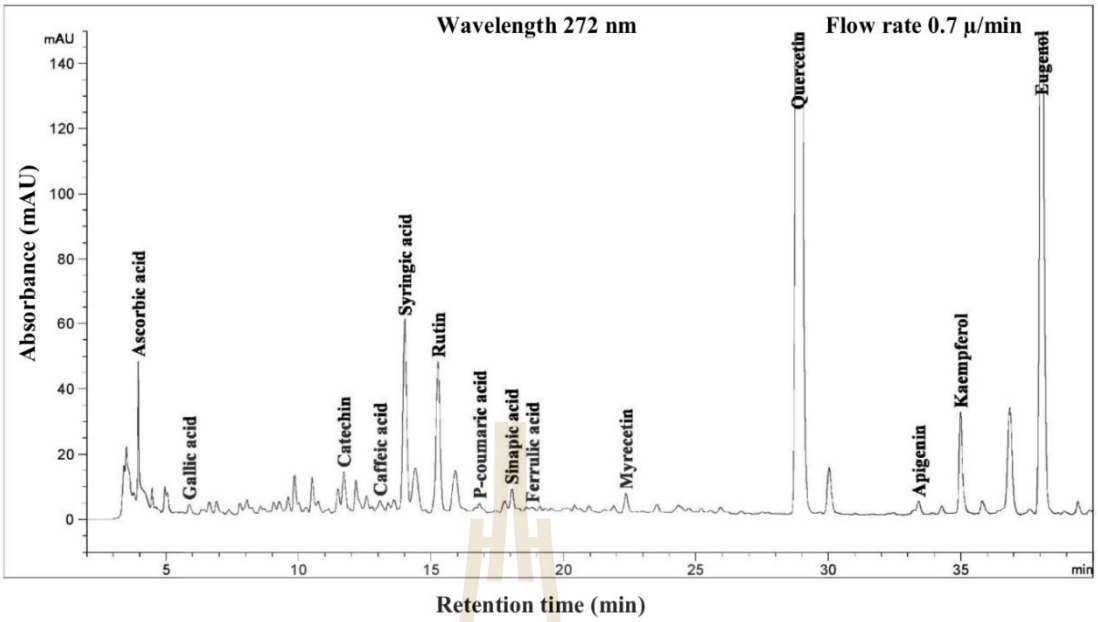

Figure 3.5 HPLC chromatogram of *Piper betle* L. crude content in ethanol extract.

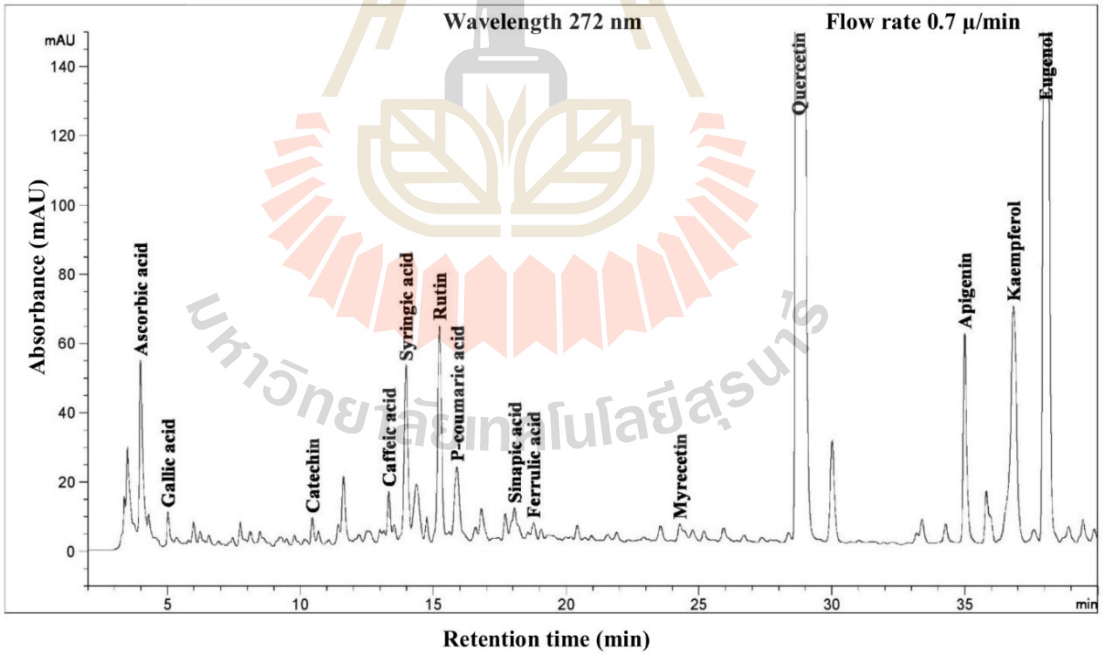

Figure 3.6 HPLC chromatogram of *Piper betle* L. crude content in chloroform extract.

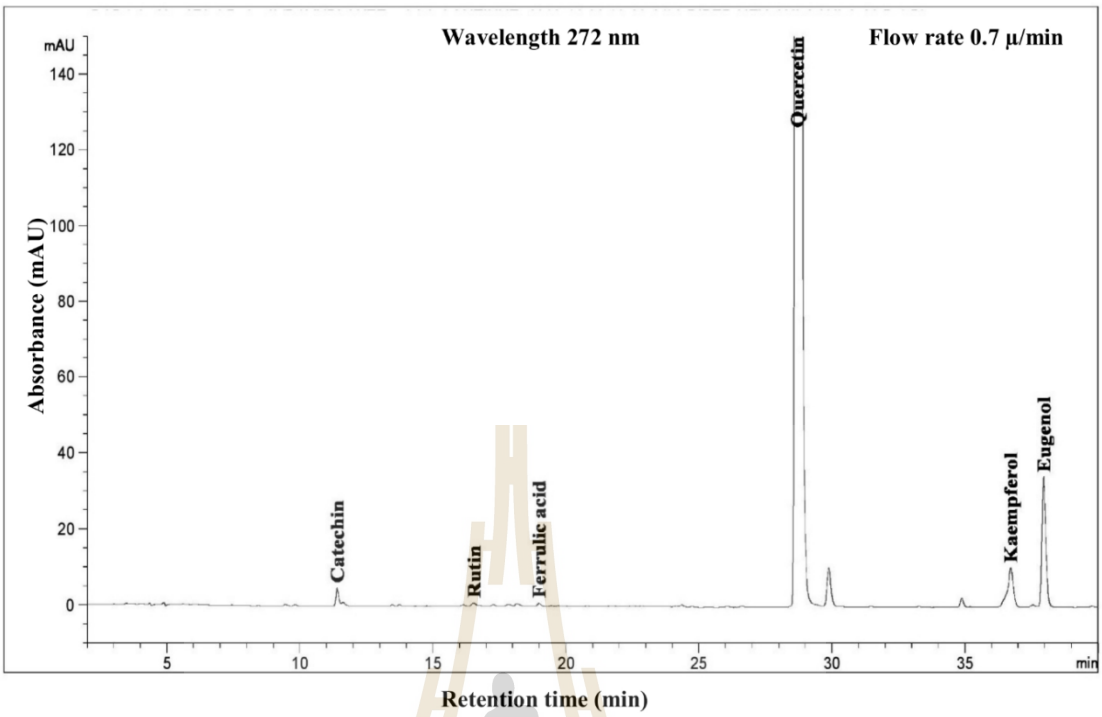

Figure 3.7 HPLC chromatogram of *Piper betle* L. crude content in hexane extract.

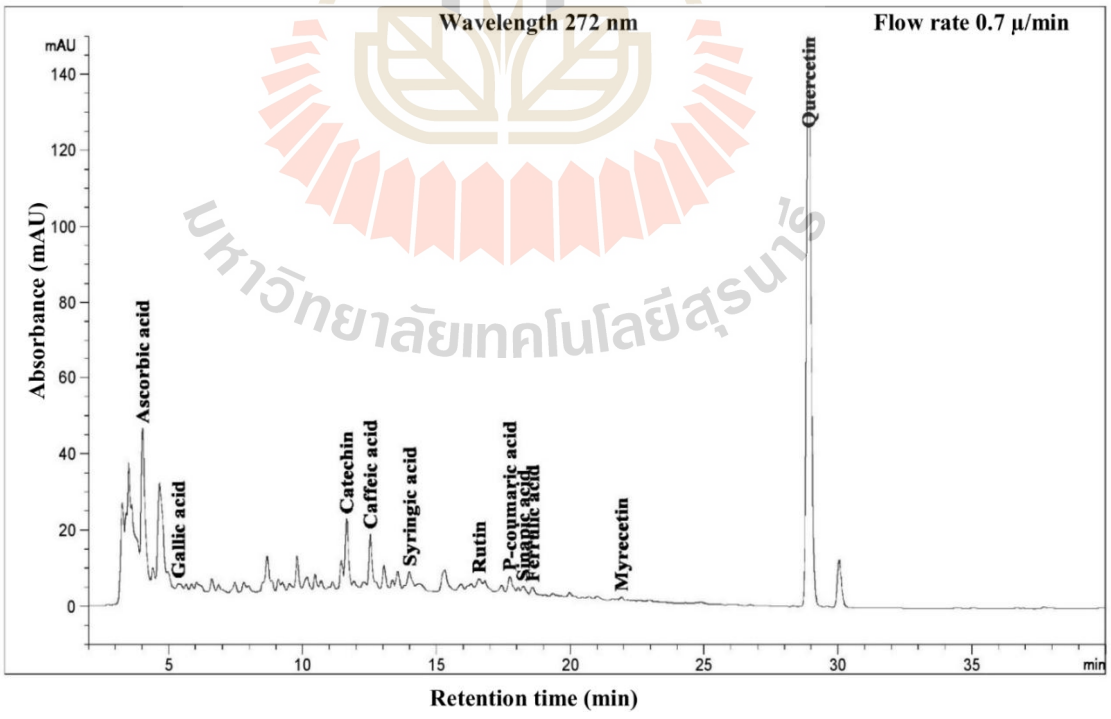

Figure 3.8 HPLC chromatogram of *Piper betle* L. residue content in water extract.

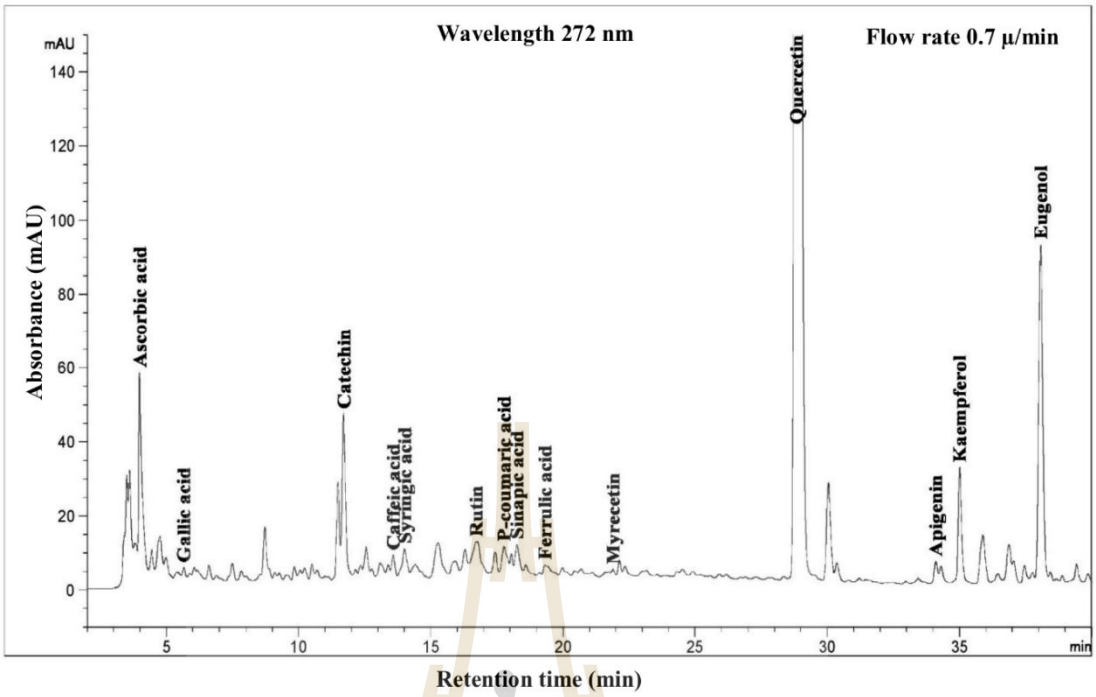

Figure 3.9 HPLC chromatogram of *Piper betle* L. residue content in methanol extract.

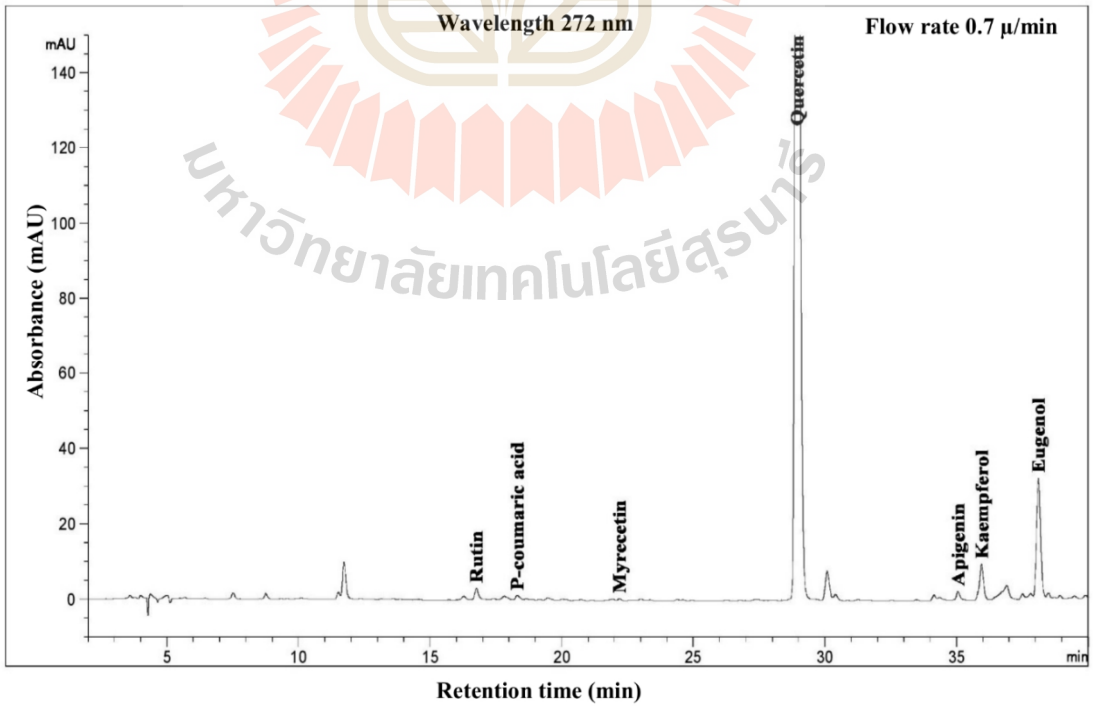

Figure 3.10 HPLC chromatogram of *Piper betle* L. residue content in ethanol extract.

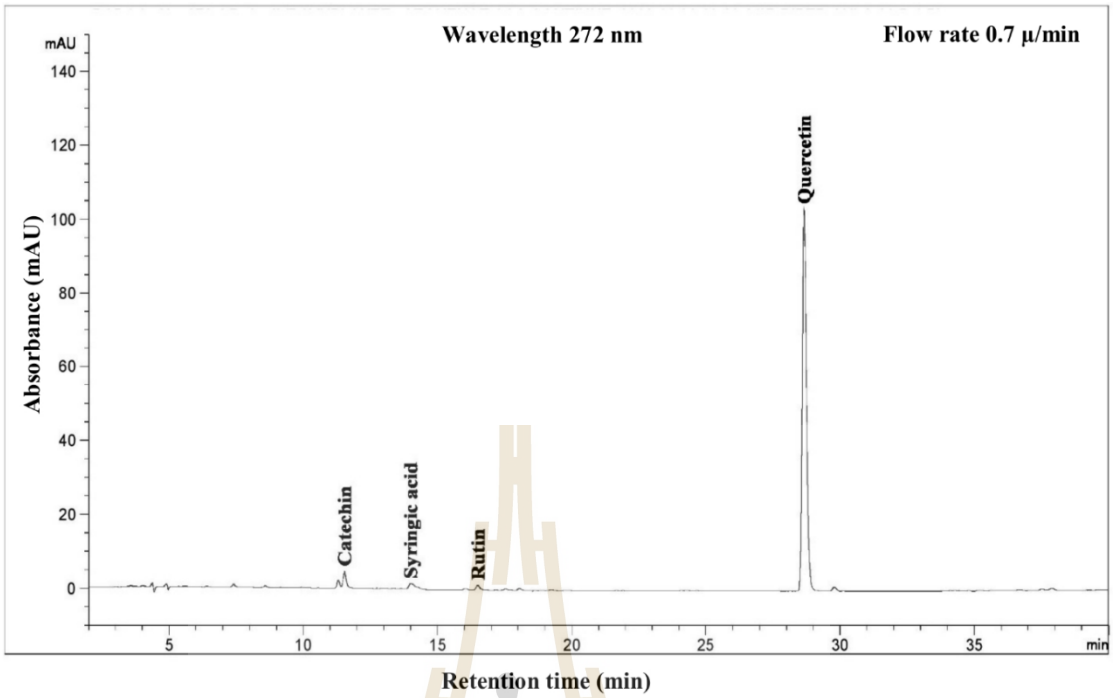

Figure 3.11 HPLC chromatogram of *Piper betle* L. residue content in chloroform extract.

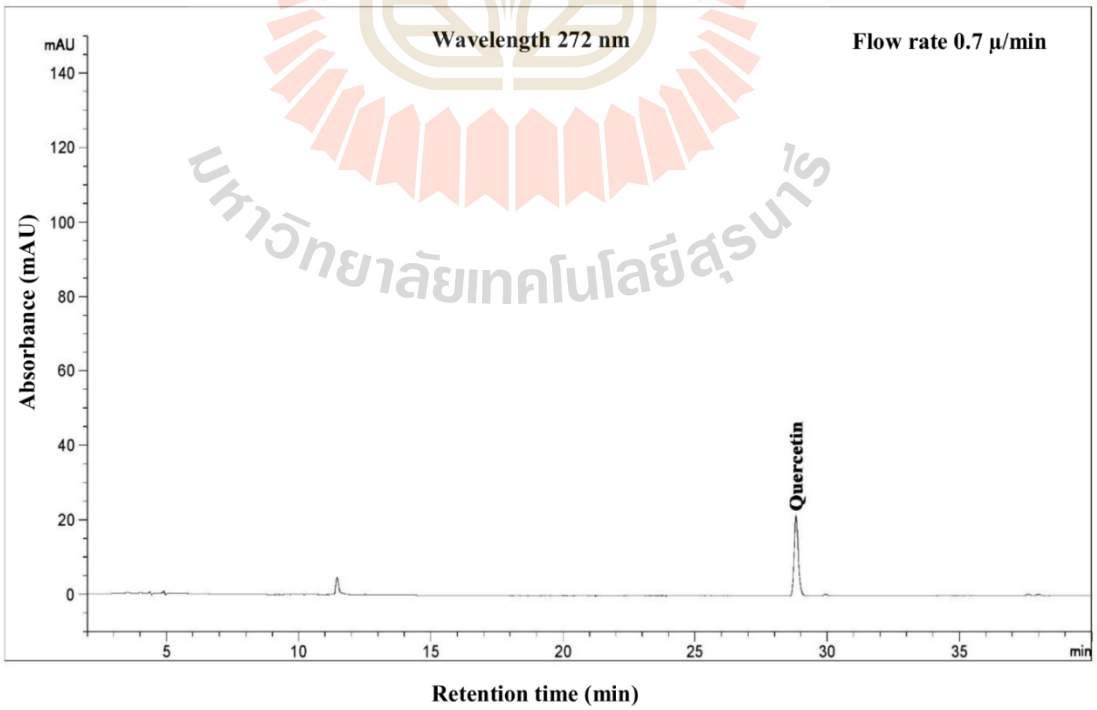

Figure 3.12 HPLC chromatogram of *Piper betle* L. residue content in hexane extract.

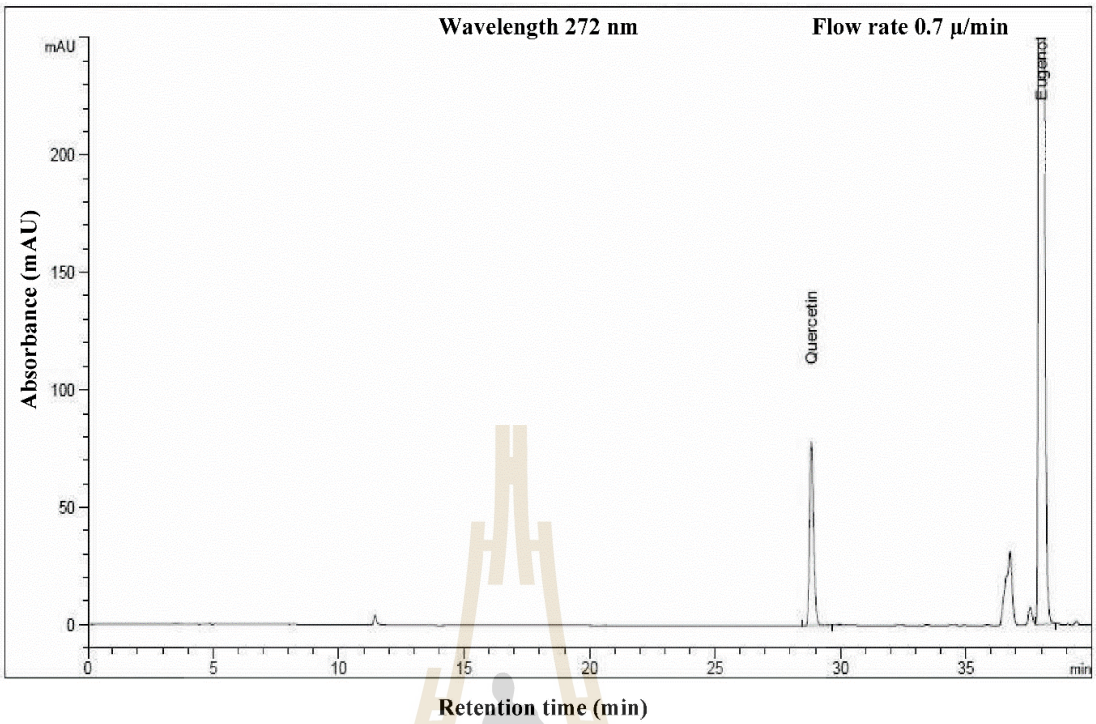

Figure 3.13 HPLC chromatogram of *Piper betle* L. oil content.

3.4.4 Quantifying compounds of phenolic compound, water-soluble vitamin, and essential oil

A completely quantitative result is presented in Table 3.3. In this present study, the proportion of *Piper betle* L. chemical compounds from highest to lowest was phenolic compounds, essential oil, and water-soluble vitamin (mg/g). The most organic compound of phenolic fraction was flavonoids 67.4% and 55.3% for crude and residue content, respectively. Eugenol as essential oil representative was only slighter number at 20% and remaining percentages confirmed as ascorbic acid (water-soluble vitamin). In previous reports, the average percentage (%) or mass (mg/g) of various *Piper betle* L. confirmed eugenol as the most major compound (Prakash et al., 2010; Begam et al., 2018), even Syahidah et al. (2017) adjusted assessment by TLC study that *Piper betle* L. might have flavonoid or other phenolic

compounds. The suitable method for extraction and detection probably provided a clearer peak in specifically *Piper betle* L. quantity (mg/g), e.g. catechin.

Catechin is difficult to extracted. The greatest reason because of catechin is lofty sensitivity in oxidation, light alkaline environment, and high temperature (Gadkari and Balaraman, 2015). However, catechin of *Piper betle* L. was successfully extracted and presented in polar and non-polar solvents using hot reflux extraction (Soxhlet apparatus), see table 3.3. There were max 0.560 mg/g using chloroform in crude content and 0.869 mg/g using methanol in residue content, where these numbers were quite low towards recommendation. Catechin were rich in tea around 20-160 mg/g and bean 70-110 mg/g (Manach et al., 2004). Meanwhile, other flavonoids were successfully detected (Table 3.3).

Along with flavonoid, gallic acid plays role as plant defence. Gallic acid is commonly found and collected from fruits and vegetables. The gallic acid content of this study was found a lesser number in both of crude and residue *Piper betle* L. content <0.06 mg/g. Karamac et al. (2006) and Bayili et al. (2011) were reported content gallic acid in plant at least 0.21 mg/g to provoke the detrimental health problem to antioxidant purpose. Hence, gallic acid was predicted greater sensitive in polar dissolver.

Table 3.3 Quantification of ascorbic acids, phenolic acids, flavonoids and essential oils of *Piper betle* L. (n=4).

Compound names	Oil content (mg/g)	*Piper betle* L. crude content (mg/g)					*Piper betle* L. residue content (mg/g)				
		W	M	E	C	H	W	M	E	C	H
Ascorbic acid	Nd	0.12±0.68	0.24±0.23	1.60±0.51	0.30±0.44	Nd	0.45±0.16	1.06±4.86	Nd	Nd	Nd
Gallic acid	Nd	0.01±0.04	0.05±0.12	0.08±0.08	0.05±0.14	Nd	0.05±0.11	0.05±0.10	Nd	Nd	Nd
Catechin	Nd	0.03±0.13	0.25±1.07	0.24±0.04	0.56±0.05	0.07±0.21	0.26±0.14	0.87±3.90	0.10±0.55	0.11±0.18	Nd
Caffeic acid	Nd	0.02±0.07	0.06±0.09	0.12±0.08	0.06±0.07	Nd	0.07±0.08	0.06±0.08	Nd	Nd	Nd
Syringic acid	Nd	0.01±0.04	0.11±0.17	0.26±0.06	0.04±0.10	Nd	0.05±0.09	0.12±0.16	Nd	0.01±0.07	Nd
Rutin	Nd	0.01±0.04	0.19±0.29	0.70±0.04	0.09±0.07	0.01±0.06	0.05±0.05	0.19±0.28	0.04±0.08	0.01±0.06	Nd
P-coumaric acid	Nd	0.01±0.04	0.06±0.16	0.19±0.13	0.06±0.11	Nd	0.05±0.11	0.07±0.16	0.01±0.08	Nd	Nd
Sinapic acid	Nd	0.01±0.04	0.06±0.03	0.23±0.17	0.05±0.08	Nd	0.04±0.07	0.10±0.25	Nd	Nd	Nd
Ferulic acid	Nd	0.01±0.02	0.03±0.08	0.06±0.04	0.03±0.06	0.01±0.04	0.01±0.05	0.03±0.14	Nd	Nd	Nd
Myricetin	Nd	0.01±0.05	0.08±0.17	0.12±0.15	0.07±0.15	Nd	0.02±0.11	0.08±0.16	0.02±0.11	Nd	Nd
Quercetin	0.05±0.04	0.20±0.13	4.40±0.56	13.61±8.13	8.70±0.12	3.04±1.34	0.54±0.19	5.63±0.73	1.18±0.26	0.39±0.36	0.15±0.24
Apigenin	Nd	0.01±0.01	0.79±1.32	4.41±0.69	0.26±0.57	Nd	Nd	0.32±0.76	0.06±0.34	Nd	Nd
Kaempferol	Nd	0.01±0.04	0.55±1.43	2.37±0.51	0.67±1.73	0.35±0.92	Nd	0.48±1.04	0.18±0.25	Nd	Nd
Eugenol	1.97±0.38	0.03±0.02	1.99±0.52	11.97±2.88	1.25±3.61	0.81±2.83	Nd	2.10±0.72	0.35±0.13	Nd	Nd

Data are presented as Mean±SEM.

ND = Non-detected.

W = water; M = methanol; E = ethanol; C = chloroform; H = hexane.

Syringic acid, a final form of benzoic acid derivative is biosynthesized by β-oxidation through shikimic pathway which is interfered massively with lignin in plant cell walls (Srinivasulu et al., 2018). Rothwell et al. (2012), reported syringic acid was present in cucumber 0.006 mg/g, parsnip 0.011 mg/g, and rosemary 0.015 mg/g, while our study addressed the vying numbers around eight times over comparing with rosemary number in both *Piper betle* L. contents of using polar solvents. In spite of effective solvent, hexane was often failed exposing this organic group. In this study, other cinnamic compounds involving caffeic acid, p-coumaric acid, sinapic acid, and ferulic acid were successfully quantified (Table 3.3).

The most famous essential oil for expressing antimicrobial and antiseptic is eugenol (Syahidah et al., 2017; Begam et al., 2018). This phenylpropanoids is ubiquitous commonly extracted from mint leaves and spices. In present study, eugenol was successfully incited by all solvents, yet, ethanol promoted a mere content about 11.971 mg/g that was incredible number examined with other eugenol sources, e.g. nutmeg 0.090 mg/g, basil 2.154 mg/g, and clove 156.951 mg/g (Rothwell et al., 2012). As expected, residue content was lesser eugenol explaining the acceptable method for aforementioned regarding essential oil extraction from *Piper betle* L.

Ascorbic acid, one of water-soluble vitamins is simplest vitamin and broadly apparent in fruit and vegetables. This essential vitamin provides functions as enzyme cofactor, hormone biosynthesis, and wound healing in plant and animal metabolism. Davey et al. (2000) reviewed the ascorbic acid played as antioxidant role solving basic oxidative stress in plant and reported Acerola (west Indian cerry) and Roseship contained the highest ascorbic acid 13 mg/g and 1 mg/g, respectively. In our study, *Piper betle* L. crude content using ethanol extract was found lesser 11.5% of

Acerola content, that was equivalent with guava content 2 mg/g, whereas *Piper betle* L. residue content using methanol was only 1 mg/g. However, the non-polar solvents were repeatedly failed to provoke ascorbic acid in crude-residue contents that indicated the slightly polar unrecommending used in this way.

3.5 Conclusion

The reversed-phase HPLC-DAD was successfully used for identification and quantification of phenolic compound, water-soluble vitamin, and essential oil of *Piper betle* L. extract in different polarity of solvents. The method demonstrated more compatible with complex compounds involving ascorbic acids, gallic acids, catechins, caffeic acids, syringic acids, rutins, *p*-coumaric acids, sinapic acids, ferulic acids, myricetins, quercetins, apigenins, kaempferols, and eugenols, thereby, promising a simple procedure with more efficiency. LC-MS and FTIR synchrotron technique may be considered for further investigation. Consequently, this method could be applied, repeated, and developed for later observation and the present value of *Piper betle* L. could be recommended for problem solving in pharmaceutical biotechnology community (quality control units), human nutrition, and animal welfare group.

Despite an important outcomes of *Piper betle* L., those organic compounds therein seem to have a close relationship with tannins. The possibly reason of this assessment could be understood to look *Piper betle* L. biosynthesis pathway (Figure 2.1). It is reported that dietary tannin in term of modulating rumen biohydrogenation is inconsistent. This is clear that next chapter of present study needs to conduct such systematic review and/or meta-analysis to obtain the strategy on supplementing *Piper betle* L. in animal diet.

3.6 Acknowledgements

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CHAPTER IV

THE LINKS BETWEEN SUPPLEMENTARY TANNIN LEVELS AND CONJUGATED LINOLEIC ACID (CLA) FORMATION IN RUMINANTS: A SYSTEMATIC REVIEW AND META-ANALYSIS

4.1 Abstract

A systematic review and meta-analysis were conducted to predict and identify ways to increase CLA formation in ruminant-derived products to treat human health issues with dietary tannins. The objective was to compare and confirm the effects of dietary tannins on CLA formation by analysing *in vitro* and/or *in vivo* studies. We reported the results of the meta-analysis based on numerical data from 38 selected publications consisting of 3712 treatments. Generally, via multiple pathways, the CLA formation increased when dietary tannins increased. Concurrently, dietary tannins increased $\triangle^9$ desaturation and the CLA indices in milk and meat ($P<0.05$ and $P< 0.001$, with average R^2 values of 0.23 and 0.44, respectively), but they did not change the rumen fermentation characteristics, including total volatile fatty acids (mmol/l) and their acid components. *In vitro* observations may accurately predict *in vivo* results. Unfortunately, there was no relationship between *in vitro* observations and *in vivo* results ($R^2<0.10$), indicating that it is difficult to predict CLA formation *in vivo* considering *in vitro* observations. According to the statistical meta-analysis results regarding animal aspects, the ranges of tannin levels required for CLA formation *in vitro* and *in vivo* were approximately 0.1-20 g/kg DM ($P<0.001$) and 2.1-80 g/kg DM

(P<0.001), respectively. In conclusion, the *in vivo* method was more suitable for the direct observation of fatty acid transformation than the *in vitro* method.

Keywords: FAs, regression, SAS statistic tool, rumen, *in vitro-in vivo*, tannin, conjugated linoleic acid, biohydrogenation.

4.2 Introduction

There is a substantial demand for ruminant-derived products, such as milk and meat, and quality, especially fat content, has become increasingly important to consumers recently (Givens, 2005). SFAs are present in higher concentrations than PUFAs in milk and meat (Lourenço et al., 2010). The relationship between dietary SFAs and the risk of CHDs is strong; CHDs result in 2155 coronary-related deaths among 344,696 persons annually and are caused by SFA accumulation in the human body when energy intake is unbalanced, resulting in unhealthy levels of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol (Jakobsen et al., 2009). Habitual alteration may result in a "healthy life" by reducing SFA intake by approximately <10%, relative to total energy and cholesterol intake, to <300 mg/d (Krauss et al., 2000). Over the past 2 decades, many nutritionists around the world have conducted relevant studies to solve this problem and have identified a natural PUFA with CLA in milk and meat as a natural key element. Most of the researchers found that CLA has a health-promoting value in humans concerning the prevention of CHDs, abating or eliminating cancer, improving immunity and treating obesity through the development of fixed lean body mass (Whigham et al., 2000; Wanders et al., 2010; Derakhshande-Rishehri et al., 2015).

Despite the considerable benefits of CLA, it can be hydrolysed and is present in limited ranges of 0.34-1.07% of total fat in milk and 0.12-0.68% of total fat in meat

(Dhiman et al., 2005). Further, CLA is an intermediate product produced by shortening LA and ALA through lipolysis, isomerization, and biohydrogenation; the dominant isomers of CLAs are C18:2 cis-9, trans-11 and C18:2 trans-7, cis-9, representing 75-90% and 3-16% of the total CLAs, respectively (Tanaka, 2005; Lourenço et al., 2010). Unfortunately, ruminants do not have the ability to endogenously synthesize LA and ALA (Nakamura and Nara, 2003). Two possible suggestions by Chilliard et al. (2007) to obtain PUFAs, including CLAs, in ruminant-derived products are as follows: an altered biohydrogenation pathway with regard to microorganism reductase and the origin of the diet in the feeding regimen. It is well documented that bacteria group A, *Butyrivibrio fibrisolvens*, which transforms LA and ALA to C18:2 cis-9, trans-11 and C18:1 trans-11 isomers (Lourenço et al., 2010), is active in the first stage of biohydrogenation. Additionally, an activated enzyme remove two hydrogen atoms with $\triangle^9$ desaturase through desaturation deposit more C18:2 cis-9, trans-11 isomers (Dhiman et al., 2005; Vasta et al., 2009; Buccioni et al., 2017a). There has been a particular interest in comparing the roles of other microorganisms in biohydrogenation, namely, protozoa and fungi. Protozoa contribute to 30-40% of lipolysis activity by adhering to the plant surface (possibly in feedstuff) by an anterior pleated zone, resulting in an efficient breakdown of cellular membranes (Huws et al., 2009). As a consequence, rumen protozoa were confirmed to be a rich source of PUFAs due to the ingestion of more chloroplasts (Kim et al., 2009). Likewise, rumen protozoa of *Epidinium* spp. were reported to have a positive association (P<0.05) with cis-9, trans-11 18:2 and trans-11 18:1 depositions (Francisco et al., 2019). However, *Epidinium* spp. were reported to be unable to induce $\triangle^9$ desaturation (Devillard et al., 2006; Kim et al., 2009) associated with bacterial lipids. Rumen fungi were represented

by the *Orpinomyces* genus, which also accelerated CLA production via the LA lipolysis isomerization stage in slow catalysis. However, the double-bonded reductase of the ALA fraction was unclear (Nam and Garnsworthy, 2007)..

The original diet in the feeding regimen can be supplemented with feeds or additional fats, such as forages, animal lipids and vegetable oils, to achieve increased CLA fractions in milk and meat, as reported in a previous meta-analysis (Glasser et al., 2008). Recently, the efficiency of the aforementioned supplementation was confirmed by its affinity for bacterial lipid group A, which is toxic to microorganism (Lourenço et al., 2010). Notably, microorganisms that interrupt lipid production seem to have a clear association with the change in the FA profiles in milk and meat. Moreover, tannins applied as phytochemicals in feeding regimens have been shown to have antimicrobial properties. Typical forms of condensed and hydrolysable tannins have been completely tested to obtain information regarding the bioactive role of target plants on rumen fermentation considering biohydrogenation (Jayanegara et al., 2011; Li et al., 2015). Additionally, tannins promoted group B bacteria (*Butyrivibrio proteoclasticus*), which convert C18-1 trans-11 forms to C18:0 forms (Costa et al., 2018). In other words, tannins could be supplemented in feed to improve CLA production via the inhibition of SA, resulting in increased $\triangle^9$ desaturation.

Furthermore, two prior meta-analyses showed that dietary nutrients containing PUFAs and tannins had a dependent relationship with feeding type (Jayanegara et al., 2012b; Khiaosa-ard et al., 2015). Grazing regimens tended to promote RA and VA depositions in ruminant-derived products. There was also a systematic literature review and meta- and redundancy analyses (Średnicka-Tober et al., 2016) that discussed organic provisions for the alteration of biohydrogenation, resulting in expected

increases in CLA deposition in milk. The abovementioned publications focus on valuable tannins, edible applications, and potential functions separately. However, none of the studies addressed the relationship between tannin supplementation in ruminant diets and biohydrogenation with the meta-analysis technique. A clear-cut method, whether using *in vitro* or *in vivo* methods, to obtain baseline data is needed. Hopefully, the results of the present meta-analysis will contribute to animal science, animal nutrition and biotechnology knowledge. The objectives of this study were (i) to elucidate the effectiveness of tannins in the modulation of CLA formation, (ii) to compare the results of *in vitro* and *in vivo* methods, and (iii) to confirm the relationship between *in vitro* and *in vivo* studies, applying meta-analysis as a statistical tool.

4.3 Materials and methods

4.3.1 Search strategy and selection criteria

A database of previous studies involving dietary tannins and CLA properties was created considering the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Figure 4.1) (Liberati et al., 2009). These publications were gathered from the Web of Knowledge, Mendeley, Scopus, PubMed and Google Scholar databases. The following keywords were applied in each database search (Appendix A): “biohydrogenation,” “conjugated linoleic acid,” ”rumen,” ”tannin,” “condensed tannin,” “hydrolysable tannin,” “meat,” “milk,” “*in vivo*” and “*in vitro.*” The databases were searched from January 1992 to March 2019, resulting in 1116442 references. The papers were published in all languages in peer-reviewed and non-peer-reviewed journals and comprised single articles, review articles, clinical trials and case report/short communications. Relevant papers were

deposited into and duplicate papers were removed by Endnote (Thompson ISI Research-Soft, Philadelphia, PA, US).

4.3.2 Search strategy and selection criteria

The study criteria, quality assessment and data extraction processes followed those in a previous meta-analysis (Jayanegara et al., 2012b; Średnicka-Tober et al., 2016). The inclusion criteria were as follows: (1) the study utilized an *in vitro* method; (2) the study utilized an *in vivo* method; (3) the study subjects were ruminants, including cows, goats, and sheep that produced dairy or meat products; (4) the study contained relevant data that was retrievable; and (5) the study was published after 1 January 1992. Authors were contacted by e-mail and through ResearchGate if the data was questionable. If contact was unsuccessful, the studies were excluded because of the inaccessibility of the data.

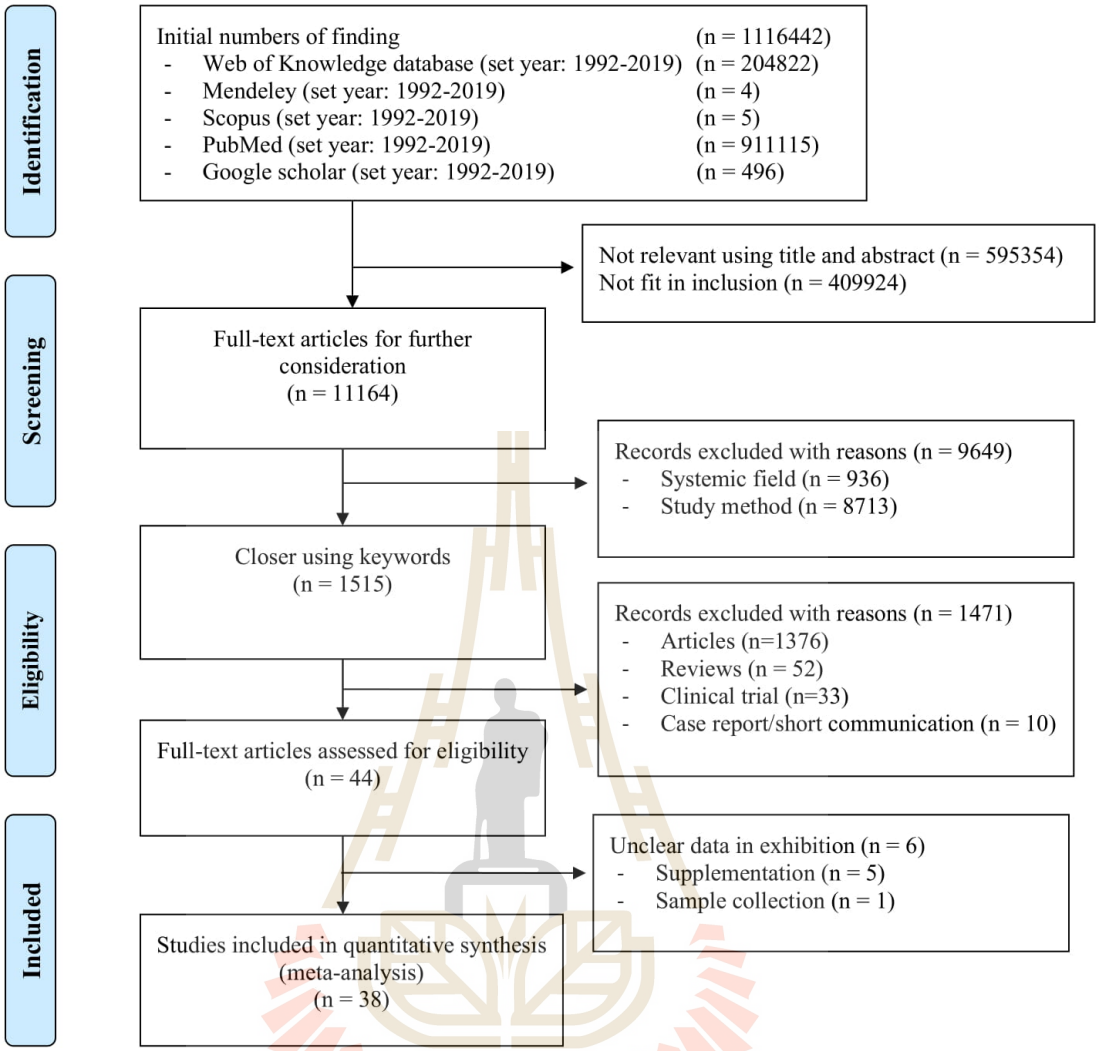


Figure 4.1 Literature retrieval flow chart using PRISMA approach.

All the numerical data from the text, tables and figures of screened papers were copied directly to create a computerized database. The raw data of 44 full-text articles were strictly screened and converted to standardized units per parameter, e.g., g/100 g fatty acid methyl esters (FAMEs) and g/kg dry matter (DM), for all FA and tannin levels, respectively. However, 6 articles were excluded because of unbalanced or unclear data. In the present meta-analysis, the tannin criteria was also established on the basis of a prior meta-analysis (Jayanegara et al., 2012b); in brief, the

condensed and hydrolysable tannins in the included studies originated from plants and had been extracted. However, a comparison of each tannin type, for instance, condensed vs. hydrolysable tannins in a single treatment, was not provided. Comparisons of these results were provided in grouping studies. The comprehensive database consisted of 3712 dietary treatments in 38 publications, as shown in Table 4.1 (11 *in vitro* experiments/2098 treatments), Table 4.2 (14 *in vivo* experiments for CLAs in milk/580 treatments) and Table 4.3 (13 *in vivo* experiments for CLAs in meat/3712 treatments). There was an article that included both *in vitro* and *in vivo* experiments on CLAs milk (Szczechowiak et al., 2016) and an article that compared CLAs in milk and meat *in vivo* (Lobón et al., 2019). Sensitivity and risk of bias analyses were performed for the selected publications. The risk of bias was based on sufficient data, the study type and the probability of confounding. Effect sizes were subjected to a meta-analysis model based on standardized mean differences (SMDs). Funnel plots, Egger's tests for funnel plot asymmetry and fail-safe number calculations were used to assess publication bias, with $P < 0.05$ indicating significant publication bias (Egger et al., 1997) (see Appendix B for further information).

4.4 Statistical analysis

The analysis of the assembled data was performed with a statistical univariate meta-analysis approach (Sauvant et al., 2008; Jayanegara et al., 2012b; Madden and Paul, 2015), using the MIXED procedure of SAS version 9.4 (SAS Institute Inc, 2015). The following model was applied:

$$Y_{ij}=P_0 + P_1Q_{ij} + R_i + p_iQ_{ij} + e_{ij} \quad (1)$$

where Y_{ij} = the dependent variable, P_0 = the overall intercept across all experiments (fixed effect), P_1= the linear regression coefficient of Y on X (fixed effect), Q_{ij} = the value of the continuous predictor variable (supplementary tannin level), R_i = the random effect of experiment $_i$, pi = the random effect of experiment i on the regression coefficient of Y on X in experiment i, and e_{ij} = the deniable residual error. For instance, we collect data regard of CLA formation. The fixed effect was means among population in which from tannins supplementation in varied animal and/or approach and the random effect was meaning for animal (cow, beef, goat and sheep). To input the CLASS statement, the variable "REFERENCENO" was applied without any quantitative information. Additionally, data were calculated according to the number of animal replicates in each experiment (SAS Institute Inc, 2015) and scaled to 1 to avoid misconception due to unequal variances among the experiments. In the fixed-effect model, small studies received lower weightings and higher weights were applied to larger studies (based on the number of measurements or samples).

Outliers were identified by examining a mixed model with a maximum likelihood (ML) approach. For example, the model included the METHOD=ML; COVTEST; and PARMS statements followed by the EQCONS=2 option. An unstructured variance-covariance matrix (type = un) was confirmed as the random part of the model. The significant differences among intercept, slope and regression coefficients were accepted.

Additionally, CLA amounts in milk and meat sources could not be compared directly in each study. It was possible to compare the total CLA data, including *in vitro* and *in vivo* observations, thoroughly. Because of incomplete data regarding the included variables, the present meta-analyses were technically performed based on the available data of individual variables.

Table 4.1 Data tabulation of *in vitro* experiments.

No exp.	Reference	*In vitro* method	Inocula donor	Basal feed	Tannins source	Tannin level (g/kg DM)	Gas sampling (h)
1	Carreño et al. (2015)	BCI	Sheep (Ewe)	TMR, Forage: Concentrate (50:50)	Chestnut (HT), Oak (HT), Quebracho (CT), Grape seed (CT)	20-80	24
2	Costa et al. (2017)	GBI	Sheep	Dehydrated Alfalfa, concentrate and sunflower oil	Chestnut (HT), Quebracho (CT), Grape seed (CT), *Cistus ladanifer* (CT)	100	6
3	Costa et al. (2018)	RBP	Sheep	Grass hay, concentrate, vegetable oil	Mimosa (CT), Chestnut (HT), Mimosa plus chestnut (MIX)	100	0
4	Guerreiro et al. (2016)	HGT	Sheep (Merino Branco ram)	Maize, concentrate and sunflower oil	*Cistus ladanifer* (CT)	100	6
5	Ishlak et al. (2015)	BCI	Cow (Holstein)	Forage: Concentrate (55:45)	Quebracho (CT)	100	24
6	Jayanegara et al. (2012a)	HGT	Cow (Brown Swiss)	Clover-ryegrass hay and concentrate	Poa alpina (HT), Achillea millefolium (HT), Alchemilla xanthochlora (HT), Capsella bursapastoris (HT), Carum carvi (HT),Chrysanthemum adustum (HT), Crepis aurea (HT), Plantago atrata (HT), Rhinanthus alectorolophus (HT), Rumex arifolius (HT), Anthyllis vulnenaria (HT), Hedysarum	1-78	24

Table 4.1 Continue.

No exp.	Reference	*In vitro* method	Inocula donor	Basal feed	Tannins source	Tannin level (g/kg DM)	Gas sampling (h)
					hedysaroides (HT), Trifolium badium (HT), Castanea sativa (HT), Fraxinus excelsior (HT), Sambucus nigra (flowers) (HT).		
7	Jayanegara et al. (2011)	*HGT*	Cow (Brown Swiss)	Hay (white clover), ryegrass and concentrate	Acacia mangium, Acacia villosa, Albizia falcataria, Artocarpus heterophyllu, Calliandra calothyrsus, Canna indica, Carica papaya, Clidemia hirta, Cycas rumphii, Erythrina orientalis, Eugenia aquea, Hibiscus tiliaceus, Ipomoea batatas, Lantana camara, Leucaena diversifolia, Leucaena leucocephala, Manihot esculenta, Melia azedarach, Mimosa invisa, Morinda citrifolia, Myristica fragrans, Paspalum dilatatum, Persea Americana, Pithecellobium jiringa, Psidium guajava, Sesbania grandiflora, Swietenia mahagoni.	2-220	24

Table 4.1 Continue.

No exp.	Reference	*In vitro* method	Inocula donor	Basal feed	Tannins source	Tannin level (g/kg DM)	Gas sampling (h)
8	Minieri et al. (2014)	*HGT*	Sheep (Ewe)	Forage and concentrate	Quebracho (CT)	49	18
9	Szczechowiak et al. (2016)	*Bag incubate of RUSITE C*	Cow (Polish Frisien Holstein)	TMR, silage and concentrate	Vaccinium vitis idaea (CT)	4.5	24
10	Toral et al. (2016)	*BCI*	Sheep (Ewe)	Hay (Alfalfa)	Onobrychis viciifolia (CT)	5-35	24
11	Vasta et al. (2008)	*GBI*	Cow (Friesian–Holstein)	Hay and Hay plus	Ceratonia siliqua (CT), Acacia cyanophylla (CT), Schinopsis lorentzii (CT)	0.06-0.01	12

GBI, glass bottle incubation; BCI, batch culture incubation; HGT, Hohenheim gas test; RBP, rumen bacteria pellets; PMR, partial mixed ration; TMR, total mixed ration; DMI, dry matter intake; CT, condensed tannins; HT, hydrolysable tannins; DM, dry matter.

Table 4.2 Data tabulation of *in vivo* milk experiments.

No Exp.	Reference	Species	Basal feed	Tannins source	Tannin level (g/kg DM)	Adaptation period/long treatment (day)	Milking (time/day)
12	Alipanahi et al. (2019)	Goat (Kurdish)	TMR, Alfalfa hay and concentrate	Oak acorn	9.1	21/42	2
13	Buccioni et al. (2015)	Sheep (Comisana ewe)	Grass hay, rolled barley and concentrate	Chestnut (HT) and Quebracho (CT)	456-750	15/30	2
14	Buccioni et al. (2015)	Sheep (Sarda ewe)	ryegrass (Lolium multiflorum), oat (Avena sativa) and white clover (Trifolium repens) (1:1:1) with grazing	Chestnut (HT)	80	90/210	2
15	Cabiddu et al. (2009)	Sheep (Sarda ewe)	Pasture sulla with grazing	*Flowering sulla (Hedysarum coronarium L.)* (CT)	25-27.4	NS/30	2
16	de Lucena et al. (2018)	Goat (Saanen)	Pornunca silage and concentrate (60:40)	Pornunca silage-based diets	11-48	20/80	2
17	Dschaak et al. (2011)	Cow (Holstein)	TMR, Forage and concentrate (59:41)	Condensed tannin extract	30	14/21	2

Table 4.2 Continue.

No Exp.	Reference	Species	Basal feed	Tannins source	Tannin level (g/kg DM)	Adaptation period/long treatment (day)	Milking (time/day)
18	Girard et al. (2016)	Cow (Holstein)	TMR, a mixture of grass hay (86:10:4 of grass, legumes, and other species, respectively)	Sainfoin (CT), BirdSfooT trefoil bull (CT), BirdSfooT trefoil polom. (CT) in pellet forms	30.4-190.9	21/52	2
19	Henke et al. (2017)	Cow (Holstein)	TMR, a mixture of grass silage, maize silage and concentrate (34:32:34)	Quabracho tannin extract (CT)	15-30	13/42	2
20	Kälber et al. (2014)	Cow (Brown Swiss)	TMR, a mixture of grass silage, maize silage and ryegrass hay (56:26:18)	Buckwheat, phacelia, chicory	4.19-14.91	10/21	2
21	Lobón et al. (2019)	Sheep (Churra Tensina ewe)	Permanent dam and pasture (composed of 68% grass, 20% leguminous plants, and 12% other species)	Quebracho (CT)	2	NS/NS	Weekly

Table 4.2 Continue.

No Exp.	Reference	Species	Basal feed	Tannins source	Tannin level (g/kg DM)	Adaptation period/long treatment (day)	Milking (time/day)
22	Maamouri et al. (2019)	Sheep (Sicilo-Sarde ewe)	Triticale pasture	*Acacia cyanophylla*	32.7	45/70	2
24	Miri et al. (2015)	Goat (crossbred: Alpine × Beetal)	Hay and concentrate	Cumin extract	1-2	21/25	2
25	Szczechowiak et al. (2016)	Cow (Polish Holstein-Friesian)	Mix silage and concentrate	*Vaccinium vitis idaea* (CT)	32.8	21/26	2
26	Toral et al. (2011)	Sheep (Assaf ewe)	TMR, forage and concentrate (40:60)	Commercial tannin	8.7	14/30	2

PMR, partial mixed ration; TMR, total mixed ration; DMI, dry matter intake; CT, condensed tannins; HT, hydrolysable tannins; DM, dry matter; NS= not specific/mentioned.

Table 4.3 Data tabulation of *in vivo* meat experiments.

No Exp.	Reference	Species	Basal feed	Tannins source	Tannin level (g/kg DM)	Adaptation period/long treatment (day)	Slaughtered period (day of age)
27	Gesteira et al. (2019)	Bulls (uncastrated Nellora)	TMR, forage and concentrate (40:60)	*Acacia mearnsii* (CT)	10-50	15/105	615 (16 h fasting)
28	Kamel et al. (2018)	Sheep (Naomi lamb)	Alfalfa hay and concentrate	Commercial quebracho (CT)	20-40	15/70	160 (direct)
29	Lobón et al. (2019)	Sheep (Churra Tensina lamb)	Permanent dam and pasture (composed of 68% grass, 20% leguminous plants, and 12% other species)	Quebracho (CT)	2	NS/35	reached 10–12 kg of BW
30	Luzardo et al. (2019)	Sheep (Texel and Australian Merino crossbreed lamb)	Pasture, intensive grazing	Lotus uliginosus cv. E-Tanin (E-Tanin) and Trifolium repens cv. Zapicán (white clover, WC)	2.1-5.8	14/28	748 (direct)
31	Marume et al. (2012)	Goat (Xhosa lop-eare)	Pasture, intensive grazing	*Acacia Karoo* (CT)	82.5	30/90	210 (direct)

Table 4.3 Continue.

No Exp.	Reference	Species	Basal feed	Tannins source	Tannin level (g/kg DM)	Adaptation period/long treatment (day)	Slaughtered period (day of age)
32	Rana et al. (2012)	Goat (crossbred (Alpine×Beetal)	Maize and concentrate	*Terminela chebula*	1.2-10.7	NS/90	270 (direct)
33	Sharifi et al. (2019)	Sheep (Baluchi lamb)	TMR, forage and concentrate (63:37)	Grape seed (CT)	0.063-0.073	42/56	252
34	Staerfl et al. (2011)	Bull (Brown Swiss×Limousin crossbred)	Maize silage and concentrate	*Acacia mearnsii* (CT)	141	24/280	reached 525 kg of BW
35	Vasta et al. (2014)	Sheep (Comisana lamb)	Herbage and concentrate	Quabracho (CT)	40.4-40.6	7/60	105
36	Vasta et al. (2007)	Sheep (Comisana lamb)	Alfalfa hay and concentrate	Carob pulp (CT)	27	7/60	105
37	Vasta et al. (2009)	Sheep (Comisana lamb)	Herbage and concentrate	Quabracho (CT)	40.3	NS/60	105
38	Vasta et al. (2010)	Sheep (Comisana lamb)	Alfalfa hay and concentrate	Quabracho (CT)	6.45	7/77	122
39	Willems et al. (2014)	Sheep (Engadine and Valaisian Black Nose ram)	Ryegrass-clover pasture, intensive grazing	Grass, legume and herb (native pasture compound) in vegetative stage	0.30-1.64	30/93	277

PMR, partial mixed ration; TMR, total mixed ration; DMI, dry matter intake; CT, condensed tannins; HT, hydrolysable tannins; DM, dry matter; NS= not specific/mentioned.

4.5 Results

4.5.1 Search results, sensitivity and bias assessments

As depicted in Figure 4.1, the present meta-analysis was derived from a strict dataset. There was a 99.99% rejection rate during the collection of publications, which exists in academic settings around the world, especially in the USA, P.R. China, Germany, Japan and India (Figure 4.2). A possible reason for this result was the inconsistent parameters of CLA synthesis and the types of observations. Regarding the selected articles (Tables 4.1-4.3), sensitivity is depicted in a forest plot (Figure 4.3), and risk of bias is presented in a funnel plot; the risk of bias for the CLA data in all the types of studies in the present meta-analysis was not significant (P=0.067) (Figure 4.4). The sensitivity and risk of bias for the other parameters is provided in the supplementary data.

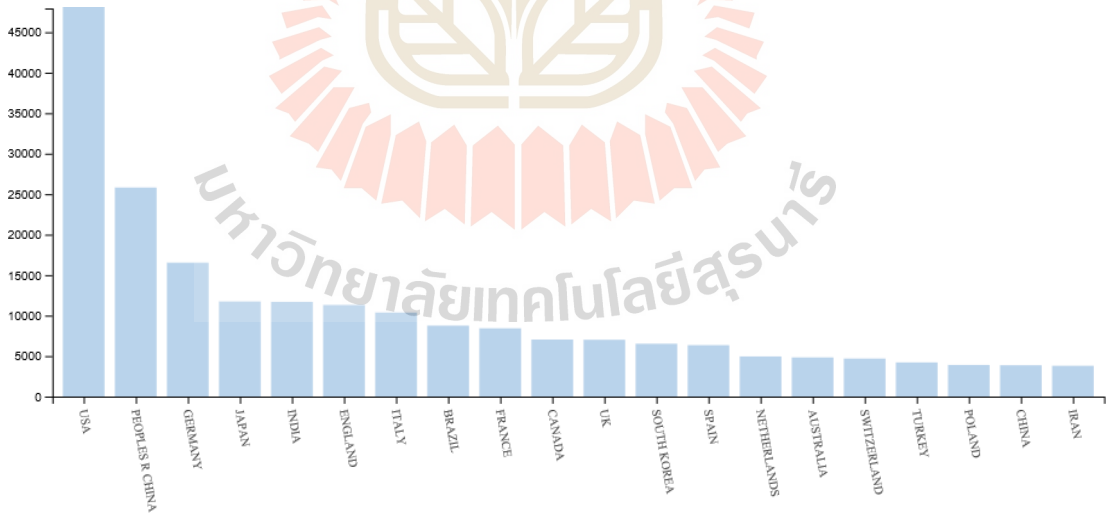


Figure 4.2 List of rejected countries during the collection of publications, which exists in academic settings around the world.

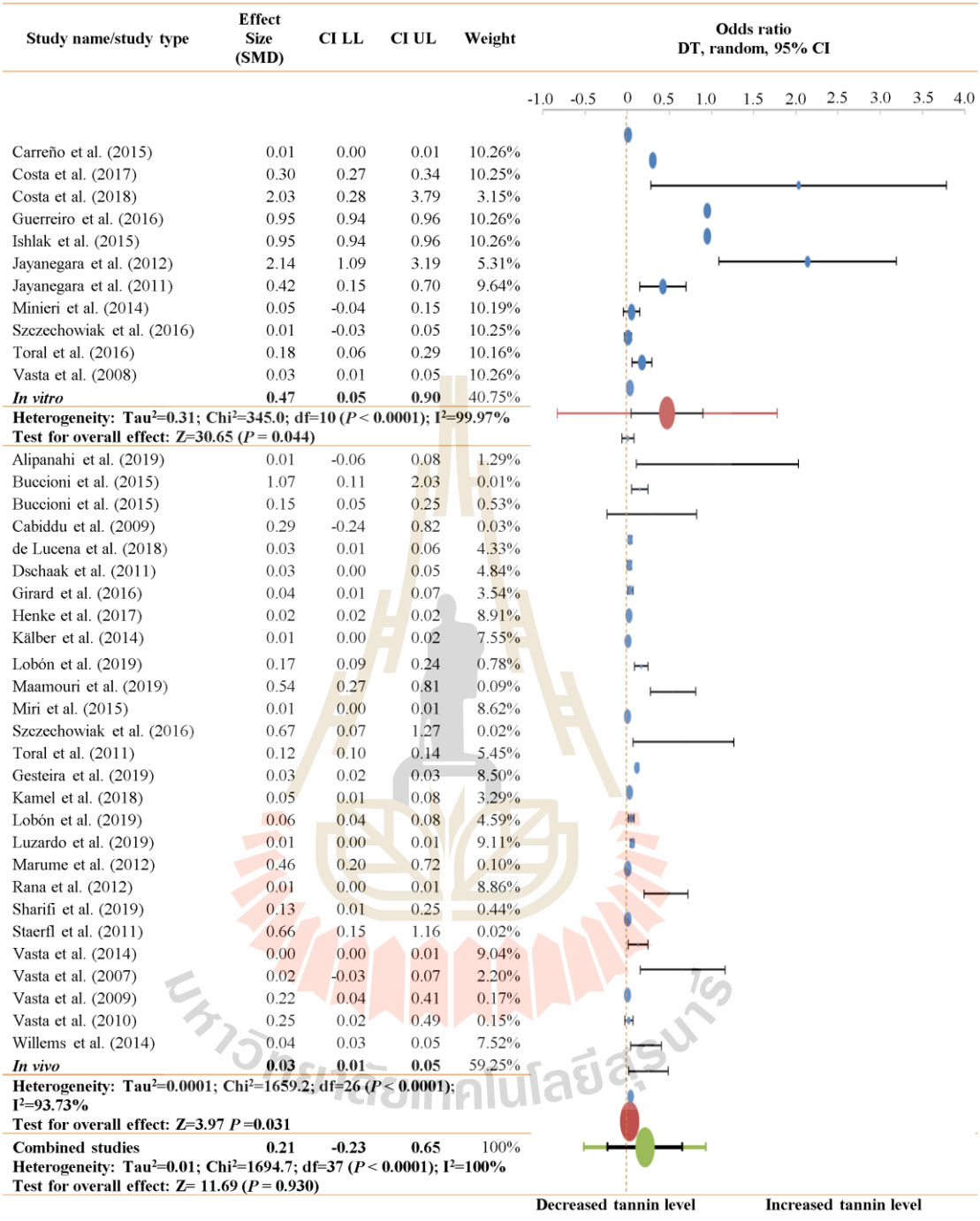


Figure 4.3 Forest plots for the links between supplementary tannin levels and CLA. It is clear that increased tannin level results in effect of intended parameter.

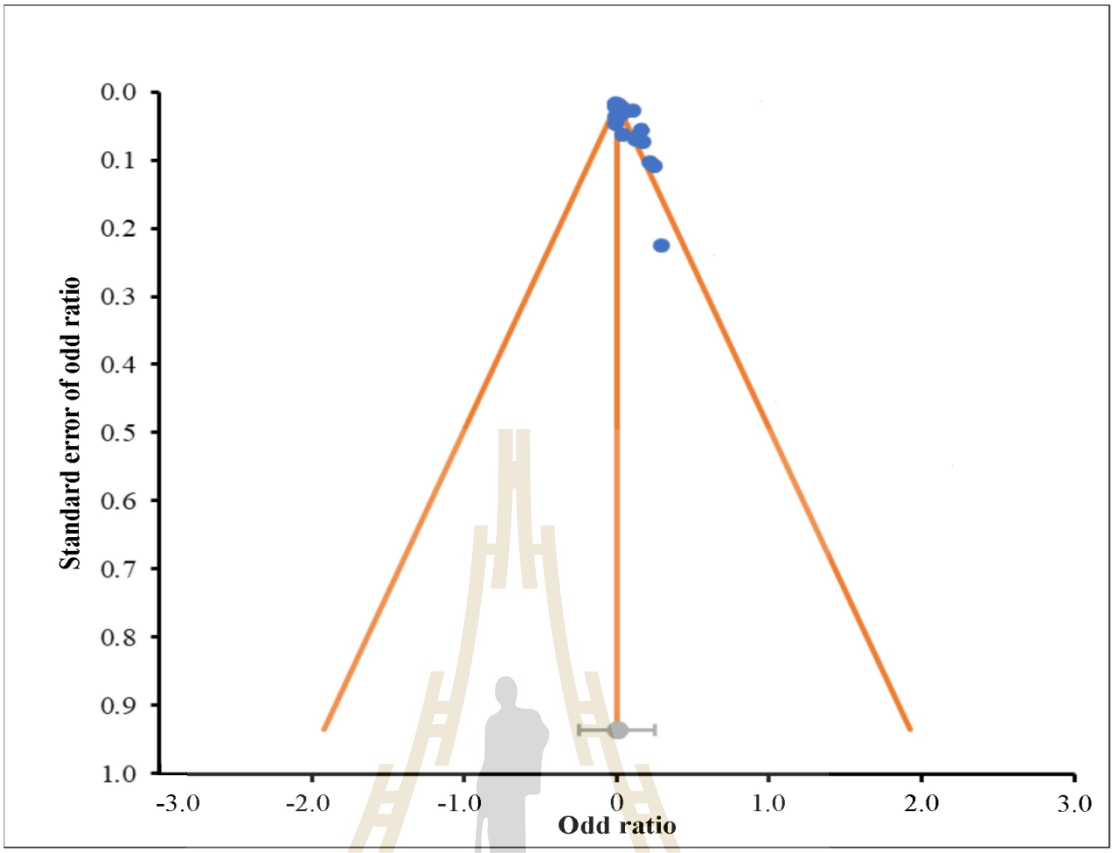


Figure 4.4 Funnel plots for the links between supplementary tannin levels and CLA. The figure addresses that risk of bias was not detected or low in study.

4.5.2 The effective level of tannins

The results of the meta-analysis of the regression lines for predicting the outcomes of *in vitro* batch culture experiments in terms of dietary tannins as a predictive variable are presented in Table 4.4. C18:2 Cis-9, trans-11 CLA increased ($P < 0.0001$ in linear equation) with increasing tannin supply, with an R^2 of 0.6422. Likewise, VA increased ($P<0.05$) with increasing tannin supply, with an R^2 of 0.6242. However, SA decreased ($P<0.001$) with increasing tannin supply, with an R^2 of 0.6701. These results supported the change in the apparent FA proportions expressed as g/100 g FAMEs, and PUFAs were increased ($P<0.001$) after tannins were

supplemented (R^2=0.5020). Dietary tannins were not associated with monounsaturated fatty acid (MUFA) and SFA proportions. In addition, total gas production decreased with increasing (P<0.0001) tannin supply, with an R^2 of 0.1736, but dietary tannins did not change the rumen fermentation characteristics, including total VFAs (mmol/l) and their acid components. According to the statistical meta-analysis results, the range of tannin levels to meet the aforementioned outcomes in the *in vitro* studies was approximately 0.1-20 g/kg DM (P<0.001).

Furthermore, the results of the meta-analysis of the regression lines to predict the outcomes of *in vivo* experiments in terms of dietary tannins as a predictive variable are presented in Table 4.5. The apparent CLA proportions in both milk and meat increased with increasing tannin supply (P<0.0001 and P<0.001, with R^2 values of 0.8352 and 0.6711, respectively). However, these results indicated that SA decreased with increasing tannin supply (P<0.01). Additionally, there was a significant, though minor, increase in VA (P<0.05) with increasing tannin supply, although the relationship was nonsignificant (R^2 <0.6). These results were similar to those in apparent FA proportions in milk or meat, expressed in g/100 g FAMEs. Interestingly, $\triangle^9$ desaturation and the CLA index, whether in milk or meat, increased with increasing tannin supplementation (P<0.05 and P<0.001, with average R^2 values of 0.2318 and 0.4382, respectively). Similar to the *in vitro* outcomes, dietary tannins did not change the rumen fermentation characteristics, including total VFAs (mmol/L) and their acid components. Therefore, the range of tannin levels necessary to obtain the abovementioned results of the *in vivo* studies was approximately 2.1-80 g/kg DM (P<0.001).

4.5.3 The regression method

The regression results between the *in vitro* and *in vivo* CLA deposition in milk are depicted in Figure 4.5a, and the results between the *in vitro* and *in vivo* CLA deposition in meat are shown in Figure 4.5b. These relationships were expressed linearly rather than quadratically. Clearly, there were no relationships among them (R^2 <0.1)

Table 4.4 The predicted equation of *in vitro* batch culture experiments[a].

Response parameter	N	Parameter estimation							
		Intercept	SE intercept	*P* intercept	Slope	SE slope	*P* slope	RMSE	R^2
FA supplementation (g/100 g FAME)									
C18:3 n-3	2098	0.1161	0.0081	0.0889	0.0349	0.0024	0.0963	17.3120	0.0891
C18:2 n-6	2098	0.0973	0.0068	0.0600	0.0012	0.0003	0.0780	14.3330	0.0899
C18:1 n-9	1858	0.1537	0.0074	0.0773	0.0034	0.0012	0.0611	15.4080	0.1870
Gas production (ml/g OM)	317	2.0662	0.2540	0.0005	-0.0005	0.0001	<0.0001	125.0300	0.1736
Total VFA (mmol/l)	1392	0.5722	0.0216	<0.0001	0.0011	0.0001	0.0622	23.1320	0.3349
C_2	1392	0.0156	0.0081	0.1198	0.0016	0.0008	0.0938	8.6198	0.2700
C_3	1392	0.0058	0.0044	0.3412	0.0065	0.0011	0.0521	7.3570	0.1200
C_4	1392	0.0024	0.0036	0.8638	-0.0100	0.0046	0.0790	3.8071	0.0300
C_5	1152	0.0301	0.0051	0.0018	0.0071	0.0052	0.2982	4.0477	0.3570
Iso-C_4+Iso-C_5	576	0.1648	0.0064	<0.0001	5.4761	5.5837	0.6116	0.1791	0.5340
FA profile (g/100 g FAME)									
C18:2 cis-9, trans-11(CLA)	2098	0.0044	0.0006	0.0008	0.2009	0.0146	<0.0001	1.3041	0.6422
C18:1 trans-1	2098	0.0077	0.0021	<0.0001	0.0392	0.0036	0.0228	4.5276	0.6242
C18:0	2098	0.0337	0.0056	0.0016	-0.0001	0.0015	0.0017	11.9020	0.6701
SFA	1842	0.0279	0.0121	0.0699	0.0008	0.0019	0.9898	22.4760	0.2973
MUFA	1842	0.0205	0.0108	0.1294	-0.0018	0.0021	0.7173	20.0980	0.1956
PUFA	1842	0.0025	0.0026	0.6038	0.0458	0.0048	0.0002	4.8936	0.5020

[a]Outcomes are averages deriving from tabulated data in Table 4.1 calculated by proc mixed.

C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; VFA, volatile fatty acid; FA, fatty acid; FAME, fatty acid methyl esters; CLA, conjugated linoleic acid; SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acid; PUFA, poly-unsaturated fatty acid; DM, dry matter; OM, organic matter; N, total data used; SE, standard error; P, *p* value; RMSE, residual mean square error; R^2, coefficient of determination.

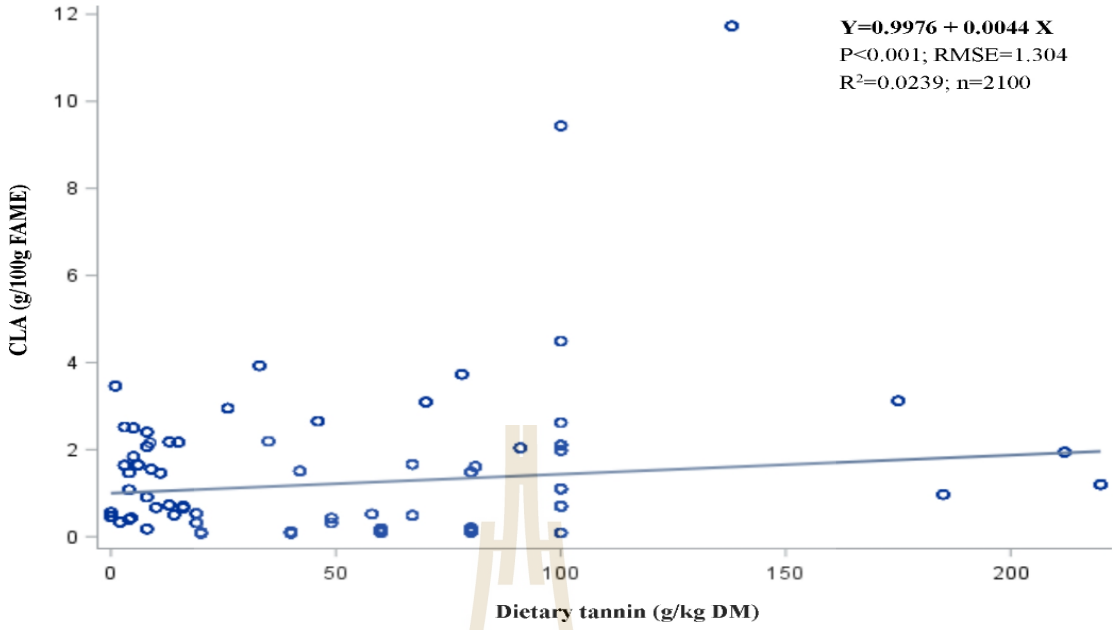
Table 4.5 The predicting equation of *in vivo* batch culture experiments[a].

Response parameter	N	Parameter estimation							
		Intercept	**SE intercept**	***P* intercept**	**Slope**	**SE slope**	***P* slope**	**RMSE**	**R^2**
FA supplementation (g/100 g FAME)									
C18:3 n-3	1284	0.0004	0.0003	15.8459	-0.0022	0.0423	0.0453	34.9540	0.0100
C18:2 n-6	1342	0.0595	0.0065	<0.0001	-0.0002	0.0001	0.0528	15.2890	0.0581
C18:1 n-9	1342	0.0125	0.0029	<0.0001	0.0160	0.0010	0.0600	6.8641	0.0133
Total VFA (mmol/L)	117	0.0429	0.0126	0.0182	0.0026	0.0013	0.2952	25.1710	0.0921
C_2	117	0.0347	0.0050	0.0010	0.0001	0.0072	0.5440	9.9188	0.2988
C_3	117	0.0006	0.0084	0.0025	0.0324	0.0043	0.0694	16.7870	0.0000
C_4	117	0.0008	0.0026	0.0098	-0.0003	0.0019	0.4956	5.1379	0.0006
C_5	73	0.0031	0.0005	0.0004	-0.0014	0.0074	0.9931	0.8895	0.3975
Iso-C_4 + Iso-C_5	31	0.0003	0.0006	0.9454	-0.0009	0.0144	0.8202	1.0553	0.0081
FA profile in milk (g/100 g FAME)									
C18:2 cis-9, trans-11 (CLA)	580	0.0159	0.0011	<0.0001	0.0303	0.0033	<0.0001	2.2603	0.8352
C18:1 trans-1	580	0.0006	0.0026	0.0188	0.0034	0.0014	0.0442	5.5867	0.4001
C18:0	580	0.0015	0.0016	0.0622	-0.0102	0.0026	0.0024	3.5275	0.5100
SFA	580	0.0044	0.0017	0.0363	-0.0131	0.0036	0.0157	3.7550	0.1070
MUFA	580	0.0017	0.0022	0.7853	-0.0037	0.0016	0.0856	4.6519	0.0010
PUFA	580	0.0036	0.0013	0.0214	0.0146	0.0024	0.0017	2.6944	0.3891
Desaturation index	580	0.00003	0.00002	0.2471	0.0239	0.0025	0.0002	0.0456	0.3500
CLA index	580	0.0022	0.0041	0.9667	0.0088	0.0012	0.0004	8.8529	0.5441

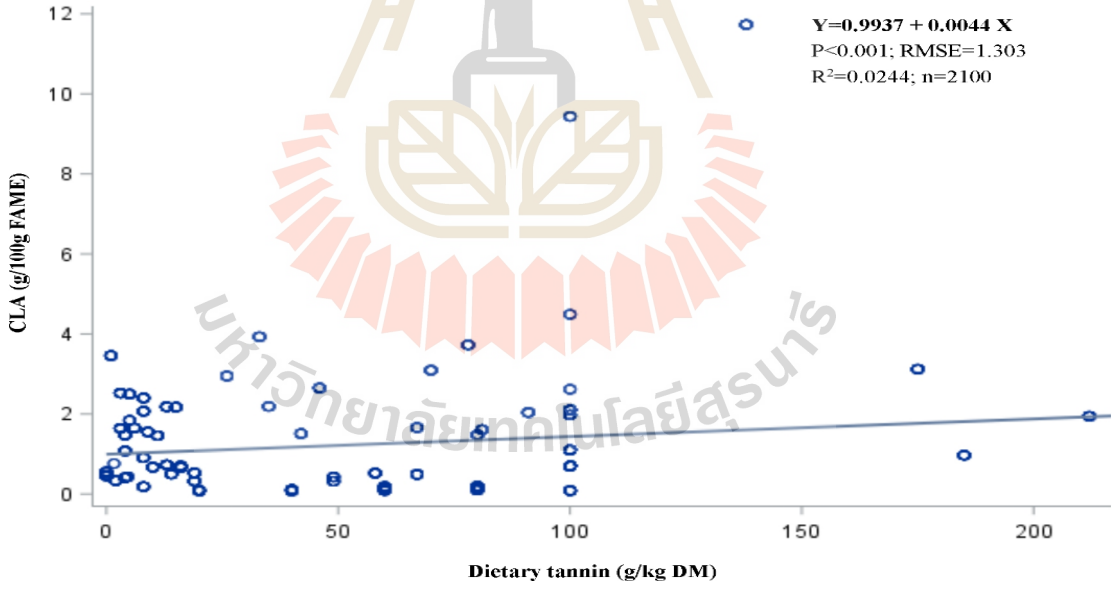
Table 4.5 Continue.

Response parameter	N	Parameter estimation							
		Intercept	SE intercept	*P* intercept	Slope	SE slope	*P* slope	RMSE	R^2
FA profile in longissimus dorsi muscle (g/100 g FAME)									
C18:2 cis-9, trans-11(CLA)	1034	0.0083	0.0007	<0.0001	0.0059	0.0018	0.0002	0.4590	0.6711
C18:1 trans-1	1034	0.0012	0.0017	0.8516	0.0072	0.0023	0.0107	1.1123	0.5350
C18:0	1034	0.1145	0.0090	<0.0001	-0.0057	0.0008	0.0001	5.8240	0.6131
SFA	1034	0.1386	0.0079	<0.0001	0.0144	0.0009	0.0653	5.0920	0.2321
MUFA	1034	0.0165	0.0092	0.0031	0.0149	0.0020	0.0001	5.9406	0.3152
PUFA	1034	0.0803	0.0115	0.0002	-0.0227	0.0009	0.2882	7.4543	0.4526
Desaturation index	1034	0.0008	0.0001	0.0031	0.0238	0.0091	0.0251	0.0454	0.1137
CLA index	1034	0.2252	0.0118	<0.0001	0.0122	0.0025	0.0009	7.2738	0.3324

[a]Outcomes are averages deriving from tabulated data in Tables 4.2-4.3 calculated by proc mixed. C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; VFA, volatile fatty acid; FA, fatty acid; FAME, fatty acid methyl esters; CLA, conjugated linoleic acid; SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acid; PUFA, poly-unsaturated fatty acid; DM, dry matter; OM, organic matter; N, total data used; SE, standard error; P, p-value; RMSE, residual mean square error; R^2, coefficient of determination. Desaturation and CLA indices were calculated as given by Corl et al. (2001) and Schennink et al. (2008) reports, respectively.



(a)



(b)

Figure 4.5 The regression relationships between the *in vitro* and *in vivo* CLA deposition. (a) in milk, (b) in meat.

4.6 Discussion

Regardless of previous publications, including meta-analyses, in which methane mitigation by dietary tannins in ruminant feeding regimens was critical, there are, to our knowledge, no investigations on altered biohydrogenation improvements in CLAs due to dietary tannin supplementation supported by numerical data collection. A prior meta-analysis regarding dietary FAs and feeding regimens found variable C18 FA amounts in milk, revealing an inextricable link with different types of feeding regimens (Khiaosa-ard et al., 2015). Consistent with other summaries, C18 FA concentrations in meat were modified through feeding approaches, such as supplementation with dietary C18 FAs (Dhiman et al., 2005). The C18 FA profiles had curvilinear relationships with CLAs, which were synthesized in an extremely limited range of 0.34-1.07% in total fat in milk and 0.12-0.68% in total fat in meat. These studies considered multiple ruminant diets; breeds; ages; antinutritional feed additives, such as ionophores; and synthetic mixtures of dietary CLAs. The results of the present meta-analysis of data on FA supplementation regimes, breeds, ages and applied techniques did not detect a bias, indicating the sole role of tannins in CLA deposition. As expected, the present results accurately predicted the suitable level tannins *in vitro* and *in vivo* and thus confirmed the results under *in vitro* to *in vivo* conditions.

4.6.1 The types of dietary tannins as regulators of ruminal biohydrogenation

The results of the present meta-analyses showed that dietary tannins in ruminant diets modulated CLA synthesis, inhibiting ruminal biohydrogenation. From a chemical standpoint, tannins are polyphenolic biomolecules that are roughly defined by broadly diverse oligomers and polymers. Frutos et al. (2004b) reviewed applied tannin classifications with regard to ruminant benefits. Before an in-depth understanding of

this potent secondary metabolite was reached, tannins had been commonly separated into two groups. HTs, or pyrogallol-type tannins, are created from carbohydrate cores and hydroxyl groups. Gallic and ellagic acids are examples of these tannin groups, and sometimes these tannins are bonded to other hydroxyl compounds such as flavonoids (Purba and Paengkoum, 2019). The second group is CTs, which can be defined as nonbranched polymers with poor carbohydrate contents; this group has a higher molecular weight than HTs (Mueller-Harvey, 1999). There is also another group of tannins characterized as catechins (i.e., tannins present in green tea leaves). Similar to condensed tannins, they also have a characteristic carbon skeleton in their structure without sugar residues (Ignat et al., 2011). A mixed tannins (MTs) (composed of HT and CT compounds) have sometimes been found in some plants.

Over the past 3 decades, CTs, HTs and MTs have been applied to ruminant diets *in vitro* and *in vivo* as regulators of ruminal biohydrogenation. Chestnut (HT), oak (HT), quebracho (CT), and grape seed (CT) at 20, 40, 60 and 80 g/kg, respectively, increased the PUFA content of FAs in the rumen content after 24 hours of incubation, whereas doses of 80 g/kg tended to promote the accumulation of cis-9, trans-11 CLA (Carreño et al., 2015). An investigation involving a shorter incubation time of 6 h by Costa et al. (2017) found that grape seed (CT) increased the disappearance of C18 FA in the diet, resulting in the increased production of cis-9, trans-11 CLA and VA, with a remaining amount of SA. It seemed likely that CTs had the ability to control the production of PUFAs and MUFAs in ruminal biohydrogenation, and the CLA transformation from dietary unsaturated fatty acids (UFAs) in the diet occurred rapidly. However, Jayanegara et al. (2012a) monitored 18 species of alpine forage plant for the disappearance and appearance of C18 profiles, and CT and HT

concentrations were compared. HTs but not CTs showed a clear positive correlation with the appearance of cis-9, trans-11 CLA. Jayanegara et al. (2011) also conducted a systematic investigation of the effects of 27 tropical forages (mainly tree and shrub leaves) on the extent of biohydrogenation. The MT contents of tropical forages had positive correlations with the production of cis-9, trans-11 CLA (R^2=0.27) and negative correlations with the production of SA (R^2 = 0.18), consistent with other reports that observed alternations in milk and meat (Toral et al., 2011; Rana et al., 2012; Willems et al., 2014; Buccioni et al., 2017b; Lobón et al., 2019).

Despite the increase in cis-9, trans-11 CLA and decrease in SA, dietary CTs, HTs and MTs had a slight impact on total VFA and ruminal microorganisms (Szczechowiak et al., 2016; Toral et al., 2016; Costa et al., 2018). Decreasing concentrations of VFA and *Butyrivibrio proteoclasticus* (SA producer) occurred with increasing cis-9, trans-11 CLA due to supplementation with CT and HT at doses of 100 g/kg DM. CTs had greater efficiency in reducing SA producers than HTs (Costa et al., 2018). However, these supplements retained cis-9, trans-11 CLA. In contrast, the same CTs dose derived from *Cistus ladanifer* extract did not change the total VFAs and increased degraded available PUFAs in the diet due to the isomerization of cis-9, trans-11 CLA (Guerreiro et al., 2016). Further, a relatively low dose of other CT sources, such as *Vaccinium vitis-idaea* dosed at 4.5 g/kg DM, was confirmed to reduce SA producers, demonstrated by the declining relative DNA abundance of *Butyrivibrio proteoclasticus* (Szczechowiak et al., 2016). In addition, other tannin levels were tested by Vasta et al. (2008) using CT sources from carob (*Ceratonia siliqua*), Acacia leaves (*Acacia cyanophylla*) and quebracho (*Schinopsis lorentzii*) at doses of 0.06-0.1 g/kg DM for their effects on ruminal biohydrogenation *in vitro*. Tannins increased VA by 23%

and decreased SA by 16% of the total FAs without altering the total VFAs, which was consistent with other observations using oak (HT) at a dose of 20 g/kg DM (Carreño et al., 2015).

Thus, the types of dietary tannins seemed to have identical effects on ruminal biohydrogenation. Considering a sufficient FA content in the diet, bioactive tannins exhibited promising antimicrobial properties in many studies and could be inhibitors of lipogenic processes, resulting in increased production of cis-9, trans-11 CLA and VA in ruminant-derived products. However, the doses of dietary tannins should be reconsidered regard of unexpected secondary metabolite behavior of FA synthesis in relation to other aspects of the rumen.

4.6.2 The effects of dietary tannins on CLA biosynthesis

CLAs are natural UFAs and are defined as primary intermediate products generated from their shortened conjugated precursors (LA and ALA) by lipolysis, isomerization and biohydrogenation by rumen lipid microorganisms. During biohydrogenation, CLAs in the cis-9, trans-11 18:2 form and their derivatives are isomerized rapidly (Tanaka, 2005; Lourenço et al., 2010); subsequently, the remaining substrate terminates biohydrogenation. Primary CLA production occurs by desaturation by removing two hydrogen atoms from trans-11 18:1 to form cis-9, trans-11 18:2 with the $\triangle^{9}$-desaturase enzyme (Dhiman et al., 2005; Vasta et al., 2009; Buccioni et al., 2017a). In other words, the method of synthesizing CLAs involves altering biohydrogenation pathways by modifying CLA precursors in the diet (Dhiman et al., 2005).

The CLA precursors as LA and ALA in forages, cereals and oils sometimes also contain cis-9 18:1 (OA: oleic acid) in a low amount as a dietary UFA.

Additionally, if the feeding regimen uses a grazing system, whether the natural polyphenols in the diet contain tannins should be reconsidered (Villalba et al., 2004). The present meta-analysis, which did not detect a bias, revealed that dietary tannins in animal diets had linear relationships with increased concentrations of cis-9, trans-11 CLA and VA in milk and meat. Forages that produce tannins could be considered additional organic matter for animal diets rich in FAs. This finding was reliable, as the results were in accordance with those of a previous meta-analysis by Średnicka-Tober et al. (2016) who observed cis-9, trans-11 CLA and VA in excessive concentrations in milk from animals maintained on organic farms that consumed more forage than those on conventional farms, consistent with other studies (Khiaosa-ard et al., 2015). Notably, other factors, such as plant wilting and leaf breakage during hay production (and to some extent prior to ensiling), in conventional farming systems may be important, as decreasing FA concentrations in forage may result from oxidative loss (Dewhurst et al., 2006). However, natural fresh tannins and other secondary metabolites of pasture plants in organic grazing systems are inevitably consumed by animals, resulting in optimum FA intake, which seems to encourage subsequent biohydrogenation (Jayanegara et al., 2011).

Lourenço et al. (2010) summarized the role of rumen microbial lipids in practical biohydrogenation. When CLAs were synthesized in the first biohydrogenation, group A of bacteria (*Butyrivibrio fibrisolvens*) was shown to produce CLAs from C18 FA with the enzyme linoleate isomerase, whereas lipolysis, isomerization and biohydrogenation were undertaken by group B of bacteria (*Butyrivibrio proteoclasticus*). Eighteen Comisana ewes at 122±6 d in milk were provided quebracho (CT) and chestnut (HT) at doses of 52.8 g/kg DM, and the presence of *Butyrivibrio*

fibrisolvens and *Butyrivibrio proteoclasticus* in the rumen liquor was observed and compared (Buccioni et al., 2017b). The presence of *Butyrivibrio fibrisolvens* increased (5-fold) in ewes fed the CT diet and (3-fold) in ewes fed HT diet, when it was compared with rumen liquor from ewes fed the control diet. The presence of *Butyrivibrio proteoclasticus* decreased (15-fold) in ewes fed the CT diet and (5-fold) in ewes fed the HT diet, similar to other results in different breed (Miri et al., 2015; Buccioni et al., 2017a). However, the mechanisms by which tannins reduce *Butyrivibrio proteoclasticus* in different dietary components (CTs vs. HTs) are not entirely clear considering various basal substrates, tannin dose (type included), age, breed and environmental farming system (Chilliard et al., 2003; Chilliard and Ferlay, 2004; Frutos et al., 2004b). In comparison, twelve multiparous Polish Holstein-Friesian cows (600±30 kg body weight) in their 5-6th month of lactation were fed a basal diet with *Vaccinium vitis-idaea* extract at 4.5 g/kg DM, which unfortunately resulted in a nonsignificantly different relative abundance of *Butyrivibrio proteoclasticus* and *Butyrivibrio proteoclasticus* DNA.

Obviously, the relatively high amounts of CLAs and VA and relatively low amounts of SA in the present meta-analysis revealed that the secondary metabolites like tannins had a greater ability to suppress *Butyrivibrio proteoclasticus* than to suppress *Butyrivibrio fibrisolvens*. The putative reason for these differences is the difference in the semipermeable membrane in these bacteria. *Butyrivibrio fibrisolvens* likely has durable impermeable barrier (Behbahani et al., 2018). As a result, the effect of dietary tannins was halted by the outer membrane of this gram-negative organism.

4.6.3 The effects of dietary tannins on de novo synthesis and endogenous desaturation

Regarding CLAs in milk synthesis, after UFA incorporation into the diet of ruminants, two consecutive metabolic processes occur. First, triglycerides from the diet (LA 85%; ALA 93%, (Chilliard et al., 2007)) are degraded into free FAs, expressed as rumen-escaped PUFAs in the mammary gland (elucidated above), thereafter undergoing *de novo* FA synthesis and secreted as short- and medium-chain FAs in milk (Sc and Mc FAs). Lower CLA and VA contents were found in milk from Saanen goats fed Pornunca silage-based diets containing different levels of tannins (11, 28, 36 and 44 g/kg DM) (de Lucena et al., 2018). It seems likely that the dietary tannins encouraged *de novo* FA synthesis to transform CLA and VA to Sc and Mc FAs. However, this was inconsistent with the results of Kälber (Kälber et al., 2014) who supplemented diets with different vegetative and reproductive stages of buckwheat, chicory, and phacelia at doses of 4.19-14.91 g/kg DM, resulting in inhibited *de novo* FA synthesis. Hence, these results could indicate that dietary tannins influence *de novo* FA synthesis irregularly in the mammary gland depending on the density of tannins (Frutos et al., 2004b; 2004a).

In secondary metabolism, SFAs that are produced by RBH are absorbed via plasma in the mammary gland. Similar to the first metabolic process, RBH partly synthesizes simple SFAs (almost all 4:0-14:0 and 50% of 16:0), which are subsequently transformed into FAs in milk. The remaining RBH substrate was initially VA that was maintained at the desaturation stage (Chilliard et al., 2007). However, in term of tannin supplementation, VA is richer in RBH due to a secondary metabolite of tannins that possesses a SA producer (Miri et al., 2015; Buccioni et al., 2017a; Buccioni et al., 2017b). In addition, other rumen microbial lipids, as well as protozoa and fungi, should

be reconsidered. Tannins have been extensively reported in previous studies (Szczechowiak et al., 2016; Vargas et al., 2017; Gomaa et al., 2018), and decreasing the total amount of ruminal protozoa was the predominant effect.

Evidence of lipolytic activity in protozoa is strongly inconsistent (Lourenço et al., 2010). Recent publications have shown that there is a positive relationship between the abundance of rumen ciliated protozoa and the proportion of cis-9, trans-11 CLA and VA; this relationship has been shown for holotrichs, entodiniomorphids, *Isotricha* and *Epidinium* (Francisco et al., 2019). Additionally, a linear relationship between decreasing SA and increasing amounts of total ciliates, holotrichs, entodiniomorphids and *Isotricha* has been shown (Francisco et al., 2019). In addition, rumen protozoa, especially *Epidinium* spp., were reported to account for approximately 30-40% of lipolysis activity by adhering to the plant surface (possibly in feedstuff) using an anterior pleated zone, resulting in the easy breakdown of cellular membranes (Huws et al., 2009). Hence, rumen protozoa were confirmed to be a rich source of PUFAs, especially CLAs and VA, due to the ingestion of more chloroplasts (Kim et al., 2009), but these organisms did not induce $\triangle^9$ desaturation (Devillard et al., 2006; Kim et al., 2009) associated with bacteria lipids. In other words, obtained CLAs and VA by protozoa were not synthesized from available FAs and/or SA (Lourenço et al., 2010). Additionally, considering only the contribution of protozoa in biohydrogenation with respect to the activity of ingested or associated bacteria, Lourenço et al. (2010) suggested limiting protozoa because *Butyrivibrio fibrisolvens* had more important role in synthesizing CLAs, affecting ruminal biohydrogenation.

In term of CLAs in meat or longissimus dorsi muscles, CLA biosynthesis occurs through a similar process, and the influence of dietary tannins is comparable to

that in CLAs in milk (Vasta et al., 2009; Toral et al., 2011; Rana et al., 2012; Kälber et al., 2014; Szczechowiak et al., 2016; Lobón et al., 2019). That is, the different rumen microorganism lipids interfered with biohydrogenation enzymes, but FA transformation was essentially equal. The FA composition, especially the CLAs in ewe milk or suckling lamb meat, was observed in thirty-nine ewes and lambs offered 10% quebracho tannins in their total diets (2 g/kg DM) (Lobón et al., 2019), and differences between pasture forage and hay in dams were included. The ewes and lambs that produced the target CLAs were compared and exhibited a 94±10.4% similarity score. According to this result, Lobón et al. (2019) suggested that the use of quebracho to improve biohydrogenation should be wisely excluded. In addition, *Acacia mearnsii* extract was investigated in serial doses at 10, 30 and 50 g/kg DM in total mixed rations (TMRs), forage and concentrate (40:60), and the concentrations of PUFA in meat were observed (Gesteira et al., 2019). The desaturase index was reported to be increased after supplementation with dietary tannins, but the amount of CLAs was decreased. This result indicated that *Acacia mearnsii* extract (CT) affected the animal performance with regard to the concentration of PUFAs in milk and meat. Regarding tannins, the differences in the desaturation rate in dairy and beef cattle were associated with FA availability in diets and feeding regimens, as shown in previous studies (Dhiman et al., 2005; Chilliard et al., 2007; Khiaosa-ard et al., 2015).

Dietary tannins have been well known not only to suppress antimicrobial properties but also to indirectly influence $\triangle^{9}$-desaturase expression by regulating fat and protein absorption (Ntambi, 1995; Vasta et al., 2009; Rana et al., 2012). Nonetheless, regulating these metabolisms depends on FA substrate availability in the mammary gland and/or adipose tissue (Urrutia et al., 2015; Szczechowiak et al., 2016).

Hence, this feature of biohydrogenation cannot be excluded. The present meta-analysis showed that desaturase and CLA indices increased when dietary tannins increased with decreasing SFA content. Notably, ruminant fat is more saturated than fat from monogastric animals. This result might suggest that the enzymes involved with SFA (i.e., acetyl-CoA carboxylase and FA synthase) have been activated by bioactive tannins, tending to have a greater performance of $\triangle^9$-desaturase. This suggestion is supported by studies (Priolo and Vasta, 2007; Vasta et al., 2009; Rana et al., 2012) showing that a $\triangle^9$-desaturase activity is fairly responsive to the dietary tannins as interpreted by increased concentrations of MUFAs and PUFAs. Ultimately, there is a putative theory that bioactive tannins (CTs and HTs) with abundant FAs in the diet are regulators that indirectly influence the endogenous desaturation process via alterations in absorbed FA and protein. In other words, bioactive tannins seem to have multiple roles in modulating the endogenous processes by changing microorganisms and enzymes with abundant FA substrates and suitable doses of tannins. The present meta-analysis also confirmed that desaturation rates between dairy and beef animals were relatively comparable to dietary tannins.

4.6.4 The susceptibility of the observed techniques to CLA biosynthesis

The results of *in vitro* observations were examined to confirm the utility of *in vivo* studies when the results are compared in the same unit. According to the present meta-analysis, the units for CLA and dietary tannins were consistently g/100 g FAMEs and g/kg DM, respectively. However, CLA production derived from *in vitro* studies represented only semi actual biohydrogenation. The diets containing FAs and tannins were isomerized by only rumen microorganisms from rumen donors in the first step of biohydrogenation. Nevertheless, the majority of CLA biosynthesis endogenously

occurs during the desaturation stage and utilizes lipid enzymes from the digestive tract (Dhiman et al., 2005; Chilliard et al., 2007; Khiaosa-ard et al., 2015). Compared to the respective controls, CLA concentrations from consecutive observations, namely, the rumen stimulation technique (RUSITEC) using cannulated cows and productive cows *in vivo*, showed inconsistent results (Szczechowiak et al., 2016). This finding indicated that it was difficult to predict *in vivo* results considering *in vitro* CLA biosynthesis. In contrast, Lobón et al. (2019) conducted *in vivo* studies on ewes and lambs and found comparable CLA production values. The *in vitro* approach provides a low-cost starting point to screen for an increase in CLA contents due to bioactive tannins; however, the provision of tannin regulators in diets enriched with FAs *in vivo* is strongly recommended.

4.7 Conclusion

This meta-analysis, which included a large amount of data from valid publications and did not detect a bias, provides a prediction of suitable dietary tannins as extracts or plants that could be supplemented in rumen diets with a fit design to modulate the effect of CLA synthesis on biohydrogenation. The recommended doses of dietary tannins did not exceed 20 g/kg DM and 80 g/kg DM for *in vitro* and *in vivo* studies, respectively. However, the ratios of forage to concentrate were similar (nearly 50:50). *In vitro* studies without animals may be rapid, simple and low-cost approaches, but the results sometimes exhibit unexpected and questionable outcomes. Hence, the *in vivo* approach was more suitable for the direct observation of FA transformation. Further, the results suggest the critical need to identify or select the origin of tannins. If tannins are derived from commercial products/extraction, the purity of tannins should

be strictly evaluated. Likewise, regarding tannins from plants, specific tannin-binding polymers and other hydroxyl groups should be widely considered. More research on other hydroxyl groups, such as flavonoids, is required to gain a better understanding of the extent of CLA synthesis (not only cis-9, trans-11 18:2 isomers but also trans-10, cis-12 18:2 isomers).

It is noteworthy that dietary tannins or active plant compounds from *Piper betle* L. to have enhancements in animal response, especially rumen biohydrogenation. However, next chapter of present study might provide observations in both of *in vitro* and *in vivo* studies. Dose, approach and suggestion of this systematic review including meta-analysis may support it fairly.

4.8 Acknowledgements

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CHAPTER V

ENHANCED CONJUGATED LINOLEIC ACID OF RUMINAL BIOHYDROGENATION AND ALTERED BIOGAS PERFORMANCE DUE TO SUPPLY OF FLAVONOID AND ESSENTIAL OIL MATRIX FROM PIPER POWDER

5.1 Abstract

The objective of present study was to investigate the influence of various PP doses on *in vitro* ruminal biogas, fermentation end-product, and biohydrogenation including lipolysis-isomerization and rumen microorganism. The study was designed as a completely randomized design with six doses of PP supplementation at 0, 5, 25, 50, 75 and 100 mg/incubation DM incubated with 400 mg basal substrate comprised of Pangola hay and concentrate (50:50). The matrix compounds (g/kg DM) of 0.27 catechin, 0.11 rutin, 3.48 quercetin, 0.41 apigenin, 0.04 myricetin, 0.27 kaempferol and 0.76 eugenol derived from PP altered the fermentation pattern: increased degradable nutrient and total volatile fatty acid, with exhibiting acetogenesis without shifting pH during fermentation. These values favoured more production of total gas kinetic, with higher carbon dioxide and lower methane production. Even though, hydrogen recovery on lipolysis-isomerization of biohydrogenation was limited, enhanced PP supply successfully possessed SA accumulation through changing biohydrogenation pathway of fatty acids referring to more C18:1 trans-11 rather than C18:2 trans-11, cis-15. As consequence, this action resulted a higher of CLA cis-9, trans-11, CLA trans-10, cis-12

and CLA trans-11, cis-13. In addition, enhanced PP supply surged total bacteria and fungal zoospore due to alleviation of rumen protozoa. In conclusion, results demonstrate that the PP is potential feed additive promising the valuable results of rumen fermentation and biohydrogenation with alleviating greatly methane production.

Keywords: Rumen, polyphenol compounds, feed additive, organic compounds, fatty acids.

5.2 Introduction

There is interest in improving quality of milk and meat produced from ruminant. Regard of fat properties, CLA is valuable fat product and one of concerned factors by consumers that could be manipulated by different feeding strategies in animal diet. From practical standpoint, feeding forages in animal diet have demonstrated an abundant lipid content (cis-9, cis-12-18:2) in the form of triacylglycerols that is the main substrate for biohydrogenation, thereby resulting a C18:2 cis-9, trans-11 as the predominant CLA isomer (Lourenço et al., 2010). Besides, there were several studies regard of feeding forage with inclusive secondary plant compounds that can affect positively on rumen metabolites, including fermentation pattern and its derivatives in nutrient degradation such as rumen methanogenesis (Cherdthong et al., 2019) and biohydrogenation (Durmic et al., 2008; Cabiddu et al., 2010; Jayanegara et al., 2012a). These might be strong evidences that supplemental feed additive containing polyphenol compounds is one of key players to modulate either rumen fermentation reflected to alternation of biohydrogenation pathway and alleviation of methane production derived from domestically ruminant.

Piper betle L. is tropical, edible and affordable plants which was reported owning a host of potent polyphenol compounds, such as flavonoids and essential oils (Purba and Paengkoum, 2019). Lourenço et al. (2014) addressed that incubated forage with quercetin and eugenol performing no affection in C18:2 cis-9, cis-12 yield, yet, exhibiting a roughly affection of alternative biohydrogenation pathway of C18:3n-3. However, Durmic et al. (2008) reported that selected Australian plants as CLA-degrading inhibitor was successfully to possess targeted ruminal bacteria, involved on biohydrogenation yielding more CLA concentration. Unfortunately, non-specific bioactive compound of this result is unknown. Since, *Piper betle* L. had other flavonoids and essential oils namely catechin, rutin, apigenin, myricetin and kaempferol, it was hypothesized that *Piper betle* L. might change potentially fermentation pathway regard of pyruvate stage and biohydrogenation. Therefore, the objective of present study was to investigate the influence of various PP doses on *in vitro* ruminal biogas, fermentation end-product, and biohydrogenation including lipolysis-isomerization and rumen microorganism.

5.3 Materials and methods

All experimental procedures were approved and completed in accordance with the Rules of Animal Welfare of Suranaree University of Technology (SUT 4/2558) for animal protection used and/or applied for experimental purposes.

5.3.1 Animal, feed, PP and experimental design

Piper betle L. leaves were planted, harvested and pooled from randomly spots at SUT organic farm, Nakhon Ratchasima, Thailand (elevation of 243 m above sea level). A material leafy was remove from earth dust and kept under 4°C overnight to

avoid any nutrient destruction (Purba and Paengkoum, 2019). The leaves were made a powder (hereafter referred to PP) as particle passed a 1-mm sieve and loaded into plastic seal dispatched to desiccator until usage time. In addition, three female Saanen goats (40±1.51 kg body weight) were prepared as rumen donor and fed with a TMR, consisting of Pangola hay and concentrate (50:50) including *ad libitum* water (NRC, 2007). This feed was also respected as basal substrate in *in vitro* experiment. Animals were assigned to feed TMR for 18 d with 15 d of adaptation period and 3 d of sampling period. Formulation and chemical composition of PP and feed are presented in Table 5.1. The study was designed as a completely randomized design with six levels of PP at 0, 5, 25, 50, 75 and 100 mg/incubation DM incubated with 400 mg of basal substrate. Each glass syringe was supplemented with sunflower oil dosed of 8 g/kg DM, which contained (g/kg FA): 16:0 (51.07), 18:0 (27.36), cis-9 18:1 (355.43), 18:2n-6 (422.24) and 18:3n-3 (1.74). PP and sunflower oil were emulsified in 1:99 v/v ethanol 96% and aqueous solution, respectively, then added into glass syringe.

5.3.2 *In vitro* experiment and sampling

In vitro experiment was conducted using Hohenheim gas test method were performed following earlier protocol by Menke and Steingass (1988) as modified by Paengkoum et al. (2015). On 16, 18 and 20 d of sampling stage, rumen fluids were suctioned from rumen via oral lavage by suction pump (Hitachi CV-SF18, Japan) before morning feeding time, avoiding saliva collection (Lodge-Ivey et al., 2009). Rumen fluids were strained using a nylon membrane (400µm; Fisher Scientific S.L., Madrid, Spain), prepared to mix with artificial buffer (1:2, ml:ml) under CO_2 and kept 39°C for 24 h. The composition of rumen-fluid buffer mixture was as follows: 474 ml rumen fluid, 0.60 g $MgSO_4.7H_2O$, 1.32 g $CaCl_2.2H_2O$, 0.10 g $MnCl_2.4H2O$, 0.10 g

$CoCl_2.6H_2O$, 0.80 g $FeCl_3$.6H2O, 35 g $NaHCO_3$, 4 g NH_4HCO_3, 5.70 g Na_2HPO_4, 6.20 g KH_2PO_4, 10 mg resazurin and 0.40 g NaOH, made up to 1000 ml by distilled water (Appendix C). The incubated preparation was finished after a hundred of glass syringe was filled with 400 mg substrate, concerned PP doses and 30 ml rumen-fluid buffer mixture. Each treatment was prepared in ten replicates in 3 runs (three glass syringes of blank containing only rumen-fluid buffer mixture per run).

After 24 h incubation, total gas production was fitted and read based on Orskov and Mcdonald (1970) model. Then, a 30 ml gas of glass syringe was forwarded to measure methane and carbon dioxide levels by injecting into gas chromatography machine (Agilent 7890A, USA). Glass syringe was kept on ice to impede fermentation (Jayanegara et al., 2012a). Once glass syringe uncapped, pH of fermented content was directly measured using pH meter (Oakton 700, USA). The fermented content was divided into 4 portions. The first portion was centrifuged at 6,000×g at 4°C for 15 min and the supernatant was stored at -20°C. For volatile fatty acid (VFA) analysis, supernatant was fixed with 25% metaphosphoric acid (Erwin et al., 1961; Filípek and Dvořák, 2009). Rumen ammonia observation was performed in accordance with the micro-Kjeldahl methods (Foss Kjeltech 8100, USA) (AOAC, 2005). The second portion was stored at -20°C for FA analysis. The third portion was treated with 10% formalin solution in a sterilized 0.9% saline solution for observation and calculation of total bacteria, protozoa and fungal zoospore using a counting chamber (Neubauer, Germany) as description by Wanapat et al. (2013). The last portion was prepared for *in vitro* dry matter degradability (IVDMD) and *in vitro* organic matter degradability (IVOMD) following previous method (Tilley and Terry, 1963) using pre-weighed Gooch crucibles.

Table 5.1 Ingredient and chemical composition of concerned treatments.

Item	**Feed**	**Piper powder**
Ingredient g/kg DM		
Dehydrated Pangola hay	500	
Cassava chip	170	
Rice bran	70	
Molasses	40	
Palm meal	100	
Soybean meal	100	
Urea	9	
Sulphur	1	
Mineral[1]	8	
Premix[2]	2	
Chemical composition, g/kg DM		
Organic matter	870.7	778.83
Crude protein	129.51	25.56
Crude fat	22.54	3.39
Neutral detergent fibre	684.73	639.36
Acid detergent fibre	545.36	509.07
Gross energy, MJ/kg DM	22.87	17.69
Total polyphenols compounds, g/kg DM		
Catechin	-	0.27
Rutin	-	0.11
Quercetin	-	3.48
Apigenin	-	0.41
Myricetin	-	0.04

Table 5.1 Continue.

Item	Feed	Piper powder
Kaempferol	-	0.27
Eugenol	-	0.76
Fatty acid profile, g/100 g FA		
C14.0	4.82	1.92
C16.0	22.01	23.54
C18.0	4.09	5.14
C18:1n-9	20.68	13.52
C18:2n-6	20.06	18.50
C18:3n-3	0.21	-

[1]Contained (g/kg): NaCl (600), P (160), Ca (240).

[2]Vitamin A (4,200.000 IU/kg), vitamin A_3 (840,000 IU/kg), vitamin E (10,000 IU/kg), vitamin K_3 (2 g/kg), vitamin B_1 (2.4 g/kg), vitamin B_2 (3.5 g/kg), vitamin B_6 (1.8 g/kg), vitamin B_{12} (0.01 g/kg), vitamin B_5 (4.6 g/kg), vitamin C (12 g/kg), folic acid (0.28 g/kg), coper (12 g/kg), manganese (40 g/kg), zinc (3.2 g/kg)), iron (42 g/kg), iodine (0.8 g/kg), cobalt (0.8 g/kg), selenium (0.35 g/kg).

5.3.3 Laboratory analysis and calculation

The samples of substrate and PP were chemically analysed for dry matter (DM), organic matter, ash, crude protein (total N × 6.25) and crude fat following prior protocol (AOAC, 2005). Acid detergent fibre and neutral detergent fibre were measured by earlier description (Van Soest et al., 1991), as residual ash included. The gross energy was determined using a bomb calorimeter with O_2 carrier (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL) according to the manufacturer's instructions. Concentration of rutin, apigenin, quercetin, kaempferol, myricetin and

eugenol was determined by a mean peak rate of signal in HPLC DAD principle (Purba and Paengkoum, 2019). All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate (Table 5.1).

FA measurement was conducted as guidelines by Folch et al. (1957), with modification (De Weirdt et al., 2013) using gas chromatography machine (Agilent 7890A, USA) with a CP-Sil88 column for FA methyl esters (100 m×0.25 mm×0.2 µm; Chrompack Inc., Middelburg, the Netherlands). Heptadecanoic acid (C17:0; Sigma-Aldrich) was applied as internal standard (Lashkari and Jensen, 2017) and C17:0 value was excluded in calculation. The column temperature was kept at 70°C for 4 min, then increased at 13°C/min to 175°C and held for 27 min, then increased at 4°C/min to 215°C and held for 17 min, then increased at 4°C/min to 240°C and held for 10 min. More detailed information about the detection and calculation method of peak area is given by Lourenço et al. (2014). The FA profile of feed and PL is presented in Table 5.1. Further, calculation of efficiency of lipolysis + isomerization (C18:2 n-6 → C18:2 cis-9, trans-11) and hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations were calculated in line with Boeckaert et al. (2007) as modified by Panyakaew et al. (2013) as shown in Table 5.4.

The fixed supernatant of 2 µl VFA sample was injected to gas chromatography machine (Hewlett Packard GC system HP6890 A; Hewlett Packard, Avondale, PA, USA) following earlier study by Erwin et al. (1961), with modification (Filípek and Dvořák, 2009). Acetic acid, propionic acid, iso-butyric acid, butyric, iso-valeric acid and valeric acid (Carlo ebra, France) were prepared with 1% formic acid and considered as standard calibration. Also, hydrogen recovery (HR) was calculated as (2 Propionate+2 Butyrate+4 CH_4)/(2 Acetate + Propionate + 4 Butyrate),

with acetate, propionate, butyrate and CH_4 expressed as net molar productions (Marty and Demeyer, 1973).

5.3.4 Statistical analysis

Statistical analysis accounted for the completely randomized design using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc 2015). Data were analysed using the model:

$$Y_{ST} = A + B_S + E_{ST} \quad (1)$$

where Y_{ST} = the dependent variable, A = the overall mean, B_S = the influence of the various PP doses ($_S$ =1–6), and E_{ST} = the residual effect. Because of value in separately run revealing unvaried, results are presented as mean values with the standard error of the mean. Differences between treatment mean was calculated by Tukey HSD (Kaps and Lamberson, 2004). Orthogonal polynomial contrasts were used to estimate the PP effect in linear and quadratic. All differences among the means with $P<0.05$ were accepted as representing statistically significant differences.

5.4 Results and discussion

5.4.1 Ruminal biogases, fermentation end-products and microorganisms

Inclusion of PP in fermented substrate incubation on ruminal biogases, fermentation end-products and microorganisms after 24 h is presented in Table 5.2. The present matrix of flavonoids and essential oils derived from PP increased gradually total VFA corresponding to a pleasure of degradability manner, especially fermented organic matter. In this study, IVDMD and IVOMD rose within the enhanced PP supply in substrate fermentation. These remarkably results increased the total VFAs. Notably, total VFAs and degradability efficiencies were earlier found in similar values,

compared to the control after substrate incubated with quercetin, rutin, catechin and eugenol (Castillejos et al., 2006; Oskoueian et al., 2013; Ramdani, 2014). These contradictions were possibly assessed due to different applied basal substrate proportion and bioactive compound plant. The former studies were subjected the fermenters a 60:40 (Castillejos et al., 2006; Oskoueian et al., 2013)/30:70 (Ramdani, 2014) forage: concentrate diet, whereas in our study, a 50:50 Pangola hay: concentrate diet was applied. Also, those reports purchased a commercially product as concerned secondary compounds. Even Ramdani (2014) successfully extracted the catechin from tea, approaching way to obtain the purity, binding behaviour and hydroxyl nutrient of PP bioactive compounds might be tenacious reason why the final results were varied (Purba and Paengkoum, 2019), see Table 5.1. Moreover, escalating value of VFA reflected a greater fermented substrate by inhabitant rumen that could also be recognized in total production of gas kinetic during fermentation. In this study, plenty of gas kinetic was occurred after PP supplemented, however, gas production volume favoured to more carbon dioxide and less methane gas. Prohibited methane production depicting to a failed methanogenesis by use of flavonoids and essential oils had been reported in earlier review (Patra and Saxena, 2010), unfortunately, a factual elaboration about rutin, apigenin, myricetin, kaempferol and caryophyllene was limited. Therefore, a weakened methane gas proportion seemed rumen methanogenesis occurred sluggishly in this study, that addressed a shift of fermented pattern of VFA referring to acetate, propionate and butyrate fractions.

In present study, existing PP during fermentation resulted in higher acetate and butyrate production, but lower propionate, valerate and branched-chain VFAs (iso-acid fraction). In calculation, this study also showed inhibited hydrogen

supply (HR) to methanogenesis and biohydrogenation. According to prior studies, propionate from pyruvate undertaken a plenty of hydrogen consumptions resulted in worse methanogenesis (Moss et al., 2000; Tavendale et al., 2005). However, there was meta-analysis reported no significantly relationship between propionate surge and methane inhibition (Jayanegara et al., 2012b). In up-to-date publication, Greening et al. (2019) confirmed that hydrogen metabolism is a more complex and widespread trait among rumen microorganisms, where it claims the hydrogen yield was uptake by other innate consumers, not only methanogens, e.g. fumarate and nitrite reduction (*Selenomonas*) and acetogenesis (*Blautia*). In present study, PP supplementations increased acetate fraction, as more evidences hydrogen was tremendously consumed by acetogenesis (*Blautia*) and hydrogen supply was depleted for extent movement. As a result, valeric and iso-acid fraction in recently study was lower as consequence of less propionic fraction (Andries et al., 1987). All changes of fermentation behaviour above were unchanged pH rumen in all treatments, while an alteration occurred in deamination stage after PP supplemented into substrate incubation (Table 5.2). A range of pH and ammonia concentration in present study was 6.8-7.0 and 19.9-20.1 mg/100 ml, which were assessed acceptable for ruminal microorganism to survive in modulating microbial growth and fermentation efficiency (Ørskov and MacLeod, 1982).

As shown in Table 5.2, enhanced PP supply alleviated protozoa rumen. A decrease of protozoa number due to supplementation of secondary compounds had been reported in many ways (Castillejos et al., 2006; Zhou et al., 2011; Oskoueian et al., 2013; Andrés et al., 2016; Szczechowiak et al., 2016) and a primary reason is ability of bioactive compounds altering cell wall synthesis or nucleic acid synthesis of protozoa.

This reduction might be reason why bacteria and fungal zoospore increased within the time. Dehority (2003) mentioned that protozoa engulfing rumen bacteria an approximately 20,000 cells per hour. Thus, increased bacteria numbers were aftereffect of eliminated protozoa. Further, Newbold et al. (2015) reported that fungal zoospore growing up with low protozoal number, which indicated narrow competition between protozoa and fungal zoospore on catching substrate during fermentation. In different reports, Cherdthong et al. (2019) confirmed that fungal zoospore had a powerful defensive manner to survive on attack mode of bioactive compounds of this study.

5.4.2 Lipolysis, isomerization and biohydrogenation

Inclusion of PP in fermented substrate on ruminal FA profile after 24 h is presented in Table 5.3. Clearly, the present matrix of flavonoids and essential oils derived from PP performed inhibited-biohydrogenation properties, where abated apparent biohydrogenation of C18:2n-6 and C18:3n-3 as PUFAs were observed. Reference of shifting biohydrogenation including its lipolysis-isomerizing FA by using flavonoids and essential oils of PP is relatively limited, only catechin, quercetin and eugenol application available in literatures (Lourenço et al., 2014; Ramdani, 2014) for *in vitro* study. Obviously, in our study, PP encouraged rumen biohydrogenation to yield more considerable PUFA accumulation, e.g. CLA cis-9, trans-11, CLA trans-10, cis-12 and CLA trans-11, cis-13, less saturated fatty acid in C18:0 and retarded mono unsaturated fatty acid in accumulation of C18:1 trans-11 rather than C18:2 trans-11, cis-15. As shown in Table 5.4, enhanced PP supply interacting to first biohydrogenation pathway of C18:2n-6, resulted in accumulation of C18:2 cis-9, trans-11 rather than C18:2 trans-11, cis-15 as observed different value in other studies (Lourenço et al., 2014).

Table 5.2 The mean value of substrate incubated with PP on ruminal biogases, fermentation end-products and microorganisms after 24 h incubation.

Parameter[1]	PP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
pH	6.90	6.84	6.80	6.80	6.85	6.90	0.013	0.438	1.000
Total gas production (ml/g OM)	30.05^{d}	36.11^{c}	36.84^{c}	38.72^{b}	42.44^{a}	42.93^{a}	0.670	<0.001	<0.001
CO_2 (ml/g OM)	14.24^{d}	18.32^{c}	18.61^{c}	20.60^{b}	26.81^{a}	27.12^{a}	0.706	<0.001	<0.001
CH_4 (ml/g OM)	11.92^{a}	10.61^{b}	10.84^{b}	9.08^{c}	8.39^{d}	7.89^{d}	0.218	<0.001	<0.001
IVDMD (%)	49.89^{c}	51.44^{b}	51.56^{b}	52.42^{a}	52.87^{a}	52.66^{a}	0.179	0.016	0.009
IVOMD (%)	50.51^{c}	65.07^{b}	65.21^{b}	66.43^{a}	65.77^{ab}	64.99^{b}	0.872	<0.001	<0.001
NH_3-N (mg/100 ml)	19.70^{b}	20.66^{a}	20.72^{a}	20.55^{a}	19.90^{b}	19.98^{b}	0.066	0.996	0.029
Total volatile fatty acid (mM)	61.11^{c}	66.10^{b}	66.37^{b}	69.44^{a}	69.12^{a}	68.78^{a}	0.460	0.004	<0.001
Acetate (C_2) (mol/100 mol)	52.12^{d}	54.20^{c}	54.28^{c}	56.49^{b}	57.87^{a}	58.21^{a}	0.342	0.001	<0.001
Propionate (C_3) (mol/100 mol)	20.10^{a}	19.48^{b}	19.45^{b}	19.24^{b}	18.81^{c}	18.01^{d}	0.105	<0.001	<0.001
Iso-butyrate (mol/100 mol)	5.55^{a}	4.95^{b}	4.92^{b}	4.16^{c}	3.59^{d}	3.63^{d}	0.109	<0.001	<0.001

Table 5.2 Continue.

Parameter[1]	PP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
Butyrate (mol/100 mol)	10.20^{d}	10.68^{cd}	10.80^{cd}	11.41^{c}	12.33^{b}	12.87^{a}	0.141	<0.001	<0.001
Iso-valerate (mol/100 mol)	4.54^{a}	4.10^{b}	4.03^{b}	3.40^{c}	2.93^{d}	2.97^{d}	0.089	<0.001	<0.001
Valerate (mol/100 mol)	7.49^{d}	6.59^{d}	6.52^{d}	5.30^{c}	4.47^{b}	4.31^{a}	0.174	<0.001	<0.001
C_2:C_3 ratio	2.59^{d}	2.78^{cd}	2.79^{cd}	2.94^{c}	3.08^{b}	3.23^{a}	0.032	<0.001	<0.001
HR (mol/mol)	0.66^{a}	0.61^{b}	0.61^{b}	0.55^{c}	0.52^{c}	0.50^{d}	0.008	<0.001	<0.001
Total bacteria, × 10^7 cells/ml	5.14^{c}	5.18^{b}	5.21^{b}	5.30^{a}	5.25^{ab}	5.20^{b}	0.021	<0.001	<0.001
Total protozoa, × 10^5 cells/ml	4.95^{a}	4.23^{b}	4.11^{b}	3.98^{c}	3.64^{d}	3.44^{d}	0.075	<0.001	<0.001
Total fungal zoospore, × 10^5 cells/ml	3.13^{c}	3.10^{c}	3.10^{c}	3.14^{c}	3.21^{b}	3.52^{a}	0.022	<0.001	<0.001

[abc] Within a row, different superscript represents the significant differences by Tukey $P<0.05$.

[1]OM = organic matter; IVDMD = *in vitro* dry matter degradability; IVOMD = *in vitro* organic matter degradability; HR = hydrogen recovery (calculated in materials and methods (Marty and Demeyer 1973).

[2]SEM =standard error of mean.

[3]Orthogonal polynomial contrast $P<0.05$.

Table 5.3 The mean value of substrate incubated with PP on ruminal fatty acid profile (g/100 g FA) after 24 h incubation.

Parameter[1]	PP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
Total SFA[4]	65.851^{a}	63.142^{b}	62.840^{b}	61.746^{c}	61.498^{c}	61.585^{c}	0.257	0.006	0.002
C14.0	0.919^{c}	0.951^{a}	0.959^{a}	0.949^{ab}	0.940^{b}	0.919^{c}	0.003	0.132	0.171
C16.0	20.570	20.440	20.621	20.591	20.480	20.611	0.035	1.000	0.741
C18.0	36.734^{a}	33.314^{b}	33.588^{b}	32.519^{c}	32.388^{c}	32.368^{c}	0.245	<0.001	<0.001
Total MUFA[5]	22.120^{c}	25.096^{b}	25.302^{b}	26.446^{a}	26.644^{a}	26.501^{a}	0.248	<0.001	<0.001
C18:1 trans-6–8	0.638^{c}	0.710^{a}	0.716^{a}	0.726^{a}	0.656^{b}	0.652^{b}	0.005	<0.001	<0.001
C18:1 trans-9	0.486^{a}	0.459^{b}	0.463^{b}	0.480^{a}	0.470^{b}	0.461^{c}	0.002	<0.001	0.529
C18:1 trans-10	0.414^{c}	0.464^{b}	0.468^{b}	0.491^{a}	0.490^{a}	0.485^{a}	0.004	0.001	<0.001
C18:1 trans-11	2.476^{e}	5.383^{d}	5.428^{d}	6.439^{c}	6.681^{b}	6.782^{a}	0.231	<0.001	<0.001
C18:1 trans-15	0.959^{c}	0.975^{b}	0.983^{b}	0.993^{b}	1.008^{a}	0.953^{c}	0.003	0.004	0.004
C18:1 cis-9	5.222	5.201	5.244	5.251	5.252	5.252	0.009	1.000	0.996
C18:1 cis-11	0.762^{c}	0.746^{c}	0.752^{c}	0.769^{b}	0.787^{a}	0.695^{d}	0.005	<0.001	<0.001
C18:1 cis-15	0.717^{c}	0.756^{b}	0.762^{b}	0.794^{a}	0.795^{a}	0.718^{c}	0.051	<0.001	0.006
Total PUFA[6]	12.028	11.761	11.858	11.808	11.858	11.914	0.022	1.000	0.660
C18:2 trans-11, cis-15	0.038^{a}	0.038^{b}	0.037^{b}	0.035^{c}	0.035^{c}	0.033^{d}	0.003	<0.001	<0.001
CLA cis-9, trans-11	0.204^{c}	0.357^{b}	0.360^{b}	0.376^{b}	0.374^{a}	0.363^{a}	0.009	<0.001	<0.001
CLA trans-10, cis-12	0.010^{d}	0.020^{c}	0.020^{c}	0.020^{c}	0.021^{b}	0.022^{a}	0.063	<0.001	<0.001

Table 5.3 Continue.

Parameter[1]	PP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
CLA trans-11, cis-13	0.505[d]	0.524[c]	0.528[c]	0.537[c]	0.659[b]	0.735[a]	0.013	<0.001	<0.001
C18:2n-6	5.869[a]	5.471[b]	5.516[b]	5.445[bc]	5.375[c]	5.364[c]	0.028	0.001	0.001
C18:3n-3	0.152[a]	0.150[b]	0.151[b]	0.150[b]	0.148[c]	0.149[c]	0.032	0.186	0.998

[abc] Within a row, different superscript represents the significant differences by Tukey $P<0.05$.

[1]SFA= saturated fatty acids; MUFA= mono unsaturated fatty acids; PUFA= mono unsaturated fatty acids.

[2]SEM=standard error of mean.

[3]Orthogonal polynomial contrast $P<0.05$.

[4]Sum of all SFA: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0 and C23:0.

[5]Sum of all MUFA: C14:1 cis-9, C15:1 cis-9, C16:1 trans-9, C16:1 cis-9, C18:1 trans-6–8, C18:1 trans-9, C18:1 trans-10, C18:1 trans-11, C18:1 trans-12-14, C18:1 trans-15, C18:1 cis-9, C18:1 cis-11, C18:1 cis-12, C18:1 cis-13, C18:1 cis-14, and C18:1 cis-15.

[6]Sum of all PUFA: C18:2 trans-11, cis-15; C18:2 trans, trans isomers; C18:2 cis, cis isomers; C18:2 cis, trans isomers; CLA cis-9, trans-11; CLA trans-10, cis-12; CLA trans-11, cis-13; C18:2n-6; C18:3n-6; C18:3n-3; C20:2n-6; C20:3n-6; C20:3n-3; C20:5n3; C24:1n-9 and C22:6n-3.

Table 5.4 The mean value of substrate incubated with PP on the efficiency of lipolysis + isomerization (C18:2 n-6 → C18:2 c9t11) and hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations.

Parameter	PP dose (mg/incubation)						SEM[1]	Contrast[2]	
	0	5	25	50	75	100		Linear	Quadratic
C18:2 n-6→ C18:2 cis-9, trans-11	70.99[b]	72.73[a]	72.73[a]	73.07[a]	73.41[a]	73.45[a]	0.319	0.053	0.095
C18:3 n-3→C18:2 trans-11, cis-15	28.10	28.51	28.31	28.52	29.22	28.47	0.126	1.000	0.149
C18:2 trans-11, cis-15→C18:1 trans-11	35.59[d]	36.47[cd]	37.52[cd]	41.13[c]	42.32[b]	43.99[a]	0.471	<0.001	<0.001
C18:2 cis-9 trans-11→C18:1 trans-11	98.58	97.55	97.56	97.46	97.48	97.56	0.396	0.981	0.956
C18:1 trans-11→C18:0	83.83[a]	53.78[b]	53.72[b]	43.41[c]	41.52[d]	40.64[d]	2.338	<0.001	<0.001

[abc] Within a row, different superscript represents the significant differences by Tukey $P<0.05$.

[1]SEM=standard error of mean.

[2]Orthogonal polynomial contrast $P<0.05$.

The former study reported quercetin and eugenol were unable to alter C18:2 cis-9 through first biohydrogenation pathway of C18:2n-6, although, it seemed successfully a slightly reduction of C18:2 trans-11, cis-15. However, later biohydrogenation regarding C18:1 trans-11 to C18:0 of these reports was similar outcomes, addressing limited supply of C18:1 trans-11 leading to decrease C18:0 accumulation. A possible reason of this difference was reflected to concerning isomer as FA input in diet. Lourenço et al. (2014) who conducted fed fermenters with more C18:3n-3 rather than C18:2n-6, this condition in contrast of present study with abundant CLA isomer, C18:2n-6. It might be true if FA component as major factor to modulate biohydrogenation. However, the biohydrogenation efficiency is trickier because this process inviting rumen biohydrogenation bacteria and should be wisely considered for other factors such as hydrogen supply (Lourenço et al., 2010). In present study seemed bacteria had responsible undertaking CLA formation that were encouraged by presence of PP, yet not occurred for stearic bacteria. Aforementioned results regarding small protozoa and big bacteria population could be additional supports to influence lipase activity (Lourenço et al., 2010). Therefore, the change of ruminal microorganisms especially bacteria and protozoa could not be excluded.

5.5 Conclusion

This study had revealed that matrix of flavonoids and essential oils being able to stimulate accumulated CLA on biohydrogenation product, without interrupting nutrient fermentation. Other, methane production is also prohibited in tremendously production. The restriction of this study consent to *in vivo* trial, feeding animal with truly these

applied bioactive compounds. Hopefully, it might be alternative strategy to develop rumen feedstuff quality and animal prosperity.

Nevertheless, the effect of solely oil derived from *Piper betle* L. seems to have potential challenging benefit compared with Piper powder. Next chapter regard of supplementing oil alone in similar initially parameter might be conducted.

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CHAPTER VI

A SURGE OF RUMINAL CONJUGATED LINOLEIC ACID AND METHANE MITIGATION WITHOUT SHIFTING RUMEN FERMENTATION DUE TO PIPER OIL SUPPLY

6.1 Abstract

CLA is naturally unsaturated fatty acid in rumen-derived products, owning health properties for consumers which could be manipulated by feeding forage and essential oil in animal diet. This study was conducted to investigate the effects of PO supplementation on lipolysis-isomerisation of ruminal fatty acid biohydrogenation and rumen fermentation including account of fermented biogases and microorganisms. A total of 50 hypodermic glass syringes was decanted 400 mg of basal substrate (Pangola: concentrate, 50:50) and incubated with PO doses at 0 5 25 50 75 and 100 mg/incubation on a dry matter basis under 39°C for 24 h, in 3 runs. Compared to the control, lower of methane production and hydrogen supply were occurred after PO supplied into the substrate. PO inhibited biohydrogenation resulting in a higher of CLA cis-9, trans-11, CLA trans-10, cis-12 and CLA trans-11, cis-13. Further, lipolysis-isomerisation of C18:1 trans-11 to C18:0 was alleviated by increased PO doses. These outcomes did not change pH and total volatile fatty acids; however, ruminal microorganisms were varied during fermentation. The results promote that the PO could be benefit in feed addictive way to develop rumen-derived product and environment nurture.

Keywords: essential oils, fatty acids, organic compounds, rumen.

6.2 Introduction

In recently years, CLA is one of biohydrogenation products and had been believed in owning indigenous health properties (Scollan et al., 2006). Lourenço et al. (2010) addressed a basic concept that feeding animal by forages containing an abundant lipid content (cis-9, cis-12-18:2) is the main substrate for biohydrogenation, resulting in a C18:2 cis-9, trans-11 as the predominant CLA isomer. However, talking regard of biohydrogenation is complex because this metabolism invites ruminal bacteria as well and corresponding to fermented nutrient manner as final pyruvate product should be more considered. Several reports had been done to modulate both of synergetic between rumen fermentation and biohydrogenation by eugenols (Lourenço et al., 2014), unsaturated fatty acids (Shingfield et al., 2006; Toral et al., 2010) and organic matters (Durmic et al., 2008; Jahani-Azizabadi et al., 2019).

Furthermore, *Piper betle* L. is edible plant which had been reported rich in essential oils (EOs), especially caryophyllene and eugenols (Das et al., 2016; Purba and Paengkoum, 2019). In chapter V, it is reported that PP has synergic dose effect on rumen fermentation and biohydrogenation. However, similar regarded parameters treated by solely Piper oil (PO) derived from *Piper betle* L. is scanty. We postulated that inclusion of PO containing eugenols would interact with substrate during fermentation. Therefore, the objective of present study was to investigate the effects of PO supplementation on lipolysis-isomerisation of ruminal fatty acid biohydrogenation and rumen fermentation including account of fermented biogases and microorganisms.

6.3 Materials and methods

6.3.1 PO preparation

PO was collected from distillate *Piper betle* L. leaves using Clevenger apparatus, ± 120°C for 4 h. *Piper betle* L. leaves was planted and harvested at the SUT organic farm (14°52’20.1072’’N, 102°1’32.574’’E: altitude of 243 m above sea level), Nakhon Ratchasima, Thailand. In addition, the fragmentation and calculation of single EO of PO were performed by HPLC DAD machine (Agilent Technologies 1260 Infinity, USA and Canada), by a reversed-phase Zorbax SB-C18 column (3.5 µm particle size, i.d. 4.6 mm × 250 mm), with mobile phase solution (1:9, HPLC-grade acetonitrile:1% acetic acid), in triplicate, commercial standards included (Purba and Paengkoum, 2019). PO contents was eugenol 0.39 g/kg DM predominantly.

6.3.2 Experiment design, *in vitro* incubation, sampling, laboratory analysis and calculation

Experiment design, *in vitro* incubation, sampling, laboratory analysis and calculation were performed as described in chapter V with PP statement changed by PO.

6.3.3 Statistical analysis

Due to unvaried values in separately runs, results are presented as mean values with the standard error of the mean. Collected data of 0 mg of PP from chapter V was used as control date in this study. Data was analysed using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc, 2015) in a completely randomised design. Data were analysed using the model:

$$Y_{ST} = A + B_S + E_{ST} \quad (1)$$

where Y_{ST} = the dependent variable, A = the overall mean, B_S = the influence of the various PO doses ($_S$ =1–6), and E_{ST} = the residual effect. Differences among treatment means were performed by Tukey HSD (Kaps and Lamberson, 2004). Orthogonal polynomial contrasts were used to estimate the PO trend in linear and quadratic. All statistically significant differences were accepted the means with $P<0.05$.

6.4 Results and discussion

6.4.1 PO effects on ruminal biogases, fermentation end-products and microorganisms

As shown in Table 6.1, PO presence in substrate incubation suggested a change of ruminal microorganisms. In this study, a gradually reduction of total protozoa was occurred after PO added, thereafter exhibited a slightly up number of total fungal zoospore. In earlier study, Sallam et al. (2011) subjected EO blend from *Mentha microphylla* plant resulting a lower protozoa number. More recently, Newbold et al. (2015) suggested an alleviated number of protozoa induced an increased ruminal fungal zoospores, as a possibly consequence of deleted action in protozoal preying and struggling for nutrients (protozoa vs. fungi). It could be accepted that presence of these hydroxyl members favoured an antimicrobial behaviour through disturbing membrane semi permeable of rumen microorganism (Benchaar and Greathead, 2011). However, the present study shown a constant number of total bacteria. Benchaar et al. (2012) reported EO did not affect neither protozoa nor bacteria population of rumen content in low and high concentrate diets. However, Wanapat et al. (2008) reported inclusion of 100-200 g lemongrass powder rich in EO promoted a low protozoa and bacteria

numbers. A possibly reason why this difference is numerous essential oils owning dose-dependent affinities on bacteria, protozoa and fungi (Greathead, 2003).

An unchanged bacteria number after PO supplementations resulted in a similar range of total gas production, carbon dioxide yield, IVDMD, IVOMD, pH, ammonia and total VFA, see Table 6.1. The comparable results of fermented yield in this study were in line with prior study (Lourenço et al., 2014) for only eugenol observation. In contrast, Sallam et al. (2011) revived that preceding low protozoa reflected by a depleted gas production and fermentable organic matter, finally producing small amount of VFA after subjected EO blend in *in vitro* incubation. Since, reference of eugenol study on concerned parameter above is literally limited, it might be accepted for this assessment. However, pH and ammonia ranges were 6.86-6.90 and 19.34-19.70 mg/100 ml in present study, which were suitable for rumen inhabitant to growth up of nutrient efficiency (Orskov and Mcdonald, 1970). Moreover, a stagnant result of total VFA by PO supply elaborated a rise of acetate, butyrate and valerate proportions, and a plunge of propionate and iso-acids proportions. These findings were expected as far as PO promoted more acetic to propionic, which was in similar with prior results (Busquet et al., 2006; Lourenço et al., 2014; Joch et al., 2016), whereas PO had successfully maintained a prominent fibre-degraders. To remind, fibre fraction was equal amount among the treatments in this study. Therefore, fermentable degradability by rumen bacteria was same effort leading to unvaried total VFA during fermentation.

Carbohydrate metabolism by anaerobic fermentation in rumen is major fermented nutrient and this metabolism produced CO_2, hydrogen (H_2) and CH_4. In this study, total gas production including CO_2 was unshifting by PO supply, however, CH_4 was limited of factual production. Mitsumori and Sun (2008) described methane-

producing appearance in rumen that was prescribed by rumen archaea and methanogen existences. Since, total protozoa relating to close association with methanogens (Li et al., 2015) was prohibited to survive, this finding could be tenacious evidence, but Ochoa-García et al. (2019) H_2 is major precursor for CH_4 formation and propionic acid had a major role to sink H_2. In other words, low propionic synthesis (as present result) caused more H_2 availability in rumen. Although, hydrogen recovery shown in lower capacity (Table 6.1). These strong arguments might be answered by Greening et al. (2019) that free-producing H_2 was primary uptake by *Blautia* as acetogenic bacteria. *Blautia* might convert available CO_2 and H_2 to acetic formation via the Wood-Ljungdahl pathway (Ni et al., 2011). In present study, acetogenic condition was occurred due to presence of PO during fermentation as aforementioned reflected by more acetate production. As consequence, H_2 was profoundly used in this pyruvate stage and there would be remaining question, of course, to other systematically metabolisms such as H_2 role of biohydrogenation.

6.4.2 The PO effects on lipolysis, isomerisation and biohydrogenation

The average value of substrate incubated with PO on ruminal FA profile after 24 h incubation is presented in Table 6.2. A higher disappearance of PUFA and appearance of remained MUFA tending to a low appearance of SFA in ruminal FA, suggested an inhibited-biohydrogenation profile occurred after PO supplemented into the substrate. It might be stronger assertion that an increased PO provoked bacteria rumen relating to biohydrogenation yielding more CLA accumulations, e.g. CLA cis-9, trans-11; CLA trans-10, cis-12; CLA trans-11, cis-13 and favoured to C18:1 trans-11 rather than C18:2 trans-11, cis-15 accumulations, thereafter C18:0 accumulation successfully diminished in terminated bioh ydrogenation. These findings were generally

Table 6.1 The average value of substrate incubated with PO on ruminal biogases, fermentation end-products and microorganisms after 24 h incubation.

Parameter[1]	PO dose (mg/incubation DM)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
pH	6.90	6.87	6.87	6.86	6.89	6.90	0.012	0.973	0.648
Total gas production (ml/g OM)	30.05	30.11	30.20	30.23	30.28	30.35	0.054	0.538	0.806
CO_2 (ml/g OM)	14.24	14.31	14.21	14.22	14.36	14.41	0.057	0.459	0.995
CH_4 (ml/g OM)	11.92^{a}	10.26^{b}	10.14^{b}	9.87^{c}	9.53^{c}	8.95^{d}	0.144	<0.001	<0.001
IVDMD (%)	49.89	50.22	50.42	50.38	50.44	50.28	0.091	0.989	1.000
IVOMD (%)	50.51	50.53	50.66	50.68	50.22	50.01	0.094	0.207	1.000
NH_3-N (mg/100 ml)	19.70	19.64	19.55	19.42	19.45	19.34	0.038	0.358	0.071
Total volatile fatty acid (mM)	61.11	60.93	60.66	60.53	60.50	60.48	0.019	0.883	0.773
Acetate (C_2) (%)	52.12^{c}	53.10^{b}	53.43^{b}	54.86^{a}	54.88^{a}	54.80^{a}	0.182	0.144	0.003
Propionate (C_3) (%)	20.10^{a}	19.84^{b}	19.61^{b}	19.46^{b}	18.93^{c}	18.40^{d}	0.090	<0.001	0.001
Iso-butyrate (%)	5.55^{a}	4.76^{b}	4.88^{b}	4.10^{c}	3.80^{d}	3.69^{d}	0.101	<0.001	<0.001
Butyrate (%)	10.20^{d}	10.69^{c}	10.72^{c}	10.94^{c}	12.08^{b}	12.91^{a}	0.142	<0.001	<0.001
Iso-valerate (%)	4.54^{a}	4.22^{ab}	3.99^{b}	3.35^{c}	3.11^{c}	3.02^{d}	0.083	<0.001	<0.001
Valerate (%)	7.49^{a}	7.39^{b}	7.37^{b}	7.29^{bc}	7.20^{c}	7.18^{c}	0.021	0.011	0.025
C_2:C_3	2.59^{d}	2.68^{c}	2.72^{c}	2.82^{c}	2.90^{b}	2.99^{a}	0.020	<0.001	< 0.001
HR (mol/mol)	0.66^{a}	0.61^{b}	0.60^{b}	0.58^{c}	0.57^{c}	0.55^{d}	0.065	<0.001	<0.001

Table 6.1 Continue.

Parameter[1]	PO dose (mg/incubation DM)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
Total bacteria (× 10^7 cells/ml)	5.14	5.11	5.10	5.09	5.10	5.12	0.009	1.000	1.000
Total protozoa (× 10^5 cells/ml)	4.95[a]	4.8[b]	4.71[b]	4.08[c]	3.77[c]	3.54[d]	0.077	<0.001	<0.001
Total fungal zoospore (× 10^5 cells/ml)	3.13[c]	3.10[c]	3.10[c]	3.12[c]	3.16[b]	3.22[a]	0.008	0.007	0.031

[abc] Within a row, different superscript represents the significant differences at *P*-value<0.05.

[1]OM= organic matter; IVDMD= *in vitro* dry matter degradability; IVOMD= *in vitro* organic matter degradability; HR= hydrogen recovery (calculated in materials and methods (Marty and Demeyer 1973)).

[2]SEM=standard error of mean.

[3]Orthogonal polynomial contrast *P*<0.05.

similar with earlier study (Gunal et al., 2014), yet, in contrast to other studies (Lourenço et al., 2014; Doreau et al., 2017) regard of depleted C18:0 accumulation, whereas the CLA and C18:1 trans-11 accumulations were dissimilar compared of which studies. Also, the type of PUFA sources in diet was found at diverge and might be a stronger concomitant to understand the influences by EO on isomerisation-hydrogenated of FA biohydrogenation.

As shown in Table 6.3, presence PO had capability of higher efficiency corresponding to the first isomerisation-hydrogenated in biohydrogenation pathway of C18:2n-6, resulted in accumulation of C18:2 cis-9, trans-11 rather than C18:2 trans-11, cis-15 as observed different value in other studies (Lourenço et al., 2014; Doreau et al., 2017). The earlier studies demonstrated that eugenol and garlic oil had a big affinity to impose the first isomerisation-hydrogenated in biohydrogenation pathway of C18:3n-3, acting more efficiencies of C18:3n-3 to C18:2 trans-11, cis-15 to C18:1 trans-11. Both of Doreau et al. (2017) and Lourenço et al. (2014) outcomes were finally resulting in similar accumulation of C18:0 compared to their respective control. However, the present study was consistent to encourage until the last of biohydrogenation scheme, elaborating at increased efficiency of C18:1 trans-11 to C18:0 (stearic acid). Clearly, these contradictions were due to the different FA input content in diet as aforementioned. In present study, substrate had abundant CLA isomer (C18:2n-6) in diet, while Doreau et al. (2017) and Lourenço et al. (2014) fed fermenter with more C18:3n-3 rather than C18:2n-6. It might be confirmed the varied isomerisation-hydrogenated result because of FA content that had a major role to alter ruminal biohydrogenation. Further, biohydrogenation was also not far by existence of bacteria biohydrogenation. For example, a higher CLA accumulation of present study suggested that CLA-degrader bacteria had been stimulated at optimum performance by PO supplementation, through distracting stearic-degrader bacteria (Durmic et al., 2008).

Table 6.2 The average value of substrate incubated with PO on ruminal fatty acid profile (g/100 g FA) after 24 h incubation.

Parameter[1]	PO dose (mg/incubation DM)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
Total SFA[4]	66.134^{a}	63.649^{b}	63.528^{b}	63.340^{b}	63.091^{b}	63.161^{b}	0.198	0.010	0.023
C14.0	0.911^{d}	1.048^{a}	1.046^{a}	1.035^{a}	1.025^{ab}	1.002^{c}	0.007	0.002	0.005
C16.0	20.400^{a}	18.440^{b}	18.405^{b}	18.378^{b}	18.279^{b}	18.396^{b}	0.121	<0.001	0.001
C18.0	36.430^{a}	35.709^{b}	35.641^{b}	35.475^{b}	35.332^{b}	35.310^{b}	0.085	0.044	0.068
Total MUFA[5]	21.937^{d}	24.380^{c}	24.524^{c}	25.040^{b}	25.580^{a}	25.575^{a}	0.198	<0.001	<0.001
C18:1 trans-6–8	0.633^{c}	0.782^{a}	0.781^{a}	0.792^{a}	0.716^{b}	0.712^{b}	0.008	0.283	<0.001
C18:1 trans-9	0.482^{d}	0.506^{bc}	0.505^{bc}	0.524^{a}	0.513^{b}	0.503^{c}	0.002	0.001	0.002
C18:1 trans-10	0.411^{c}	0.511^{b}	0.510^{b}	0.536^{a}	0.535^{a}	0.529^{a}	0.007	<0.001	<0.001
C18:1 trans-11	2.456^{d}	4.664^{c}	4.844^{c}	5.747^{b}	5.963^{b}	6.053^{a}	0.193	<0.001	<0.001
C18:1 trans-15	0.951^{d}	1.075^{b}	1.073^{b}	1.084^{ab}	1.100^{a}	1.040^{c}	0.008	0.041	0.264
C18:1 cis-9	5.179	5.220	5.210	5.218	5.219	5.218	0.009	0.995	0.976
C18:1 cis-11	0.756	0.756	0.755	0.758	0.755	0.755	0.001	0.864	0.998
C18:1 cis-15	0.711^{d}	0.746^{c}	0.745^{c}	0.777^{a}	0.762^{b}	0.760^{b}	0.003	1.000	<0.001
Total PUFA[6]	11.929^{a}	11.971^{a}	11.948^{a}	11.620^{b}	11.329^{c}	11.264^{c}	0.046	0.004	0.005
C18:2 trans-11, cis-15	0.038	0.038	0.038	0.038	0.038	0.038	0.000	1.000	1.000

Table 6.2 Continue.

Parameter[1]	PO dose (mg/incubation DM)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
CLA cis-9, trans-11	0.202^{c}	0.322^{b}	0.321^{b}	0.336^{a}	0.334^{a}	0.324^{b}	0.007	<0.001	<0.001
CLA trans-10, cis-12	0.010^{d}	0.018^{c}	0.018^{c}	0.018^{c}	0.019^{b}	0.020^{a}	0.001	<0.001	<0.001
CLA trans-11, cis-13	0.501^{d}	0.573^{c}	0.572^{c}	0.580^{bc}	0.589^{b}	0.656^{a}	0.007	<0.001	<0.001
C18:2n-6	5.820^{a}	5.511^{b}	5.501^{b}	5.450^{bc}	5.420^{bc}	5.380^{c}	0.024	0.001	0.001
C18:3n-3	0.151	0.151	0.151	0.151	0.151	0.151	0.000	1.000	1.000

^{abc} Within a row, different superscript represents the significant differences at *P*-value<0.05.

[1]SFA= saturated fatty acids; MUFA= mono unsaturated fatty acids; PUFA= mono unsaturated fatty acids.

[2]SEM=standard error of mean.

[3]Orthogonal polynomial contrast *P*<0.05.

[4]Sum of all SFA: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0 and C23:0.

[5]Sum of all MUFA: C14:1 cis-9, C15:1 cis-9, C16:1 trans-9, C16:1 cis-9, C18:1 trans-6–8, C18:1 trans-9, C18:1 trans-10, C18:1 trans-11, C18:1 trans-12–14, C18:1 trans-15, C18:1 cis-9, C18:1 cis-11, C18:1 cis-12, C18:1 cis-13, C18:1 cis-14, and C18:1 cis-15.

[6]Sum of all PUFA: C18:2 trans-11, cis-15; C18:2 trans, trans isomers; C18:2 cis, cis isomers; C18:2 cis, trans isomers; CLA cis-9, trans-11; CLA trans-10, cis-12; CLA trans-11, cis-13; C18:2n-6; C18:3n-6; C18:3n-3; C20:2n-6; C20:3n-6; C20:3n-3; C20:5n3; C24:1n-9 and C22:6n-3.

Table 6.3 The average value of substrate incubated with PO on the efficiency of lipolysis+isomerisation (C18:2 n-6 → C18:2 c9t11) and hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations.

Parameter	PO dose (mg/incubation DM)						SEM[1]	Contrast[2]	
	0	5	25	50	75	100		Linear	Quadratic
C18:2 n-6→ C18:2 cis-9, trans-11	70.987[b]	72.525[a]	72.577[a]	72.832[a]	72.981[a]	73.180[a]	0.143	0.043	0.061
C18:3 n-3→C18:2 trans-11, cis-15	28.095	27.959	28.095	28.048	28.143	28.095	0.057	0.910	1.000
C18:2 trans-11, cis-15→C18:1 trans-11	35.593	34.985	35.424	35.314	35.195	35.085	0.073	0.397	0.602
C18:2 cis-9, trans-11→C18:1 trans-11	98.581	97.787	97.793	97.702	97.719	97.793	0.200	1.000	0.998
C18:1 trans-11→C18:0	83.832[a]	61.470[b]	59.529[b]	50.286[c]	48.332[d]	47.940[d]	0.954	<0.001	<0.001

[abc] Within a row, different superscript represents the significant differences at *P*-value<0.05.

[1]SEM=standard error of mean.

[2]Orthogonal polynomial contrast *P*<0.05.

6.5 Conclusion

This study had demonstrated that PO provision of eugenol results in a higher CLA accumulation, without interrupting nutrient fermentation. In addition, methane production is suppressed in profoundly manner. The results promote that the PO could be benefit in feed addictive way to develop rumen-derived product and environment nurture. In spite of this study reveals a host of advantages, *in vivo* trial and feeding animal investigation could be more considered.

Despite in PO benefit on rumen biohydrogenation, it seems that PO have a different effect against PP and there will be a new hypothesis. A different form of *Piper betle* L. could be expected to have non-similar effect during fermentation and rumen biohydrogenation. According to chapter III, the remaining form of *Piper betle* L., PRP that is substrate non contained oil supposed to have different effect. Therefore, next chapter could deliver PRP study elaborating its effect on rumen fermentation and biohydrogenation.

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CHAPTER VII

PIPER RESIDUE POWDER RICHER IN FLAVONOIDS PROMOTES METHANE MITIGATION AND MODULATES CONJUGATED LINOLEIC ACID OF RUMINAL BIOHYDROGENATION

7.1 Abstract

The objective of present study was to investigate the influence of various doses of PRP on *in vitro* ruminal biogas, fermentation end-product, and biohydrogenation including lipolysis-isomerization and rumen microorganism. The study was designed as a completely randomized design with six doses of PRP supplementation at 0, 5, 25, 50, 75 and 100 mg/incubation DM incubated with 400 mg basal substrate comprised of Pangola hay and concentrate (50:50). The matrix compounds (g/kg DM) of 0.36 catechin, 0.08 rutin and 1.72 quercetin derived from PRP changed the fermentation pattern: escalated degradable nutrient and total volatile fatty acid, with showing acetogenesis without changing pH during fermentation. These values regarded that fermentation producing more gases of total gas kinetic and carbon dioxide, but less release of methane. Hydrogen recovery on lipolysis-isomerization of biohydrogenation was reduced, increased provision of PRP remarkable reduced C18:0 accumulation through shifting biohydrogenation pathway of fatty acids referring to more C18:1 trans-11 rather than C18:2 trans-11, cis-15. As consequence, this occur produced a higher of CLA cis-9, trans-11, CLA trans-10, cis-12 and CLA trans-11, cis-13. Further,

increased provision of PRP increased total bacteria and fungal zoospore due to alleviation of rumen protozoa. In conclusion, results show that the PRP is one of beneficial waste from agricultural biomasses promoted methane mitigation and enhanced ruminal conjugated linoleic acid that are potential expectations to achieve health sustainable agriculture practices.

Keywords: Agricultural by-products, environment, flavonoids, plant polyphenols, rumen, biohydrogenation.

7.2 Introduction

Untreated and unutilized agricultural biomasses/residues including roots, fruits, stalks and leaves which be produced in large amounts annually from harvestable yield, leading to have significant negative environmental impacts. Those said, some practical managements, such as normally discarded or burned agricultural residues are in practice a potential valuable supply of greenhouse gases, especially methane accumulation (Sadh et al., 2018; Tripathi et al., 2019). Notably, some of these residues are reported to have a beneficial use of nutrient sources for animal farming (Teferedegne, 2000). For instance, untreated and unutilized leaves could suggest to provision of additional feed-stock material for ruminants, where these supplies with abundance of forage are predominantly precursors to maintenance body requirements and to produce meat and milk. Besides, there is a considerable concern by consumers during 2 last decades in quality of rumen-derived products, especially conjugated linoleic acids (CLA) (Ferlay et al., 2017). CLA have a well-documented health benefit value in humans regarding to the prohibition of coronary heart disease, deletion of cancer, enhancing immunity and treating obesity through the development of fixed lean body mass (Whigham et al.,

2000; Wanders et al., 2010; Derakhshande-Rishehri et al., 2015). Therefore, there are some challenges in attempting to determine the extent of crop-produced biomass in relation to what is a benefit in sustainable environment, where plants, animal and humans are included.

Methane mitigation and enhanced CLA content of fatty acid milk that are produced from dairy ruminants could be varied by diverse feeding strategies in diets (Lourenço et al., 2010). For illustration, feeding forages for animal rations incorporated with an abundant lipid content (cis-9, cis-12-18:2) as CLA precursors and inclusion of secondary metabolites from plants had been reported to have a positive impact on rumen metabolites, fermentation characteristics and its derivatives in nutrient degradation such as reducing methane production (Cherdthong et al., 2019) and achieving CLA content of ruminal biohydrogenation (Alipanahi et al., 2019; Gesteira et al., 2019; Vasta et al., 2019). Given by those, a natural mode of secondary metabolites suggests to have antimicrobial value for rumen inhabitants by passively movement into membrane semi-permeable of microorganism. This occurred resulted in direct deteriorations for methanogens as methane producer and *Butytivibrio proteocalsticus* as CLA-degraded bacteria on ruminal biohydrogenation. It seems likely that ruminant performances including fibre and fat degradations could be modulated by forming feeding regimens. As consequence, in recently years, efforts in utilizing leaves richer in remaining secondary metabolites which are from harvestable yield could be alternative way to approach these achievements.

Use of *Piper betle* leaves (*Piper betle* L.) in industrial practices to provide the essential oils for consumers produces tons of garbage remained utilized. It is reported that essential oils were extracted from these leaves which are the source for medicine,

stimulant, antiseptic, tonic and other ayurvedic formulations (Das et al., 2016). The natural polyphenols had been suggested to have these health-promising properties (Cardona et al., 2013). In addition, the mixture of polyphenols namely phenolic acids, flavonoids and essential oils formed in *Piper betle* leaves showed the varied quantities based on processing and extraction (Purba and Paengkoum, 2019). Hence, remaining leaves or Piper Residue Powder (PRP) may still have abundant polyphenols to be used on further purposes. We postulated that those remaining leaves incorporated into animal diets might alter beneficially fermentation pathway on pyruvate stage and biohydrogenation by demonstrating antimicrobial towards ruminal inhabitants. Therefore, the objective of present study was to investigate the influence of various doses of PRP on *in vitro* ruminal biogas, fermentation end-product, and biohydrogenation including lipolysis-isomerization and rumen microorganism.

7.3 Materials and methods

7.3.1 PRP preparation

Piper betle L. leaves were collected from different spots at SUT organic farm, Nakhon Ratchasima, Thailand (14°52’20.1072’’N, 102°1’32.574’’E: altitude of 243 m above sea level). The collected leaves were free from a material leafy and then were stored under 4°C overnight to prevent any nutrient destruction (Purba and Paengkoum, 2019). Clevenger apparatus at approximately 120°C for 4 h was used to extract essential oils from *Piper betle* L. leaves (PO collection). Remaining leaves were dried at 45°C for 3 d, powdered as particle passed a 1-mm sieve and loaded into plastic seal dispatched to desiccator until usage time (hereafter referred to PRP). The quantification of polyphenol contents in PRP was performed by protocol of Purba and

Paengkoum (2019) by preparing extracted and assayed in water, methanol, ethanol, chloroform and hexane. In present study, averaged values (g/kg DM) of those solvents were 0.36, 0.08 and 1.72 for catechin, rutin and quercetin, respectively.

7.3.2 Experiment design, *in vitro* incubation, sampling, laboratory analysis and calculation

Experiment design, *in vitro* incubation, sampling, laboratory analysis and calculation were performed as described in chapter V with PP statement changed by PRP.

7.3.3 Statistical analysis

Due to unvaried values in separately runs, results are presented as mean values with the standard error of the mean. Collected data of 0 mg of PP from chapter V was used as control date in this study. Data was analysed using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc, 2015) in a completely randomised design. Data were analysed using the model:

$$Y_{ST} = A + B_S + E_{ST} \quad (1)$$

where Y_{ST} = the dependent variable, A = the overall mean, B_S = the influence of the various PRP doses ($_S$ =1–6), and E_{ST} = the residual effect. Differences among treatment means were performed by Tukey HSD (Kaps and Lamberson, 2004). Orthogonal polynomial contrasts were used to estimate the PRP trend in linear and quadratic. All statistically significant differences were accepted the means with $P<0.05$.

7.4 Results and discussion

7.4.1 Ruminal biogases, fermentation end-products and microorganisms

Inclusion of PRP in fermented substrate incubation on ruminal biogases, fermentation end-products and microorganisms after 24 h is presented in Table 7.1. The present matrix of flavonoids derived from PRP remarkable increased total VFAs, as regards of an enhancement of degradability outcomes, particularly fermented organic matter. In current study, IVDMD and IVOMD increased within the increased provision of PRP in substrate fermentation. As result, these outcomes induced the total VFAs. However, degradable hydroxyl compounds such quercetin, rutin and catechin leaded to a similar outcome of degradability linked to fermentation end-product such a VFA, when those compounds were supplemented in fermented incubations (Oskoueian et al., 2013; Ramdani, 2014). These conflicting results were possibly reviewed because of different applied basal substrate proportion and bioactive compound plant. The previous studies were subjected the fermenters a 60:40 (Oskoueian et al., 2013)/30:70 (Ramdani, 2014) forage: concentrate diet, whereas in our study, a 50:50 Pangola hay: concentrate diet was applied. More elaboration of reasons may suggest that those observations used a commercially product as concerned secondary compounds. A typically effort by Ramdani (2014) who accomplish to extract the catechin from tea could be reviewed. Although, suitable way to achieve the purity, binding behaviour and hydroxyl nutrient of PRP likely PP (mentioned above) bioactive compounds might be acceptable reason why the final results were varied (Purba and Paengkoum, 2019). Furthermore, increase of VFA concentration indicated an optimal fermented substrate by inhabitant rumen tending to more production of gas kinetic during fermentation. In present study, a gradual gas kinetic containing more carbon dioxide and less methane

gas was occurred after PRP supplemented. Alleviated methane production seemed likely that methanogenesis could be possessed by presence of flavonoids from PRP which was consistent with earlier review (Patra and Saxena, 2010). Observation in flavonoids studies regard of methanogenesis is scanty. Comparison of data tabulation, however, PRP expressed a weaker mode to alleviate methane production than PP. This notion is strong by the fact that PRP has lesser polyphenol content than PP that may make a different effect. Therefore, an attenuated methane gas proportion reflected rumen methanogenesis occurred roughly in this study, that showed a change of fermented pattern of VFA referring to acetate, propionate and butyrate fractions.

In present study, incorporated PRP into substrates during fermentation resulted in higher acetate and butyrate productions, but lower propionate, valerate and branched-chain VFAs (iso-acid fraction). In calculation, this study also showed inhibited HR to methanogenesis and biohydrogenation. According to earlier observations, propionate from pyruvate undertaken a large of hydrogen consumptions tending to a weakened methanogenesis (Moss et al., 2000; Tavendale et al., 2005). But, prior meta-analysis showed no significantly relationship between a greater propionate and methane reduction (Jayanegara et al., 2012b). More recently, Greening et al. (2019) confirmed that hydrogen metabolism is a more complex and widespread trait among rumen microorganisms, where it claims the hydrogen yield was uptake by other innate consumers, not only methanogens, e.g. fumarate and nitrite reduction (*Selenomonas*) and acetogenesis (*Blautia*). In present study, PRP supplementations increased acetate fraction, as more evidences hydrogen was tremendously consumed by acetogenesis (*Blautia*) and hydrogen supply was prevented for extent movement. Hence, valeric and iso-acid fraction in current study was lower as consequence of less propionic fraction

(Andries et al., 1987). All shifts of fermentation outcomes above were unvaried pH rumen in all treatments, while an initially change occurred in deamination side after PRP supplemented into substrate incubation (Table 7.1). A range of pH and ammonia concentration in present study was 6.7-6.9 and 19.7-20.7 mg/100 ml, which were pointed to ruminal microorganism to survive in improving microbial growth and fermentation efficiency (Ørskov and MacLeod, 1982).

Regard of microorganism, increased provision of PRP reduced protozoa rumen (Table 7.1). A depletion of protozoa number due to supplementation of secondary compounds had been reported in many ways (Patra and Saxena, 2009; 2010; Santra et al., 2012; Wencelova et al., 2014; Ma et al., 2017). Given those reports, a major reason is ability of bioactive compounds interrupting cell wall synthesis or nucleic acid synthesis of protozoa. This might be reason why bacteria and fungal zoospore duplicated in colony within the time. According to Dehority (2003), protozoa engulfing rumen bacteria an approximately 20,000 cells per hour. This occurs leaded to increase bacteria numbers were aftereffect of eliminated protozoa. In addition, Newbold et al. (2015) reported that fungal zoospore growing up with low protozoal number, which indicated rare competition between protozoa and fungal zoospore on penetrating their substrate during nutrient degradation and fermentation. Cherdthong et al. (2019) confirmed that fungal zoospore had a powerful defensive manner to defend against passively diffusion-osmosis of bioactive compounds from flavonoids.

Table 7.1 The mean value of substrate incubated with PRP on ruminal biogases, fermentation end-products and microorganisms after 24 h incubation.

Parameter[1]	PRP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
pH	6.90	6.87	6.86	6.86	6.88	6.90	0.028	1.000	0.400
Total gas production (ml/g OM)	30.05^{d}	37.25^{c}	38.25^{c}	39.40^{b}	40.80^{a}	41.05^{a}	0.152	<0.001	<0.001
CO_2 (ml/g OM)	14.24^{d}	14.90^{c}	14.70^{c}	15.15^{b}	16.16^{b}	16.44^{a}	0.061	<0.001	0.408
CH_4 (ml/g OM)	11.92^{a}	9.16^{b}	8.85^{b}	8.60^{c}	7.92^{d}	7.70^{d}	0.037	<0.001	<0.001
IVDMD (%)	49.89^{c}	50.66^{b}	51.23^{ab}	51.64^{a}	51.65^{a}	52.04^{a}	0.205	<0.001	0.597
IVOMD (%)	50.51^{c}	64.43^{b}	64.88^{ab}	65.13^{ab}	65.51^{a}	65.53^{a}	0.252	<0.001	<0.001
NH_3-N (mg/100 ml)	19.70^{c}	20.66^{a}	20.68^{a}	20.51^{ab}	20.11^{b}	20.02^{b}	0.081	0.647	<0.001
Total volatile fatty acid (mM)	61.11^{b}	63.77^{a}	63.99^{a}	64.01^{a}	64.11^{a}	64.27^{a}	0.254	<0.001	0.001
Acetate (C_2) (mol/100 mol)	52.12^{c}	53.36^{b}	54.17^{ab}	54.24^{ab}	54.37^{a}	54.53^{a}	0.215	<0.001	0.023
Propionate (C_3) (mol/100 mol)	20.10^{a}	19.67^{b}	19.53^{b}	19.38^{bc}	19.11^{c}	18.86^{d}	0.078	<0.001	0.907
Iso-butyrate (mol/100 mol)	5.55^{a}	5.04^{b}	4.57^{d}	4.59^{b}	4.74^{c}	4.97^{b}	0.020	<0.001	<0.001
Butyrate (mol/100 mol)	10.20^{d}	10.57^{c}	10.80^{b}	10.97^{ab}	11.02^{a}	11.14^{a}	0.043	<0.001	0.015
Iso-valerate (mol/100 mol)	4.54^{a}	4.12^{b}	3.74^{d}	3.76^{d}	3.88^{c}	4.07^{b}	0.016	<0.001	<0.001
Valerate (mol/100 mol)	7.49^{a}	7.24^{b}	7.19^{b}	7.06^{bc}	6.88^{c}	6.43^{d}	0.028	<0.001	0.001

Table 7.1 Continue.

Parameter[1]	PRP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
C_2:C_3 ratio	2.59^{d}	2.71^{c}	2.77^{b}	2.80^{b}	2.85^{a}	2.89^{a}	0.011	<0.001	0.026
HR (mol/mol)	0.66^{a}	0.58^{b}	0.56^{c}	0.55^{c}	0.53^{d}	0.53^{d}	0.002	<0.001	<0.001
Total bacteria, × 10^7 cells/ml	5.14^{b}	5.26^{a}	5.29^{a}	5.29^{a}	5.28^{a}	5.28^{a}	0.210	0.003	0.011
Total protozoa, × 10^5 cells/ml	0.049^{a}	0.044^{b}	0.042^{b}	0.040^{c}	0.038^{c}	0.036^{d}	0.167	<0.001	<0.001
Total fungal zoospore, × 10^5 cells/ml	0.031^{c}	0.031^{c}	0.031^{c}	0.031^{bc}	0.032^{b}	0.033^{a}	0.126	<0.001	<0.001

^{abc} Within a row, different superscript represents the significant differences by Tukey P<0.05.

[1]OM= organic matter; IVDMD= *in vitro* dry matter degradability; IVOMD= *in vitro* organic matter degradability; HR= hydrogen recovery (calculated in materials and methods (Marty and Demeyer 1973).

[2]SEM=standard error of mean.

[3]Orthogonal polynomial contrast $P < 0.05$.

7.4.2 Lipolysis, isomerization and biohydrogenation

Inclusion of PRP in fermented substrate on ruminal FA profile after 24 h is presented in Table 7.2. Clearly, the present matrix of flavonoids derived from PRP showed inhibited-biohydrogenation properties, where shifted apparent biohydrogenation of C18:2n-6 and C18:3n-3 as PUFAs were observed. Reference of shifting biohydrogenation including its lipolysis-isomerizing FA by using flavonoids of PRP is relatively limited, only catechin and quercetin application available in literatures (Lourenço et al., 2014; Ramdani, 2014) for *in vitro* study. They reported consistent results that supplied flavonoids into substrate fermenters resulted in a change of ruminal biohydrogenation. However, deposited efficiency corresponding to CLA isomers counted towards the molecular weight of flavonoid itself. Obviously, in our study, PRP induced rumen biohydrogenation to yield more considerable PUFA accumulation, e.g. CLA cis-9, trans-11, CLA trans-10, cis-12 and CLA trans-11, cis-13, less saturated fatty acid in C18:0 and increased mono unsaturated fatty acid in accumulation of C18:1 trans-11 rather than C18:2 trans-11, cis-15.

As shown in Table 7.3, increased provision of PRP seemed successful to interact on the first biohydrogenation pathway of C18:2n-6, resulted in accumulation of C18:2 cis-9, trans 11 rather than C18:2 trans-11, cis-15 as observed different value in other studies (Lourenço et al., 2014). The former study reported quercetin and eugenol were unable to alter C18:2 cis-9 through first biohydrogenation pathway of C18:2n-6, although seemed successfully a slightly reduction of C18:2 trans-11, cis-15. However, later biohydrogenation regarding C18:1 trans-11 to C18:0 of these reports was similar outcomes, addressing limited supply of C18:1 trans-11 leading to decrease stearic acid (C18:0) accumulation. It is noteworthy that a possible reason of this inconsistent was

reflected to highlight of available isomer as FA input in diet. Lourenço et al. (2014) who conducted fed fermenters with subjecting more C18:3n-3 rather than C18:2n-6, this condition completely opposite to the current study with abundant CLA isomer of C18:2n-6. It is believed that FA properties leading to predominantly impact on modulating ruminal biohydrogenation. However, the biohydrogenation efficiency is unpredictable parameter, where this transformation connect to rumen biohydrogenation bacteria and should be wisely considered for other factors such as hydrogen supply (Lourenço et al., 2010). This study seemed bacteria to have a major role undertaking CLA formations, which were changed by presence of PRP, but it did not affect to stearic bacteria. Aforementioned results about alleviated protozoa and escalated bacteria populations could be the best references to induce lipase activity (Lourenço et al., 2010). The change of ruminal microorganisms especially bacteria and protozoa could not be excluded.

To last of *in vitro* observation, there were critically different outcomes on transformation of C18:2 t11c15 to C18:1 t11 compared among PP, PO and PRP. In PP and PRP study, flavonoids may bind other compounds likely phenolic acids and essential oils, resulting in a greater degradation of C18:2n-6 and C18:3n-3 formed in C18:1 t11. Although, absence of flavonoids and phenolic acids in PO study suggested a critically different antimicrobial of eugenols. This assessment may revive that eugenols had stronger compound to interrupting *Butyrivibrio fibrisolvens* on intermediate of ruminal biohydrogenation (Ramos-Morales et al., 2013).

Table 7.2 The mean value of substrate incubated with Piper residue powder (PRP) on ruminal fatty acid profile (g/100 g FA) after 24 h incubation.

Parameter[1]	PRP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
Total SFA[4]	66.134^{a}	63.649^{b}	63.528^{b}	63.340^{b}	63.091^{b}	63.161^{b}	0.255	<0.001	0.001
C14.0	0.911^{d}	1.048^{a}	1.046^{a}	1.035^{ab}	1.025^{b}	1.002^{c}	0.004	<0.001	<0.001
C16.0	20.400^{a}	18.440^{b}	18.405^{b}	18.378^{b}	18.279^{b}	18.396^{b}	0.075	<0.001	<0.001
C18.0	36.430^{a}	35.709^{b}	35.641^{b}	35.475^{b}	35.332^{b}	35.310^{b}	0.143	0.001	0.031
Total MUFA[5]	21.937^{d}	24.380^{c}	24.524^{c}	25.040^{b}	25.580^{a}	25.575^{a}	0.098	<0.001	<0.001
C18:1 trans-6–8	0.633^{c}	0.782^{a}	0.781^{a}	0.792^{a}	0.716^{b}	0.712^{b}	0.003	<0.001	<0.001
C18:1 trans-9	0.482^{d}	0.506^{c}	0.505^{c}	0.524^{a}	0.513^{b}	0.503^{c}	0.002	0.001	<0.001
C18:1 trans-10	0.411^{c}	0.511^{b}	0.510^{b}	0.536^{a}	0.535^{a}	0.529^{a}	0.002	0.001	<0.001
C18:1 trans-11	2.456^{d}	4.664^{d}	4.844^{c}	5.747^{b}	5.963^{a}	6.053^{a}	0.020	<0.001	<0.001
C18:1 trans-15	0.951^{d}	1.075^{b}	1.073^{b}	1.084^{ab}	1.100^{a}	1.040^{c}	0.004	<0.001	<0.001
C18:1 cis-9	5.179	5.220	5.210	5.218	5.219	5.218	0.021	0.370	0.508
C18:1 cis-11	0.756	0.756	0.755	0.758	0.755	0.755	0.003	0.994	0.991
C18:1 cis-15	0.711	0.746	0.745	0.777	0.762	0.760	0.003	<0.001	<0.001
Total PUFA[6]	11.929^{a}	11.971^{a}	11.948^{a}	11.620^{b}	11.329^{c}	11.264^{c}	0.047	<0.001	0.013
C18:2 trans-11, cis-15	0.038	0.038	0.038	0.038	0.038	0.038	0.000	0.993	0.842
CLA cis-9, trans-11	0.202^{c}	0.322^{b}	0.321^{b}	0.336^{a}	0.334^{a}	0.324^{b}	0.041	<0.001	<0.001

Table 7.2 Continue.

Parameter[1]	PRP dose (mg/incubation)						SEM[2]	Contrast[3]	
	0	5	25	50	75	100		Linear	Quadratic
CLA trans-10, cis-12	0.010[d]	0.018[c]	0.018[c]	0.018[c]	0.019[b]	0.020[a]	0.069	<0.001	<0.001
CLA trans-11, cis-13	0.501[d]	0.573[c]	0.572[c]	0.580[bc]	0.589[b]	0.656[a]	0.002	<0.001	0.907
C18:2n-6	5.820[a]	5.511[b]	5.501[b]	5.450[b]	5.420[c]	5.380[c]	0.022	<0.001	<0.001
C18:3n-3	0.151	0.151	0.151	0.151	0.151	0.151	0.001	0.989	0.842

[abc] Within a row, different superscript represents the significant differences by Tukey $P<0.05$.

[1]SFA= saturated fatty acids; MUFA= mono unsaturated fatty acids; PUFA= mono unsaturated fatty acids.

[2]SEM=standard error of mean.

[3]Orthogonal polynomial contrast $P<0.05$.

[4]Sum of all SFA: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0 and C23:0.

[5]Sum of all MUFA: C14:1 cis-9, C15:1 cis-9, C16:1 trans-9, C16:1 cis-9, C18:1 trans-6–8, C18:1 trans-9, C18:1 trans-10, C18:1 trans-11, C18:1 trans-12–14, C18:1 trans-15, C18:1 cis-9, C18:1 cis-11, C18:1 cis-12, C18:1 cis-13, C18:1 cis-14, and C18:1 cis-15.

[6]Sum of all PUFA: C18:2 trans-11, cis-15; C18:2 trans, trans isomers; C18:2 cis, cis isomers; C18:2 cis, trans isomers; CLA cis-9, trans-11; CLA trans-10, cis-12; CLA trans-11, cis-13; C18:2n-6; C18:3n-6; C18:3n-3; C20:2n-6; C20:3n-6; C20:3n-3; C20:5n3; C24:1n-9 and C22:6n-3.

Table 7.3 The mean value of substrate incubated with Piper residue powder (PRP) on the efficiency of lipolysis + isomerization (C18:2 n-6 → C18:2 c9t11) and hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations.

Parameter	PRP dose (mg/incubation)						SEM[1]	Contrast[2]	
	0	5	25	50	75	100		Linear	Quadratic
C18:2 n-6→ C18:2 cis-9, trans-11	70.99^{b}	72.73^{a}	72.73^{a}	73.07^{a}	73.41^{a}	73.45^{a}	0.319	0.053	0.095
C18:3 n-3→C18:2 trans-11, cis-15	28.10	28.51	28.31	28.52	29.22	28.47	0.126	1.000	0.149
C18:2 trans-11, cis-15→C18:1 trans-11	35.59^{d}	36.47^{cd}	37.52^{cd}	41.13^{c}	42.32^{b}	43.99^{a}	0.471	<0.001	<0.001
C18:2 cis-9, trans-11→ C18:1 trans-11	98.58	97.55	97.56	97.46	97.48	97.56	0.396	0.981	0.956
C18:1 trans-11→C18:0	83.83^{a}	53.78^{b}	53.72^{b}	43.41^{c}	41.52^{d}	40.64^{d}	2.338	<0.001	<0.001

^{abc} Within a row, different superscript represents the significant differences by Tukey $P<0.05$.

[1]SEM=standard error of mean.

[2]Orthogonal polynomial contrast $P<0.05$.

7.5 Conclusion

This study had demonstrated that matrix of flavonoids composed of catechin, rutin and quercetin seemed to have a beneficial property on accumulation of CLA during ruminal biohydrogenation, on rumen fermentation by increasing fermentation end-product without changing pH and tremendously declining methane production. These achievements could be beneficial practices for building a sustainable agriculture in crop, farm and human concerns. The limitation of current study was to prove that the selected dose of PRP that could resulted in similar outcomes in *in vivo* trial, feeding animal with truly these applied bioactive compounds.

To select the best level and form of *Piper betle* L. on rumen fermentation and rumen biohydrogenation without giving negative impact on animal, chapters 5, 6 and 7 are compared fairly. As a result, 5 mg of Piper powder (PP) is the best option to undergo feeding trial in next chapters. Hopefully, this selected dose gives abundant effect on animal performance including milk biohydrogenation and animal response to antioxidant donor to alleviate lipid peroxidation and oxidative stress.

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CHAPTER VIII

MILK FATTY ACID COMPOSITIONS, RUMEN MICROBIAL POPULATIONS AND ANIMAL PERFORMANCES IN RESPONSE TO DIETS RICH IN LINOLEIC ACID SUPPLEMENTED OR WITHOUT POLYPHENOLS FROM *PIPER BETLE* L. LEAVES IN SAANEN GOATS

8.1 Abstract

The aim of the study was to evaluate milk fatty acid profile, rumen microbial population and animal performance in response to diets containing sunflower oil supplemented or without polyphenols from PP in dairy goats. Twelve multiparous Saanen goats (42±1.00 kg) were randomly assigned to two experimental groups in an experiment which lasted for 6 weeks. The two experimental diets formulated as TMR were: CTH diet (containing 0% PP) and DPB diet (containing 1.3% PP dry matter basis). Dry matter intake (DMI) and milk yield were recorded daily. Samples of feed, milk and rumen liquor were collected and analysed weekly after 15 days of adaptation period. Results show that inclusion of phenolic acids, flavonoids and essential oils from PP in diet (DPB) resulted in a greater milk yield and altered the composition of milk. DMI was not affected among treatments (P>0.05), but apparent digestibility in fermented organic matter was a greater percentage (P<0.05) by inclusion of PP in DPB diet. Compared with the control diet (CTH), the DPB diet decreased saturated fatty acid content and increased unsaturated fatty acid content in milk. Inclusion of PP decreased

C18:0 production (P=0.001), tended to increase C18:1 trans11 and C18:2 cis9 trans11 (P<0.05). Overall, total CLA in goats fed by DPB was found a double increasing (1.77 to 2.62 g/100 g FA) than goats fed by CTH diet (P<0.05). The desaturase rate (except desaturase for carbon 18, P<0.05), atherogenic and thrombogenic indices were not affected by inclusion of PP in DPB diet. Moreover, the DPB diet escalated total VFA and altered VFA profile. DPB diet showed affinity to alter the relative abundances of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in rumen liquor. Compared with rumen liquor from goats fed with CTH diet, the presence of *Butyrivibrio fibrisolvens* increased about 5-folds, although, the presence of *Butyrivibrio proteoclasticus* declined by about 11-fold in goats fed the DPB diet. The use of sunflower oil dosed of 17.6 g/kg and a practical dose of flavonoids and essential oils from valuable leaves (*Piper betle* L.) inclusion of 12.8 g/kg in the diet of dairy goats can be an efficient approach to improve the nutritional quality of milk.

Keywords: phenolic acids, flavonoids, essential oils, milk fatty acids, rumen, microbial populations, goats.

8.2 Introduction

A great deal of efforts has been focused on developing nutritional quality of rumen-derived products in the last decade, including beneficial fatty acids in milk (Chilliard et al., 2007). CLA in milk is a beneficial fatty acid because of its potential health promoting effects in human (Pariza, 2004). RA (C18:2 cis-9, trans-11) is represented as a major precursor of CLA and is mostly produced endogenously via rumen biohydrogenation (Tanaka, 2005). To achieve the above, dietary polyunsaturated sources, especially those from vegetable oils or marine oils incorporated into ruminant

diets may induce rumen biohydrogenation resulting in an accumulated content of C18:2 cis-9, trans-11 (Shingfield et al., 2013). This achievement due to abundant precursors of C18:2 cis-9, trans-11 in diets, such as C18:2 cis-9, cis-12 and C18:3 cis-9, cis-12, cis-15 which could support isomerisation and hydrolysation on ruminal biohydrogenation (Chilliard et al., 2000). Previous study showed that inclusion of vegetable oils in diet offered to goats for 5 weeks resulted in about 2- and 3-folds increase in the content of C18:2 cis-9, trans-11 and C18:1 trans-11 in milk fat, respectively (Chilliard and Ferlay, 2004). Even though, there is a limitation for goats and sheep to transform less than 60 to 100 g/head/day of supplemented lipid to C18:2 cis-9, trans-11 and C18:1 trans-11 in milk, thereby making oil supplementation costly (Buccioni et al., 2015). Among 80 vegetable oils observed to meet a good safety record of potential nutritional and their possibly oxidation for dairy animals, and sunflower oils, richer in C18:2 cis-9, cis-12 had been suggested to have those characteristics which contain more C18:1 cis-9 and less C18:3 cis-9, cis-12, cis-15 (Shingfield et al., 2006; Dubois et al., 2007). Besides, prolonged lipid supplementation with unbalanced forage intake may lead to depression of milk fat in animals (Glasser et al., 2008; Shingfield et al., 2013). Hence, in recent years, the focus has shifted to formulating feed ration with lesser amount of lipids for animals, thereby achieving optimal rumen biohydrogenation of dietary PUFA. This achievement could lead to a significant accumulation of desaturation of C18:1 trans11 in mammary tissue, which is originated from ruminal FA absorption in rumen.

Numerous *in vitro* studies had been conducted on the use of phenolic acids (PAs), flavonoids (FOs) and essential oils (EOs) from plant origins for ruminal methane mitigation and inhibition of rumen biohydrogenation (Jayanegara et al., 2011; Jayanegara et al., 2012b; Durmic et al., 2014; Lourenço et al., 2014). It has been

reported that PAs with FOs and/or PAs with EOs were metabolised in rumen by hydrolysing their glycosides and cleavages of heterocyclic compounds, and these degradations resulted in di- or monohydroxyphenolics, phloroglucinol and volatile fatty acid, especially acetate and butyrate (McSweeney et al., 2001). In addition, secondary metabolites seemed to interact with fibre and protein in the rumen through hydrogen and hydrophobic bonds, respectively, which were stretch-induced alteration of the proportion of total bacteria, methanogens, Butyrivibrio group and cilia protozoa (Oskoueian et al., 2013; Cobellis et al., 2016). Previous *in vivo* studies showed that supplementary FOs derived from mulberry leaf increased population of total rumen bacteria (Ma et al., 2017), whereas animals supplemented with EOs affected the population of Proteobacteria (Poudel et al., 2019). Study in goats by dietary Papaya leaf richer in PAs source affected abundance of *Butyrivibrio fibrisolvens* and modulates biohydrogenation of C18 polyunsaturated fatty acids (Jafari et al., 2018). Also, long term addition of rutin to dairy animals tended to increased milk yield and feed digestibility, but leaded to diminish microbial crude protein production and serum lysozyme content (Cui et al., 2015). It seems likely that FOs and EOs had a similar effect like tannin as member of PAs which could protect feed protein from rumen microbial degradation (Frutos et al., 2004a). Hence, provision of plant polyphenols in animal diets may have a direct antimicrobial affection for inhabitant rumen by interfering cell wall synthesis or nucleic acid synthesis, leading to direct deteriorations for rumen microorganism activity (Cherdthong et al., 2019). Although studies on the influence of polyphenols as regards to incorporated of FOs and EOs on rumen microbial population are still scanty, it is reported that *Butyrivibrio fibrisolvens* and *Butyrivibrio*

proteoclasticus play key roles in rumen biohydrogenation (Lourenço et al., 2010; Vasta et al., 2010).

Furthermore, a low correlation between *in vitro* and *in vivo* may lead to contradictory results in how animals' response is, particularly in terms of intermediate products in the rumen during biohydrogenation (Lourenço et al., 2010; Lourenço et al., 2014). Susceptibility to the origin and dose for plant polyphenols with abundant lipid contents in feeding regimens could be the principal factors to modulate rumen biohydrogenation. We hypothesized that, ruminant fed diet supplemented with sunflower oil plus plant polyphenols inhibits ruminal biohydrogenation (PUFA contents > SFA contents) and suggests to improve rumen fermentation compared with those supplemented with only sunflower oil. Therefore, the aim of the present study was to evaluate the effect of moderate amount (<2%) of the plant polyphenols from PP in diet supplemented with sunflower oil on milk composition and its fatty acid profile and on the relative abundance of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in the rumen microbial community over different sampling times.

8.3 Materials and methods

All experimental procedures were approved and completed in accordance with the Rules of Animal Welfare of Suranaree University of Technology (SUT 4/2558) for animal protection used and/or applied for experimental purposes.

8.3.1 Animals and diets

Twelve lactating (second week of early lactation) multiparous Saanen goats with average body weight of 42±1.00 kg from the SUT goat and sheep research farm, Thailand, were randomly assigned to two experimental groups (n=6). The goats

were housed individually in metabolic cage (length 2.2 m×width 1.3 m×height 2 m) throughout 2 weeks adaptation followed by 6 weeks of feeding trial. Feed intake was recorded and calculated by the difference between the amount of feed offered and the amount of residual feed daily. The goats were milked twice daily at 07:00 and 16:00 h using a portable HL-JN02 milking machine (50 kPa; 60/40 pulsation ratio) (Hailian packaging equipment, Jiangsu, China), and daily milk yield of individual goat was recorded every day of the 6 weeks.

The experimental diets were a TMR formulated according to NRC (2007) requirement for goats (Table 8.1). The PP was prepared from *Piper betle* L. leaves harvested from several plants in the SUT organic farm, Nakhon Ratchasima, Thailand (elevation of 243 m above sea level) which were latter pooled together. The collected leaves were cleaned with deionized water and kept overnight at 4°C before drying in oven at 40°C for 3 days, ground to powder, filled in sealed plastic bags and kept pending for use for the experiment. The TMR was prepared every alternate day and the nutritive value and secondary metabolite content of TMR (Table 8.2). The dosage level of PP was selected based on previous studies considered safe for the animal and practical for the farmers (De Nardi et al., 2014; Olagaray and Bradford, 2019; Purba et al., 2020). The TMR was offered to the experimental goats twice daily (ca. 0.8 times the voluntary feed intake previously determined *ad libitum* during adaptation period) after each milking sessions and clean drinking water was offered *ad libitum*.

Table 8.1 Ingredient (% DM) of the CTH and the TMR with DPB.

Item	Diet	
	CTH	**DPB**
Pangola hay	23.81	23.63
Cassava chip	10.98	10.90
Cassava pulp	18.48	17.85
Mineral mix	1.01	1.01
Molasses	1.63	1.63
Palm meal	13.28	12.85
Piper	0.00	1.28
Premix	2.02	2.02
Rice bran	13.60	13.70
Soybean meal	11.60	11.52
Sulphur	0.26	0.26
Sunflower oil	1.76	1.76
Urea	1.58	1.57

8.3.2 Sampling and analysis

Feed samples, including orts were recorded daily and polled by week and stored at -20°C. Samples were dried in oven at 55°C for 2 days and then ground (Retsch SM 100 mill; Retsch Gmbh, Haan, Germany) with a mesh size of 1 mm. The samples were analysed for dry matter, organic matter, ash, crude protein (total N × 6.25) and crude fat following published protocol (AOAC, 2005) and acid detergent fibre and neutral detergent fibre (Van Soest et al., 1991). Gross energy was determined using a bomb calorimeter with O_2 carrier (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL) according to the manufacturer's instructions. Concentration of FOs and EOs was assayed in water, methanol, ethanol, chloroform and hexane extracts and measured by quantifying a mean signal of peak rate under photo diode array UV

detector set wavelength at 272 nm using HPLC (Purba and Paengkoum, 2019). Content of fatty acid was extracted according to Folch et al. (1957) with modification (De Weirdt et al., 2013) equipped to gas chromatography principle with 17:0 (Sigma Chemical Co., St Louis, MO) as the internal standard, and identified using the same procedure described below for FA of milk samples. All feed analysis was carried out in triplicate (Table 8.2).

From day 16 onwards, completely sampling including feeding intake and excretions of faeces were collected and polled within a week. Apparent digestibility of each nutrient was calculated by calculating the feed intake and faeces excreted, as formulated:

$$\text{apparent digestibility (\%)} = \frac{(\text{amount of nutrient intake - amount of nutrient in feces excreted})}{(\text{amount of nutrient intake})} \times 100$$

where each analysis of nutrient in feed and faecal samples was performed using identical procedures in earlier protocol for nutrient determination of chemical analysis.

On days 22 29 36 43 50 and 57 goats were milked and given free access to their ration for 1 h (Tian et al. (2018). Then, feeds were removed and rumen liquor samples were collected from each goat using a stomach tube connected to a manual pump (Lodge-Ivey et al., 2009) 3 h post-feeding. The pH of each rumen liquor sample was immediately measured and then partitioned and kept in 2 Falcon tubes and stored at -80°C until analysis for total volatile fatty acid (VFA) content (10 ml) and microbiological assay (5 ml). The rumen liquors for VFA analysis were centrifuged at 6,000×g at 4°C for 15 min (Sorvall Legend XT/XF Centrifuge Series, Thermo Fisher Scientific, Waltham, MA) and the supernatant was stored at -20°C until analysis. The

supernatant was fixed with 25% metaphosphoric acid (Erwin et al., 1961; Filípek and Dvořák, 2009). The fixed supernatant of 2 µl VFA sample was injected to gas chromatography machine in triplicate (Hewlett Packard GC system HP6890 A; Hewlett Packard, Avondale, PA, USA) following the procedure of Erwin et al. (1961), with modification (Filípek and Dvořák, 2009). Acetic acid, propionic acid, iso-butyric acid, butyric, iso-valeric acid and valeric acid (Carlo ebra, France) were assayed in 1% formic acid solution and considered as standard calibration. Quantification was done using an external calibration curve based on the standards described above. Data were expressed in millimoles per litre for total VFA and in mol per 100 mol for VFA composition. In addition, methane production was estimated using previous protocol (Moss et al., 2000).

Total DNA was isolated and purified from 1 ml of rumen liquor using the QIAamp DNA Stool Mini Kit by performing the Repeated Bead Beating Plus Column (RBB+C) method systematically (Yu and Morrison, 2004). The DNA concentration was performed by measuring the 260/280 absorbance ratio with a NanoDrop NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA). The DNA was eluted in 50 µl of nuclease-free water and quality were verified by 1% agarose gel electrophoresis.

Relative abundances of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in rumen liquor samples were measured by real-time quantitative PCR (RT-qPCR), using total bacterial DNA as reference (Makkar and McSweeney, 2005). The primers used in this study were identified from the literature to amplify partial 16S rRNA gene of total bacteria (Maeda et al., 2003), while for *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* were given by Potu et al. (2011). For each primer pair,

reaction efficiencies were derived from a standard curve generated from a 6-fold serial dilution of pooled.

The RT-qPCR analysis was performed using a Roche Lightcycler 480-II (Roche Applied Science, Switzerland) in a total volume of 20 µl. For *Butyrivibrio fibrisolvens*, total bacteria and *Butyrivibrio proteoclasticus*, 2.5 ng, 2.5 ng and 25 ng of DNA, respectively were added to 10 µl of QuantiTect SYBR Green RT-PCR Kit (full master mix) containing 400 nM each primer. Each sample was done prepared in triplicate using LightCycler® 480 Multiwell Plate 96 (LifeScience, Thailand). The RT-qPCR setting was set according to earlier investigation (Buccioni et al., 2015). Amplification conditions were 95°C for 3 min, 40 cycles of 95°C for 15 s, and 60°C (*Butyrivibrio fibrisolvens* and total bacteria) or 55°C (*Butyrivibrio proteoclasticus*) for 30 s. The melting curve was set the range of 60°C to 95°C to determine amplification specificity, following all non-probe-based qPCR reactions. Cycle threshold values were converted into normalized relative quantities, corrected by PCR efficiency using LightCycler 480 software version 1.2.9.11. The *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* 16S rRNA gene values were expressed as a relative of percentage of total bacteria.

8.3.3 Statistical analysis

Since observations were performed on the same experimental unit (goat), a repeated measures ANOVA with one experimental factor (diets) and one repeated factor (sampling time) has been considered. Animals were treated as simultaneously replications and linear mixed effects models were set with both `diets' and `time' as fixed effects and `animals' as random effect to obtain suitable data for ANOVA. A full

factorial model was considered with the two fixed effects by using the MIXED procedure of SAS (SAS Institute Inc, 2015):

$$Y_{ijkl}=\mu + A_i + B_j + C_k\,(A) + (A\times B)_{ij} + e_{ijkl}$$

where Y_{ijkl} is the observation; μ is the overall mean; A_i is the fixed effect of diet (i=1 to 2); B_j is the fixed effect of sampling time (j=1 to 6); C_k is the random effect of the goat nested within the diet (k=1 to 6); $(A\times B)_{ij}$ is the interaction between diet and sampling time; and e_{ijkl} is the residual error. Akaike's information criterion of the mixed model of SAS was selected to fit the covariance structure was compound symmetry. Statistical significance of the diet effect was tested against variance of goat nested within diet according to repeated measures design theory (Kaps and Lamberson, 2004). Least square means are reported and significance was declared at $P<0.05$. Differences among diets and sampling time in each grouping of parameter was performed using Tukey HSD at $P<0.05$

8.4 Results and discussion

Ingredient compositions of the control (CTH) and the treatment (DPB) diets are presented in Table 8.1. Diets was composed of Pangola hay (particle size >4 cm) and concentrate. Sunflower oils used for both diets were supplemented in same amount (ca. 1.76% DM) to provide abundance supply of PUFA. The two diets were iso-caloric and iso-nitrogenous with the main differences been the CTH diet was without the polyphenol compounds from the PAs, FOs and EOs contents (Table 8.2).

Table 8.2 Nutritive value, gross energy content, PAs (g/kg DM), FOs (g/kg DM), EOs (g/kg DM) and FAs profile (g/100 g FA) of the TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item	Diet (g/kg DM)	
	CTH	**DPB**
Organic matter	900.47	906.39
Crude protein	172.75	172.74
Ether extract	40.03	40.05
Neutral detergent fibre	513.31	501.00
Acid detergent fibre	349.26	340.47
Gross energy (MJ/kg DM)	20.68	20.65
PAs		
Gallic acid	0	1.13
Caffeic acid	0	1.01
Syringic acid	0	1.25
P-coumaric acid	0	0.70
Sinapic acid	0	0.72
Ferulic acid	0	0.34
FOs		
Catechin	0	5.65
Rutin	0	2.36
Quercetin	0	73.04
Apigenin	0	8.70
Myricetin	0	0.89
Kaempferol	0	5.75
EOs		
Eugenol	0	15.96
Caryophyllene	0	5.27
FAs		
C16:0	12.98	14.06
C18:0	3.08	2.88

Table 8.2 Continue.

Item	Diet (g/kg DM)	
	CTH	DPB
C18:1 cis-9	23.80	24.07
C18:2 cis-9, cis-12	36.36	36.28
C18:3 cis-9, cis-12, cis-15	1.88	1.86

8.4.1 Outcomes of apparent digestibility

Apparent of digestibility of goats received CTH and DPB diets is presented in Table 8.3. Clearly, supplementation of PP (DPB diet) affected nutrient digestibility of dry matter, organic matter, crude protein, NDF and gross energy ($P<0.05$). However, digestibility of fat and ADF did not affected among the treatment.

8.4.2 Milk composition

Supplementation of PP (DPB diet) increased ($P<0.05$) milk yield and 3.5% fat-corrected milk (FCM) (Table 8.4). Milk component such pH, total solid, acidity and density were similar among treatment and time sampling ($P>0.05$). Fat, protein, lactose, casein and solid non-fat of yield production increased with PP supplementation (DPB). There was interaction between diet and time sampling for somatic cell count. Inclusion of PP in diet (DPB) decreased about 5-folds ($P=0.028$) somatic cell counting compared with goats fed the CTH diet.

FA profile in milk was significantly affected by the dietary treatment (Table 8.5). Compared with the control diet (CTH), the DPB diet decreased ($P<0.05$) content of SFA and increased ($P<0.05$) that of UFA. C18:0 synthesis was inhibited ($P=0.001$) by PP supplementation. In contrast, C18:1 trans11 ($P<0.05$) and C18:2 cis-9, trans-11 increased ($P<0.05$) over time. Overall, total CLA in goats fed by DPB was found a double increasing (1.77 to 2.62 g/100 g FA) than goats fed by CTH diet ($P<$

0.05). The desaturase rate (except desaturase for carbon 18, $P<0.05$), atherogenic and thrombogenic indices were not different among the treatments (Table 8.6). The effects of time observed in milk yield and milk composition including FA concentrations were different ($P<0.05$), especially data collected at terminated period (at 5-6 week of sampling time).

8.4.3 Animal performance and relative abundance of lipid-degraded bacteria

The average pH value of rumen liquor was 6.4 and 6.5, ($P>0.05$) respectively of CTH and DPB diets (Table 8.7). Compared with the CTH diet, DPB diet had higher ($P<0.05$) total VFA but lower iso-valerate ($P<0.05$) with unchanged ratio for acetic to propionic. Supplementation of PP (DPB diet) did not affected methane production by initially goats.

Inclusion of PP in diet (DPB) showed affinity the relative abundances of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in rumen liquor. The proportion of *Butyrivibrio fibrisolvens* ranged from 0.016 to 0.108% of total bacteria, whereas *Butyrivibrio proteoclasticus* ranged from 0.033 to 0.454%. Compared with rumen liquor from goats fed with CTH diet, the presence of *Butyrivibrio fibrisolvens* increased about 5-folds ($P<0.05$), although, the presence of *Butyrivibrio proteoclasticus* decreased about 11-folds ($P<0.01$) in goats fed the DPB diet (Table 8.7).

Table 8.3 Nutrient intake and total-tract digestibility of lactating Saanen goats fed a TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item	Diet		Time (Weeks)						SEM[1]	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Intake (kg/d)												
Dry matter	1.21	1.22	1.23	1.23	1.23	1.23	1.23	1.23	0.001	0.080	0.250	0.464
Organic matter	1.09	1.10	1.11	1.11	1.11	1.11	1.11	1.11	0.031	0.055	0.059	0.248
Crude protein	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.005	0.062	0.689	0.464
Fat	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.003	0.139	0.565	0.421
Neutral detergent fibre	0.62	0.62	0.63	0.63	0.63	0.63	0.63	0.63	0.017	0.241	0.990	0.938
Acid detergent fibre	0.42	0.42	0.43	0.43	0.43	0.43	0.43	0.43	0.011	0.279	0.464	0.689
Gross energy (MJ/kg)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.001	0.066	0.061	0.382
Digestibility (%)												
Dry matter	70.94^{p}	71.38^{q}	71.87^{a}	71.77^{a}	71.86^{a}	71.52^{a}	71.81^{a}	72.01^{b}	0.833	0.036	0.011	0.062
Organic matter	76.62^{p}	77.56^{q}	77.58^{a}	78.07^{a}	78.18^{a}	78.75^{a}	79.09^{b}	79.55^{b}	0.962	0.024	0.001	0.670
Crude protein	77.96^{p}	78.82^{q}	78.77^{a}	79.19^{a}	79.63^{a}	79.88^{a}	79.84^{a}	80.50^{b}	1.632	0.037	0.002	0.511
Fat	96.13	96.04	96.16	96.03	96.15	95.69	95.94	95.68	0.104	0.213	0.716	0.105
Neutral detergent fibre	69.52^{p}	69.98^{q}	70.17^{a}	70.41^{a}	70.50^{b}	70.64^{b}	70.45^{b}	70.51^{b}	0.760	0.031	0.015	0.818
Acid detergent fibre	54.65	55.67	54.22	56.28	55.44	54.60	53.83	53.61	0.133	0.100	0.057	0.792
Gross energy	61.82^{p}	64.12^{q}	63.03	62.94	63.02	62.72	63.14	62.97	0.357	0.013	0.757	0.473

Different superscript lowercase letters in each row within a treatment variable indicate significant differences.
[1]SEM, standard error of measurement.

Table 8.4 DMI, milk yield and milk composition from lactating Saanen goats fed a TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item[1]	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Milk yield (kg/d)	1.08^{p}	1.18^{q}	1.13^{a}	1.13^{a}	1.13^{a}	1.13^{a}	1.12^{b}	1.12^{b}	0.149	0.011	0.013	0.571
FE (kg milk/kg DM)	0.89^{p}	0.94^{q}	0.92^{a}	0.92^{a}	0.92^{a}	0.92^{a}	0.91^{b}	0.91^{b}	0.146	0.021	0.019	0.096
3.5% FCM (kg/d)	1.10^{p}	1.24^{q}	1.19^{a}	1.18^{a}	1.17^{a}	1.17^{ab}	1.16^{b}	1.15^{b}	0.054	0.023	0.001	0.996
Milk composition												
pH	6.43	6.44	6.43	6.42	6.37	6.42	6.36	6.42	0.135	0.390	0.546	0.175
Fat (g/kg)	36.16^{p}	38.28^{q}	38.04^{a}	37.53^{a}	37.59^{a}	37.29^{ab}	36.72^{b}	36.15^{b}	0.235	0.027	0.001	0.757
Protein (g/kg)	32.05^{p}	34.90^{q}	33.68^{a}	33.52^{a}	33.52^{a}	33.45^{a}	33.38^{b}	33.31^{b}	0.285	0.008	0.003	0.258
Casein (g/kg)	25.35^{p}	28.96^{q}	31.07^{a}	26.59^{ab}	26.45^{ab}	26.33^{b}	26.28^{b}	26.22^{b}	0.161	0.003	0.025	0.455
Lactose (g/kg)	37.53^{p}	45.90^{q}	42.24^{a}	41.78^{a}	41.78^{a}	41.76^{a}	41.37^{b}	41.37^{b}	0.159	0.015	0.029	0.594
Total solid (g/kg)	125.92	124.16	125.07	125.04	125.04	125.04	125.03	125.03	0.122	0.488	0.060	0.482
Solid not fat (g/kg)	80.99^{p}	89.57^{q}	88.67^{a}	89.03^{a}	83.63^{b}	83.63^{b}	83.45^{b}	83.27^{b}	0.166	0.015	0.002	0.143
Milk acidity, °D	11.13	11.19	11.16	11.16	11.16	11.16	11.16	11.16	0.031	0.390	0.833	0.968
Milk density, kg/l	1.58	1.53	1.56	1.56	1.56	1.56	1.56	1.56	0.037	0.517	0.650	0.827
Urea (mg/dl)	26.55^{p}	37.29^{q}	33.95	31.55	31.55	31.55	31.48	31.42	0.065	0.044	0.352	0.584
Somatic cell ($\times 10^3$/ml)	1744.73^{p}	427.08^{q}	1646.78^{a}	1283.22^{ab}	1074.33^{b}	838.79^{b}	837.03^{b}	835.27^{b}	1.805	0.033	0.041	0.028

Different superscript lowercase letters in each row within a treatment variable indicate significant differences. SEM, standard error of measurement.
[1]FE, feed efficiency for producing milk. FCM, fat-corrected milks.

Table 8.5 Milk fatty acids (g/100 g) from lactating Saanen goats fed a TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item[1]	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Saturated												
C6:0	3.970	3.710	3.840	3.840	3.840	3.840	3.840	3.840	0.130	0.388	0.896	0.141
C8:0	3.309	3.413	3.223	3.223	3.223	3.223	3.223	3.223	0.106	0.678	0.160	0.228
C10:0	8.091^{p}	4.872^{q}	6.482^{a}	6.478^{b}	6.478^{b}	6.477^{b}	6.476^{b}	6.476^{b}	0.274	0.044	0.006	0.812
C11:0	0.212	0.127	0.170	0.170	0.170	0.170	0.170	0.170	0.778	0.075	0.219	0.819
C12:0	6.212	4.559	5.386	5.385	5.386	5.385	5.385	5.386	0.154	0.076	0.861	0.178
C13:0	0.141^{p}	0.083^{q}	0.112	0.112	0.112	0.112	0.112	0.112	0.103	0.015	0.062	0.302
C14:0	9.377	7.932	8.655	8.645	8.635	8.655	8.655	8.655	0.585	0.104	0.759	0.090
C15:0	0.661^{p}	0.550^{q}	0.606	0.605	0.605	0.605	0.605	0.606	0.434	0.032	0.657	0.665
C16:0	24.989	23.917	24.518	24.541	24.555	24.540	24.545	24.551	0.439	0.006	0.239	0.668
C18:0	13.995^{p}	11.994^{q}	12.986^{a}	12.980^{b}	12.979^{b}	12.977^{b}	12.976^{bc}	12.975^{c}	0.156	0.006	0.002	0.668
C20:0	0.351	0.329	0.340	0.340	0.340	0.340	0.340	0.340	0.422	0.155	0.110	0.122
C21:0	0.041	0.047	0.044	0.044	0.044	0.044	0.044	0.044	0.247	0.310	0.074	0.060
C22:0	0.214	0.275	0.245	0.245	0.245	0.245	0.245	0.245	0.623	0.083	0.663	0.775
C23:0	0.039	0.018	0.110	0.110	0.110	0.110	0.110	0.110	0.118	0.126	0.135	0.123
C24:0	0.049	0.034	0.042	0.042	0.042	0.042	0.042	0.042	0.110	0.061	0.554	0.111
Monounsaturated												

Table 8.5 Continue.

Item[1]	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
C14:1 cis-9	0.322	0.413	0.367	0.367	0.367	0.367	0.367	0.367	0.027	0.239	0.769	0.917
C16:1 cis-9	0.622	0.664	0.643	0.643	0.643	0.643	0.643	0.643	0.197	0.371	0.052	0.137
C18:1 trans-11	18.694^{p}	21.823^{q}	20.259^{a}	20.249^{b}	20.246^{b}	20.244^{b}	20.242^{bc}	20.240^{c}	0.136	0.022	0.001	0.508
C18:1 cis-9	3.181	8.265	12.502	12.491	12.494	12.492	12.493	12.496	0.377	0.092	0.234	0.080
C20:1	1.309^{p}	0.143^{q}	0.726^{a}	0.726^{a}	0.726^{a}	0.725^{b}	0.725^{b}	0.725^{b}	0.037	0.042	0.006	0.663
Polyunsaturated												
C18:2 cis-9, cis-12	1.341^{p}	2.690^{q}	2.026^{a}	2.025^{a}	2.024^{b}	2.024^{b}	2.024^{b}	2.024^{b}	0.038	0.023	0.004	0.282
C18:3 cis-9, cis-12, cis-15	0.317^{p}	0.951^{q}	0.634^{a}	0.634^{a}	0.634^{a}	0.634^{a}	0.633^{b}	0.633^{b}	0.026	0.024	0.010	0.158
C18:2 cis-9, trans-11	1.351^{p}	2.155^{q}	1.753^{a}	1.752^{ab}	1.752^{ab}	1.751^{b}	1.751^{b}	1.751^{b}	0.082	0.026	0.001	0.782
C18:2 trans-9, cis-11	0.120	0.137	0.128	0.128	0.128	0.128	0.128	0.128	0.027	0.132	0.060	0.856
C18:2 trans-10, cis-12	0.144	0.172	0.158	0.158	0.158	0.158	0.158	0.158	0.935	0.259	0.349	0.721
C18:2 trans-9, trans-11	0.160	0.158	0.159	0.159	0.159	0.159	0.159	0.159	0.056	0.949	0.103	0.058
C20:5 n-3	0.035	0.063	0.049	0.049	0.049	0.049	0.049	0.049	0.305	0.089	0.076	0.157
C22:5 n-3	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.496	0.720	0.273	0.993
C22:6 n-3	0.687	0.421	0.554	0.554	0.554	0.554	0.554	0.553	0.623	0.189	0.192	0.803
Total CLA	1.775^{p}	2.622^{q}	2.198^{a}	2.197^{b}	2.197^{b}	2.197^{b}	2.197^{b}	2.196^{b}	0.765	0.037	0.004	0.387
SFA	71.650^{p}	61.861^{q}	66.756^{b}	66.758^{b}	66.761^{ab}	66.764^{a}	66.767^{a}	66.770^{a}	0.412	0.031	0.002	0.370

Table 8.5 Continue.

Item[1]	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
UFA	28.350^{p}	38.139^{q}	33.244	33.242	33.239	33.236	33.233	33.230	0.393	0.038	0.771	0.963
MUFA	24.128^{p}	31.309^{q}	27.718	27.718	27.718	27.718	27.718	27.718	0.352	0.040	0.730	0.826
PUFA	4.222^{p}	6.830^{q}	5.526^{a}	5.523^{a}	5.521^{ab}	5.518^{b}	5.515^{b}	5.512^{b}	0.896	0.028	0.048	0.292
n−3	1.106	1.498	1.303	1.302	1.302	1.302	1.302	1.302	0.870	0.179	0.060	0.254
n−6	1.341^{p}	2.710^{q}	2.026^{a}	2.025^{a}	2.024^{b}	2.024^{b}	2.024^{b}	2.024^{b}	0.381	0.023	0.004	0.285
SCFA	15.441^{p}	12.040^{q}	6.823^{b}	6.837^{b}	6.838^{ab}	6.838^{a}	6.839^{a}	6.839^{a}	0.183	0.015	0.020	0.897
MCFA	42.464^{p}	38.200^{q}	40.398	40.410	40.414	40.419	40.424	40.428	0.216	0.041	0.316	0.065
LCFA	42.094^{p}	49.760^{q}	52.779^{a}	52.753^{a}	52.748^{a}	52.743^{b}	52.738^{b}	52.732^{b}	0.216	0.039	0.049	0.759

Different superscript lowercase letters in each row within a treatment variable indicate significant differences. SEM, standard error of measurement.

[1]CLA, conjugated linolenic acid. SFA, saturated fatty acid. UFA, unsaturated fatty acid. MUFA, monounsaturated fatty acid. PUFA, polyunsaturated fatty acid. SCFA, short chain fatty acid. MCFA, medium chain fatty acid. LCFA, long chain fatty acid. n-3, omega-3 FA. n-6, omega-6 FA.

Table 8.6 Desaturation (DI), thrombogenic and atherogenicity indices of milk from lactating Saanen goats fed a TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item[1]	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Total DI	0.260	0.305	0.261	0.261	0.261	0.261	0.261	0.261	0.543	0.097	0.117	0.327
DI C14:1	0.033	0.049	0.041	0.041	0.041	0.041	0.041	0.041	0.167	0.104	0.849	0.186
DI C16:1	0.024	0.027	0.026	0.026	0.026	0.026	0.026	0.026	0.096	0.312	0.155	0.861
DI C18:1	0.572^{p}	0.645^{q}	0.609	0.609	0.609	0.609	0.609	0.609	0.479	0.044	0.670	0.830
DI C18:2 cis-9, trans-11	0.298	0.207	0.123	0.123	0.123	0.123	0.123	0.123	0.159	0.077	0.665	0.779
Atherogenicity	2.424	1.579	1.941	1.940	1.940	1.942	1.942	1.943	0.782	0.067	0.108	0.219
Thrombogenicity	2.865	1.988	2.376	2.377	2.377	2.378	2.378	2.378	0.310	0.051	0.056	0.838

Different superscript lowercase letters in each row within a treatment variable indicate significant differences. SEM, standard error of measurement.

[1]DI, desaturation index, calculated following to Brogna et al., (2011); Atherogenicity, calculated following to Ulbricht and Southgate (1991); Thrombogenicity, calculated following to Chilliard et al. (2003).

Table 8.7 VFA composition and relative abundances of Butyrivibrio species in rumen liquor from lactating Saanen goats fed a TMR with sunflower oil (CTH) and TMR with sunflower oil and PP (DPB).

Item	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
pH	6.61	6.82	6.83	6.83	6.73	6.68	6.68	6.68	0.901	0.345	0.079	0.054
Total VFA, mM	80.81^{p}	98.40^{q}	90.59	90.61	89.35	88.13	89.03	89.92	1.191	0.044	0.111	0.073
Individual VFA, mol/100 mol												
Acetate	62.04	62.66	62.35	62.36	62.37	62.38	62.38	62.39	0.512	0.052	0.171	0.437
Propionate	19.82	20.06	19.94	19.94	19.93	19.93	19.93	19.92	0.170	0.369	0.268	0.051
Iso-butyrate	0.55	0.44	0.50	0.50	0.50	0.50	0.50	0.50	0.835	0.201	0.083	0.313
Butyrate	10.14	10.29	10.22	10.21	10.21	10.21	10.21	10.21	0.331	0.065	0.263	0.727
Iso-valerate	2.02^{p}	1.11^{q}	1.56	1.56	1.56	1.56	1.56	1.56	0.217	0.026	0.504	0.054
Valerate	5.43	5.43	5.43	5.42	5.42	5.42	5.42	5.42	0.115	0.415	0.064	0.199
Acetate/Propionate ratio	3.13	3.12	3.13	3.13	3.13	3.13	3.13	3.13	0.029	0.309	0.544	0.120
Methane by moss, ml/100 ml	26.65	26.83	26.70	26.72	26.75	26.68	26.80	26.79	0.032	0.137	0.544	0.785
Population[1]												
Butyrivibrio fibrisolvens	-0.86 (0.02)^{p}	-0.61 (0.10)^{q}	-0.11 (0.05)^{b}	-0.11 (0.05)^{b}	-0.10 (0.06)^{a}	-0.10 (0.06)^{a}	-0.10 (0.06)^{a}	-0.10 (0.06)^{a}	0.161	0.016	0.006	0.171
Butyrivibrio proteoclasticus	-1.00 (0.44)^{p}	-1.54 (0.04)^{q}	-0.440 (0.243)^{a}	-0.441 (0.243)^{a}	- 0.445 (0.240)^{b}	-0.448 (0.239) ^{c}	-0.453 (0.236)^{c}	-0.453 (0.236)^{c}	0.122	0.002	0.020	0.208

Different superscript lowercase letters in each row within a treatment variable indicate significant differences. SEM, standard error of measurement.

[1]Log10 of % 16S rRNA gene of total bacteria (observed values in parentheses).

8.5 Discussion

In present study, the PP inclusion was observed to modulate animal performances and dairy characteristics by initial secondary metabolites such as PAs, FOs and EOs. Since, PA study has been exceedingly in chapter IV and free-access references with earlier reports (Scalbert, 1991; Cabiddu et al., 2010; Cardona et al., 2013; Cutrim and Cortez, 2018; Vasta et al., 2019), the later discussion is highlighted in FO and EO concerns.

In practices regard of incorporated secondary metabolites such as polyphenols in animal diets suggested to consider the acceptable condition for animal itself (Jayanegara et al., 2012b).

FOs are one of secondary compounds with variable phenolic structures and promote good flavour. However, preserving FOs with EOs in diet might provide a slightly sour-bitter taste leading to an affinity in eating rate and feed intake (Patra et al., 2019). In the present study, DM intake of goats fed with 13 g FOs-EOs daily was not affected, suggesting that inclusion of PP at the rate for this study did not affect palatability of the TMR. This result was consistent with those of De Nardi et al. (2014) and Ma et al. (2017) who evaluated the effect of polyphenol-EO mixture and FO-rich diet in dairy cows and sheep.

In ruminants, fermentation products, metabolites, microbiome and pH are affected by diets (Mamuad et al., 2019). In the present study, ruminal pH was stable with a mean value of 6.7 for the entire 6 weeks measurement, suggesting that inclusion of FOs-EOs in diet did not drastically change the ecosystem of the gut microbiota. This finding was in agreement with previous studies (De Nardi et al., 2014; Lourenço et al., 2014; Ma et al., 2017). Ruminal pH is closely related with fermentation products, such

as ammonia deposition, lactic acid and VFA. Although, there was no close relationship between pH and VFA in present study, total VFA increased with the presence of FO-EO. Our result differed with earlier findings (De Nardi et al. (2014), who evaluated polyphenols-EOs in diet which failed to influence on total VFA production in the rumen. However, our results showed no change in acetate, propionate and butyrate proportion but PP supplementation increased the acetic to propionic ratio. More recently, Ma et al. (2017) reported supplementation of FOs derived from Mulberry leaf increased total VFA and only the proportion of iso-valeric was affected. In contrast, Joch et al. (2019) mentioned that lower VFA concentration was observed in rumen fluids derived from lactating cows fed by 1.2 g/head/d of essential oil blend. The inconsistent findings seems to suggest that a reduction of total VFA might be due to a strong antimicrobial property of EOs which can regulate and modified rumen fermentation (Wallace, 2004). The bioactive compounds of EOs could interfere membrane semi-permeability of microorganism in rumen, thereby inhibiting their activity especially on carbohydrate degradation (Calsamiglia et al., 2007).

Moreover, instead of antimicrobial role, FOs seemed to have a positive effect on nutrient fermentation. Among the FOs, rutin and quercetin have been reported to able to modulate microorganism activity to enhance nutrient degradation in rumen, thus, leading to a greater ruminal VFA concentration (Berger et al., 2015; Cui et al., 2015). In the present study, the content of FOs was higher than EOs and this fact might create a new inhibitory affinity to the microorganisms in the rumen. Evaluation of antimicrobial properties of FO-rich fractions and EOs had been conducted to understand the effect of their activity against different bacteria (Mandalari et al., 2007). The results showed that combination of those compounds decreased inhibitory activity against several bacteria.

It was suggested that EOs inhibit microorganism growth, and although FOs may compete with EOs to select the target sites through their hydrophobic interaction, this occurrence caused a change in the structural conformation between FOs and EOs tending to lower accessibility of EOs to suspend microbial activity. Therefore, an increase in the total VFA recorded in the present study suggested a lack of EOs because of the interruption by FOs for interrupting the process of fermentable nutrient in rumen.

Dietary FOs with other phenolic compounds in feeding regimens suggested to the inconsistent results on milk yield and milk composition due to lactation stage, time observation and status of animal health (Olagaray and Bradford, 2019). In the current study, inclusion of FOs-EOs in diet increased production of milk yield accompanied by varied contents of milk composition. These results were in accordance with earlier study by Winkler et al. (2015), who reported a higher content of lactose in milk from dairy cows received green tea and curcuma extract (GTCEx) compared with the initial control diet on early lactation stage. However, the content of fat, protein and casein was not affected by inclusion of GTCEx. In addition, a higher content of fat and protein in present study might be due to that PP having a higher efficiency of digestibility rate in the rumen (Berger et al., 2015) and thus modulate the various hydrolysing and conjugating enzymes in the small intestine as primary substrates (Abbas et al., 2017). In current study, PP demonstrated a greater digestibility of fermented organic matter and protein that could improve activity of synthesizing enzymes in dairy goats. Furthermore, these compounds decreased the somatic cells in milk in the current study, which was similar to the results found in dairy cows fed flax meal (Schogor et al., 2013). In many reports, *Piper betle* L. is well-known as potential agent of antioxidant and anti-inflammatory (Arambewela et al., 2006; Alam et al., 2013; Das et al., 2016).

Therefore, the possibility of increasing milk yield in similar DMI intake attributes to the capability of PP to reduce metabolic stress and inflammation to achieve a more efficient utilization of energy and crude protein for milk production (Winkler et al., 2015).

It is worthy to note that results of present study showed that inclusion of PP increased milk yield and several milk constituents; including fat, protein, casein, lactose. The above suggests the advantage of supplementing PP in lactating goats. However, almost all of the above parameters were lower at week 6 as compared to 5-time samplings beforehand. The decline in milk yield and nutrients composition is expected as Clark and García (2017) who reviewed goat milk data collected over the last 100 years reported that milk yield, and fat and protein components normally increase during the first 60 days of lactation but decrease thereafter. Several relevant factors namely breed, age, parity, management and lactation stage can influence milk production and milk composition (Chilliard et al., 2003). Results of our study showed goats received DPB diet had lower number of milk somatic cell counts suggesting they have a higher physiological mammary health as compared to those fed CTH diet. It was reported that inflammation rate of mammary gland could affect the quality and quantity of milks, and polyphenols are the best-known antioxidant to treat alleviating stress-induced inflammation on animal (Schogor et al., 2013; Clark and García, 2017; Olagaray and Bradford, 2019). Thus, results of this study showed that secondary metabolites derived from PP provide the needed antioxidant for inflammation suppression in lactating goats (Das et al., 2016; Purba and Paengkoum, 2019).

To our knowledge, only limited information is available about the effect of FOs and/or EOs on FA milk profile and relatively abundance of bio-hydrogenation related microbiota, especially *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus*

(Oskoueian et al., 2013; Kim et al., 2015; Ma et al., 2017). In the current study, inclusion of PP in diet basically maintained the total bacteria population. This result is in agreement with previous *in vitro* studies, which reported supplementation of FOs (Oskoueian et al., 2013) and EOs (Patra and Yu, 2012; Cobellis et al., 2016) did not influence the population of total bacteria in rumen. These findings corroborated with an *in vivo* study which supplemented FOs from Mulberry leaf (Ma et al., 2017). As regards of basic function of phenolic compounds, FOs and EOs have natural phytochemicals to behave as potent antimicrobial, although, the effect of their biological properties is depending on their bioavailability (Abbas et al., 2017; Olagaray and Bradford, 2019). In the current study, microbiologic characterisation of rumen liquor showed that inclusion of PP in diet resulted in a surge in the relative abundance of *Butyrivibrio fibrisolvens*, while *Butyrivibrio proteoclasticus* number dropped drastically. Since, the reference about inclusion of FOs-EOs in diet is scarce, those compounds might be assessed as similar as polyphenols interfere. To the best of our knowledge, there were two consecutive observations supplemented polyphenols derived from Papaya leaf in goat diets to investigate the relative abundance of *Butyrivibrio fibrisolvens*. In an *in vitro* experiment, supplementation of 6% DM of papaya leaf in fermenters reduced population of *Butyrivibrio fibrisolvens* (Jafari et al., 2016) but supplementing the same material at 50% DM to replace concentrate diet in goats resulted in an escalated the number of *Butyrivibrio fibrisolvens* (Jafari et al., 2018). It seemed likely that diet composition plays a fundamental role in the affinity of rumen microorganisms. Hence, a change of milk FA profile observed in the current study represented an enhancement of rumen BH, as regards of inclusion of PP in diet altering microbial ecosystem. Even tough, the factor stimulating rumen BH is not only

known by observing *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus*, but also other groups of ruminal bacteria, e.g. *Prevotella, Clostridiales* and *Ruminococcaceae* which could be altered by diet formulation (Halmemies-Beauchet-Filleau et al., 2013; Castro-Carrera et al., 2014). Further studies are needed to better clarify the effect of FOs and EOs on particular bacterial strains included in BH scheme.

As expected, rumen BH in present study was inhibited by inclusion of FOs-EOs in diet, resulting milk SFA content in lower amount than UFA content. Several studies in *in vitro* and *in vivo* described that supplementing phytochemical substrates with abundant lipid content could change a rumen BH and produce a limited content of C18:0 (Jayanegara et al., 2011; Buccioni et al., 2015; Miri et al., 2015). In current study, C18:0 content in milk was produced in lower amount and C18:2 cis9 trans11 and C18:1 trans11 were increased by dietary DPB. It is well documented that ability of mammary tissue to undertake hydrogenation and stearoyl Co-A desaturase enzyme (SCD) activity regulated a final step of BH, which convert readily C18:1 trans11 to C18:0 directly and intermediated process of converting C18:1 trans11 to C18:2 cis9 trans11. In the mammary tissue, the SCD activity helps to catalase systematically oxidation of C18: trans11 and this process is fundamentally important resulting in production of 70–95% of the C18:2 cis9 trans11 present in the milk. In earlier references, Khiaosa-ard et al. (2015) and Średnicka-Tober et al. (2016) summarized that C18:2 cis9 trans11 and C18:1 trans11 in excessive concentrations in milk from animals raised in organic farms, which might be considered the forage source in grazing system providing plants rich in secondary metabolites. Among a list of desaturations observed in the current study, the FOs-EOs influenced $\triangle^9$ desaturation. In other words, escalated amount of C18:0 cis9 trans11 and C18:1 trans11 in milk were highlighted due to a change of microbial-lipid

population as aforementioned, especially possessed stearic producer (*Butyrivibrio proteoclasticus*). However, whether FOs-EOs affect directly or indirectly (for instance, by modulating the substrate availability to the mammary gland) the activity of SCD enzyme needs further investigation. In regard to this last point, a change of major bacteria community particularly limited population of *Butyrivibrio proteoclasticus* and dietary prolonged PUFA in the diet had been reported leading to milk fat depression (Glasser et al., 2008; Lourenço et al., 2010; Shingfield et al., 2013). However, it is rare phenomena founded in present study that increased C18:2 cis9 trans11 with low amount of C18:2 trans10 cis12 in milk fat production. This result indicating no massive *trans*-10 shift had occurred in the rumen BH pathways. Regardless of antimicrobial and antioxidant roles, PP was reported in different references owning antidiabetic properties (Arambewela et al., 2005; Das et al., 2016). This role to be pointed to rutin content of PP could express gastroprotective effects exerting a limited activity on lipid peroxidation (Abdel-Raheem, 2010; Hosseinzadeh and Nassiri-Asl, 2014).

8.6 Conclusion

The use of sunflower oil in the diets of dairy goats with practical doses of combination of PAs, FOs and EOs in leaves resulted in significant alternations of milk yield, milk composition and milk FA profile, without affecting DMI intake and feed efficiency to produce milk daily. Specifically, the DPB diet seemed to be more efficient in impeding rumen BH of PUFA to SFA with decreasing stearic acid content, thereby increasing the a total CLA, especially RA content. Also, an unexpected result of C18:2 trans10 cis12 as inducer milk fat depression is absent from goats fed with DPB diet. Obviously, this occurs due to desaturation stage of carbon-18 was affected, resulting in

an escalated content of vaccenic acid. Further, a change of microbial rumen related to BH was indirectly proved by presence of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in rumen liquor. Notably, this combination of PAs, FOs and EOs in plant mostly shown positive effects. A present evidence of a greater content of total VFA either suggested no negative effects on fermentation is equally important to highlight of this study. However, dose–response study is needed to elucidate the different origin of organic compounds, whether crude or extract forms needed to be tested fairly to achieve the reliable and reproducible effects of LA on BH to maximize the advancement of CLA and vaccenic acid contents in milk fat exerting lower amounts of lipid inclusion. Ultimately, experiment of this regard is needed to confirm these results over a longer period (early, mid and late of lactation).

Despite promising outcomes by PP (DPB) diet on animal response including CLA content in milk, metabolic status due to higher lipid inclusion should be observed, especially during early lactation. Next chapter regarding antioxidant status of dairy goats is presented.

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CHAPTER IX

MAMMARY GENE EXPRESSIONS AND OXIDATIVE INDICATORS WITH ANTIOXIDANT ENZYME ACTIVITIES IN THE RUMEN FLUID, BLOOD, MAMMARY TISSUE AND MILK OF DAIRY GOATS FED PIPER POWDER

9.1 Abstract

In early lactation, dairy animals have been characterized by more oxidative stress and low antioxidant defence. However, there are limited data focussing on the role of plant polyphenols on regulating antioxidant in dairy animals. In this study, we evaluated the effect of PP on the activity of antioxidant enzymes (SOD, GPx and CAT) in the ruminal fluid, mammary tissue, milk and blood, and oxidative stress indicators (DPPH and TBARS) in the ruminal fluid, mammary tissue, milk and plasma. The mRNA abundance of the antioxidant enzymes and oxidative stress-related genes was measured in mammary tissue. Twelve Saanen goats (body weight (BW), 42±1.00 kg; DIM, 14±3d) were allotted into two experimental groups, which received two experimental diets formulated in a TMR: CON diet (no PP supplementation) and RDP diet (inclusion of 12.83 g/kg DM PP). The trial lasted 6 weeks, after a 2 weeks adaptation period. Compared with goats receiving CON diet, dietary RDP increased SOD activity in ruminal fluid, mammary tissue, milk and blood. GPx and CAT in those samples were similar activity among throughout experiment. DPPH inhibition observed in plasma, milk, ruminal fluid and mammary tissue were not affected by dietary RDP.

Besides, the dietary RDP affected TBARS production in plasma, milk, ruminal fluid and mammary tissue, with declination values of approximately 1-fold, 1-fold, 2-fold, and 1-fold, respectively. Furthermore, alleviated TBARS production was associated with dwindling mRNA expression of NFKB but greater expression of SOD1, SOD2, SOD3 and NFE_2L_2 in mammary tissue. The results of present study suggest that inclusion of PM in diet (RDP) can improve the oxidative status of Saanen goats in early lactation, however, stability motion of PP in later lactation stage is still questionable.

Keywords: phenolic acids, flavonoids, essential oils, milk fatty acids, rumen, microbial populations, goats.

9.2 Introduction

Although improving the nutritional quality of dairy products through the provision of prolonged lipid in ruminant diets, this activity increases a level of lipid peroxidation and is prone to oxidative stress, as consequence of failed metabolic requirements for maintenance and production (Gobert et al., 2009). Activity of this peroxidation resulted in oxidative damage, which lipid substrate containing carbon-carbon double bond (s) is attacked by a free radical and other reactive oxygen (Vasilaki and McMillan, 2011). In term of immune response such specific cellular metabolic circumstances and repair capacities, the cells promote cell survival or induce cell death (Ayala et al., 2014). A survival cell in animal body exerts the extent of natural antioxidant system in correspondence with intracellular enzymes such as SOD, GPx and CAT, which have a fundamental role to delete superoxide's and peroxides for further deteriorated reaction (Miller et al., 1993).

A plunged oxidative stress associated with enhanced antioxidant properties on lactation performance of dairy ruminants could be obtained from supplementary antioxidant agent in diets, which could help to mitigate an initiation of lipid peroxidation (Celi, 2010). In general, a couple of potential antioxidant agents pointed to supplementary vitamin C and/or vitamin E in diets protects lipids and lipid structures against peroxidation (Buettner, 1993). Further, plant polyphenols associated with vitamin E had been reported to decrease plasma lipoperoxidation in dairy cows (Gobert et al., 2009). Solely vitamin E supplementation as antioxidant replacer had no effect on lipoperoxidation, whereas vitamin E in association with polyphenol-containing diets resulted in lowered lipoperoxidation by boosting the resistance time against peroxidation (+47%) and by alleviating the oxidation rate (-48%). More recently, Côrtes et al. (2012) and Schogor et al. (2013) who corroborated Gobert's results, that dietary polyphenols from plant lignans without combination of vitamin C or E contribute to increase antioxidant properties and shelter mammary tissue and other tissues of dairy cows from oxidative stress damage. Hence, plant polyphenols richer in antioxidant substrates could be assessed as alternative way to promise enhancement of metabolic defence against oxidative stress coming along with positive side effect in abundance of immunity, reproductive and health benefits.

In last decade, research on contemporary antioxidants has concerned on natural molecules to delight consumers to be much interested in a 'clean label' trend regard of safety and toxicity, without leading to deterioration on the environment (Asioli et al., 2017). Polyphenol-rich plants are naturally strong antioxidants and antidiabetics, and Piper betle L. leaf is prominent as the richest dietary source of flavonoids, essential oils and phenolic acids (Purba and Paengkoum, 2019). This Piperaceae leaf is edible and

eligible for human and animal, with preserving nutrient of protein, fat, carbohydrate and mineral about of 3-3.5%, 0.4-1.0%, 0.5-6.10% and 2.3-3.3% of total nutrient as dry matter (DM), respectively (Guha, 2006). However, it is highly perishable commodity, thus, always subject to environment as valuable waste. Furthermore, polyphenols likely flavonoids could be degraded on nutrient fermentation through an intermediate metabolite (McSweeney et al., 2001) and then is transferred to milk and plasma in higher of antioxidant fraction (Tian et al., 2018). A typically flavonoids in diet such anthocyanin was observed in rumen fluid, mammary tissue, milk and plasma of Saanen goats (Tian et al., 2018; Tian et al., 2019). The levels of DPPH scavenging activity and SOD in plasma, abundance of NFE2L2) SOD2, GPX1, and GPX2 mRNA expression in the mammary gland were increased with a higher content of anthocyanin in milk. To our knowledge, there is scarce information about the effect of PP on the activity and expression of antioxidant enzymes in dairy goats during early lactation. We hypothesized that dietary PP attenuates oxidative stress indicators in physiological fluids (e.g. blood, ruminal fluid and milk) and physiological tissues (e.g. tissue in mammary gland). Therefore, the present study investigated the effect of PP on the activity of antioxidant enzymes (SOD, GPx and CAT) in the ruminal fluid, mammary tissue, milk and blood, and oxidative stress indicators DPPH and TBARS in the ruminal fluid, mammary tissue, milk and plasma of dairy goats during early lactation.

9.3 Materials and methods

9.3.1 Animals, diet and experimental procedures

Animals, diet and experimental procedure were performed as described in chapter VIII with DPB statement changed by RDP.

9.3.2 Experimental procedures

Altogether remained feed included ort and actual corrected data of milk yield were recorded daily throughout the experiment, and data from 15 days onwards were averaged and inputted to ANOVA. Samples of the feed were collected weekly and were frozen at -20°C for subsequent drying at 55°C and further related analysis. On day 21, 29, 37 and 45, milk samples were collected from morning and afternoon milkings, sampled freshly in proportion to milk yield (100 g per kg of recorded), pH checked (Oakton 700, USA) and pooled together. Milk samples were allotted into 2 aliquots: the first aliquot was directly stored at -80°C fixed with 0.02% (w/w) of sodium azide for analysis of milk antioxidant (DPPH, TBARS, SOD, CAT and GPx). The second aliquot was kept frozen at -20°C with a preservative (bronopol tablet; D&F Control Systems Inc., Dublin, CA) for milk analysis, e.g. fat, protein, casein, lactose, total solid, urea of milk composition and somatic counting score. Data about of feed intake, milk composition (except somatic counting cell) was not shown in this chapter (see chapter VIII).

On day 23, 30, 37, 44, 51 and 58 rumen fluids at 0 h (before feeding), 3 h and 6 h were collected from each goat using a stomach tube connected to a manual pump (Lodge-Ivey et al., 2009). Immediately after collection, each sample of rumen fluids was measured for pH using a portable pH meter (Eutech Pc 700, Italia), then strained through four layers of cheesecloth, and filtered ruminal fluids of each sampling time were stored at -80°C for DPPH, TBARS, SOD, CAT and GPx analyses. On the day of observation, samples of frozen rumen fluids were thawed on ice box and abundant volume of those were directly used for TBARS determination. To determine the activity of antioxidant enzymes (SOD, CAT and GPx), remained thawed rumen fluids were

cleaned from protozoa (centrifuged at 800 g for 15 min at 4°C) and bacteria debris (second centrifuged at 13700 g for 25 min at 4°C). For DPPH analysis, 75 ml of the supernatant of second centrifuged were mixed with methanol (2.55 ml) during 60 s and subsequently centrifuged at 9809 g for 15 min, and the upper layer was carried on performing DPPH analysis. Besides, blood samples (15 ml) were collected immediately before the morning feeding (0 h), 3 h and 6 h post feeding from jugular vein into Vacuette tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) containing K_3-EDTA (0.47 mol/L). Plasma was isolated from the blood by centrifugation at 3000 g for 15 min at 4°C (Sorvall Legend XT/XF Centrifuge Series, Thermo Fisher Scientific, Waltham, MA) and stored at -80°C to determine plasma antioxidant (DPPH, TBARS, SOD, CAT and GPx). The remaining erythrocyte was stored at -80°C for subsequent analysis of enzyme activity.

On days 24 31 38 45 52 and 59 biopsies of the mammary gland were performed following to earlier method by Farr et al. (1996) and minor modification given by Tian et al. (2018). Briefly, a midpoint (about 10 cm^2 area of skin) on the left or right rear quarter gland was chosen as biopsy area. The area was strictly checked about 2-3 cm from a recent scar that indicated earlier biopsy area. Before offering anaesthesia procedure, the selected area was cleaned and shaved. The samples of biopsy were taken using a semi-automatic biopsy needle (16 G×90 mm, SAG-16090, TSK Corporation, Tochigi, Japan). This activity caused a minor inflammation, although wound area was completely disappeared within 3 d. Biopsy samples of mammary tissue were subsequently rinsed in sterile saline solution to remove all traces of blood and then divided into three parts: the first part kept into 1.5 ml microcentrifuge tube was instantly frozen in liquid N_2 and stored consecutively at -80°C for gene expression analyses. The

second part was ground promptly with a rotor–stator homogeniser (IKA-T50 ultra-turrax, USA) and kept at -80°C for further analysis of antioxidant enzyme activity.

9.3.3 Chemical analysis

Chemical analysis of milk composition (except somatic counting cell) and feed including its organic compound was performed by given in chapter VIII. Somatic cells were counted using an optical somatic cell counter (Lactoscan SCC 6010; (Milkotronic, Milkotronic, Nova Zagora, Bulgaria).

Determination of DPPH in plasma, milk, ruminal fluid and mammary tissue was performed spectrophotometrically (Varioskan-LUX multimode microplate reader, Thermo Scientific, USA) using a stable free-radical DPPH as descripted in previous study (Martinez et al., 2006), with minor modification. Briefly, 200 mM solution of DPPH (Sigma-Aldrich, St. Louis, MO; Pcode: 101845869) was assayed in methanol and made freshly (±60 min before used) as described by Tian et al. (2018). All samples of milk, rumen fluid, mammary tissue and plasma were received this concentration and all reactions were prepared and read by polypropylene ninety-six-well plates. Plasma samples were prepared and performed completely according to Martinez et al. (2006), while milk and rumen fluid samples were in accordance to Schogor et al. (2013). The samples of mammary tissue were prepared from 10% mammary tissue homogenate. About 1 ml of homogenised mammary tissue was mixed with 9 ml of normal saline on ice, then centrifuged at 6000 g for 20 min at 4°C, and supernatant was collected to perform DPPH observation. The assay in DPPH was determined at 515 nm after 30, 30, 25 and 30 min for plasma, milk, ruminal fluid and mammary tissue, respectively. The assay was performed in quadruplicate. Radical scavenging capacity was expressed as percentage DPPH (DPPH%) and calculated

using the following equation: DPPH%= [(Abs control -Abs sample)/ (Abs control)] × 100. A calibrated curve of antioxidant capacity was calculated as described by Li et al. (2009) and a linear relationship of antioxidant and volume dilution including the 50% effective concentration (EC50) was performed according to previous reports (Smet et al., 2008; Chen et al., 2013).

Lipid peroxidation was assessed in plasma, milk, ruminal fluid and mammary tissue from the original samples using a commercially available TBARS assay kit (CN: E BC K184 OXI-TEK; Elabscience, USA) under the florescence procedure (λex/em = 560 nm/585 nm) and was performed according to the manufacturer's instructions. The assay was performed in quadruplicate.

The activity of GPx, CAT and SOD in plasma, erythrocytes, ruminal fluid, milk and mammary tissue was determined enzymatically under monitoring absorption by microreader (Varioskan-LUX multimode microplate reader, Thermo Scientific, USA). The activity of GPx was calculated according to the changing rate of absorbance value of catalyzing NADPH and $NADP^+$ under absorbance peak at 340 nm using a GPx assay kit (CN: E-BC-K148). The activity of CAT was calculated according to the changing rate of absorbance value of decomposing H_2O_2 by CAT under absorbance peak at 240 nm using a CAT assay kit (CN: E-BC-K106). The activity of SOD was measured based on colorimetric analysis of WST-1 products after samples in microplate reader incubated at 37°C for 20 min. The activity of SOD was determined at 450 nm using a SOD assay kit (CN: E-BC-K020). The sensitivity, intra assay CV, inter assay CV and recovery were 0.2 U/ml, 2.9, 3.7 and 96.6%, respectively.

9.3.4 Quantitative real-time RT-PCR amplifications of gene expression

Total RNA was extracted and purified from the mammary biopsy samples using the TRIzol reagent method described by Labrecque et al. (2009), with a minor modification (Tian et al., 2018). Briefly, 100-150 mg of mashed mammary biopsy and 0.2 g of 0.5-mm dia. Zirconia/silica (Cat. No. 11079105z, BioSpec Products) were assayed in 1 ml of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), then were run in a homogenizer for 45 s, interval 5 s and repeated at 6 runs. The precipitation of RNA from assay was performed using phenol: chloroform (50:50). Purity of the extracted RNA was performed by measuring the 260/280 absorbance ratio with a NanoDrop NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA). Abundant purity of extracted RNA obtained in average 260/280 ratio throughout the experiment was 1.99 (1.98-2.04). Integrity of the RNA was performed by verifying the presence of 18S and 28S RNA bands using an ImageQuant LAS 500 imager (GE Healthcare BioSciences) after electrophoresis on a 1% agarose gel. The RNA was diluted to a consistent concentration (150 ng/µL) with DEPC water (Ambion by Life Technologies, NY) for cDNA synthesis. The cDNA synthesis was performed as described by the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI).

The relative mRNA abundance of the selected genes was conducted using Real Time quantitative PCR (RT-qPCR). Quantitative PCR amplification, detection and data analyses were equipped using a Roche Lightcycler 480-II (Roche Applied Science, Switzerland). Reference of selected genes as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeper gene, SOD1, SOD2, SOD3, GPx1, GPx2, GPx3, CAT and nuclear factor (erythroid-derived 2)-like 2

(NFE_2L_2) have been published previously (Ma et al., 2018; Tian et al., 2018); κ light polypeptide gene enhancer in B-cells (NFKB) and three additional housekeeper genes (peptidylpropyl isomerase A (PPIA), actin β (ACTB), Polyubiquitin) were given by Schogor et al. (2013). In present study, GAPDH was identified as the best reference gene for normalisation of datasets. The primers set of selected genes were designed including their sequencing using the GenScript real-time PCR (TaqMan), which were purchased from the Vivantis Technologies Sdn Bhd company (explicit due to company concern).

The RT-qPCR amplifications were assayed in a 10 µl reaction volume, which consisted of 5 µl of 2×Roche 04707516001 LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 2 µl of 10×diluted cDNA and 1 µl of forward and reverse primers. The mixed assay was prepared in the PCR plates (LightCycler 480 multiwell plate 96, white; Roche Diagnostics GmbH) with plastic cover. The plates were centrifuged at 4°C, 1,500 rpm for 3 min (Universal 320, Hettich Zentrifugen, Germany). The cycling conditions were 10 min at 95°C for pre-incubation, 40 cycles of 30 s at 95°C for amplification, and 55 to 59°C for 1 min and cooling at 40°C for 30 s. Amplifications were performed in quadruplicate for each gene. Amplification efficiency (AE) of each gene was calculated using the standard curve method according to Fink et al. (1998): $AE = (10^{-1/s}-1)$, where S is the slope of the generated standard curve. The calculated values of AE were 94.67-104.11%. The relative mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ as given by Tian et al. (2018).

9.3.5 Statistical analysis

Since observations were performed on the same experimental unit (goat), a repeated measures ANOVA with one experimental factor (diets) and one repeated

factor (sampling time) has been considered. Animals were treated as simultaneously replications and linear mixed effects models were set with both `diets' and `time' as fixed effects and `animals' as random effect to obtain suitable data for ANOVA. A full factorial model was considered with the two fixed effects by using the MIXED procedure of SAS (SAS Institute Inc, 2015):

$$Y_{ijkl}=\mu + A_i + B_j + C_k\,(A) + (A\times B)_{ij} + e_{ijkl}$$

where Y_{ijkl} is the observation; μ is the overall mean; A_i is the fixed effect of diet (i = 1 to 2); B_j is the fixed effect of sampling time (j=1 to 6); C_k is the random effect of the goat nested within the diet (k = 1 to 6); $(A\times B)_{ij}$ is the interaction between diet and sampling time; and e_{ijkl} is the residual error. The Shapiro-Wilk test was used to check normality between the data obtained at different sampling times. Statistical significance of the diet effect was tested against variance of goat nested within diet according to repeated measures design theory (Kaps and Lamberson, 2004). Least square means are reported and significance was declared at $P<0.05$. Differences among diets and sampling times in each grouping was performing using Tukey HSD at $P<0.05$. Comparison of varied sampling in hours of feeding times were performed using contrast statement ($P<0.05$).

9.4 Results

After 6 weeks of trial observations, all goats were lively health without changing behaviour. There was interaction between diet and sampling time for somatic cell which was decreased about 4-folds ($P<0.001$) in goats fed the RDP diet. Radical-scavenging activities observed in plasma, milk, ruminal fluid and mammary tissue were not affected by dietary RDP, with range of 29.66-68.50% of DPPH inhibition (SE 0.99).

The dietary RDP resulted in similar value of the EC50 among diet and time, with average values of 1.86, 0.45, 0.60, and 1.21 µl/ml, respectively, in plasma, milk, ruminal fluid and mammary tissue. The dietary RDP varied TBARS production in plasma, milk, ruminal fluid and mammary tissue, with declination values of approximately 1-fold, 1-fold, 2-fold, and 1-fold, respectively (Table 9.1). The effect of sampling time was occurred in samples of mammary tissue and milk. There were effect of feeding time for rumen fluids and plasma, where feeding at 0 h and 6 h compared feeding at 3 h was significantly different (P<0.001), feeding at 3 h compared feeding at 6 h was not changed (P>0.05), and feeding at 0 h compared feeding at 3 h was varied (P<0.05), are depicted at Figure 9.1.

The activity of GPx and CAT profiles in rumen fluid, mammary tissue, milk, plasma and erythrocyte was not affected by dietary RDP (Table 9.2). In contrast, the dietary RDP increased significantly (P<0.001) for SOD in rumen fluid, mammary tissue, milk, plasma and erythrocyte. Regardless of treatments, there was effect in sampling time of rumen fluid, plasma and erythrocyte. The RDP diet escalating the activity of SOD in rumen fluids (RFs) compared to the CON diet was ranked in descending order as follows: RF 0 h after feeding < RF 3 h after feeding < RF 6 h after feeding. However, the activity of SOD in erythrocyte from goats fed with dietary RDP was founded in a greater effect (P<0.05) compared goats fed with dietary CON on 0, 3, and 6 h post-feeding. Times at 3 and 6 after feeding were similar. The except only adhered in plasma SOD was similar among treatments of diets and feeding times (Figures 9.2).

There were interactions (P<0.001) between diet and time for SOD1, SOD2, SOD3, and NFE_2L_2 (except NFKB, figure 9.5) mRNA abundance in mammary tissue, with a

significant increase with dietary RDP (Figures 9.3-9.4). The mRNA abundance of the CAT, GPx1, GPx2 and GPx3 genes was not affected by the dietary RDP (Figures 9.6-9.7).

9.5 Discussion

In the present study, we evaluated the effect of PP in diet (RDP) on oxidative stress indicator of dairy goats by the performance of radical-scavenging activity. The radical-scavenging activity was assessed by DPPH assay that is commonly used to evaluate antioxidants in straightforward way (Mensor et al., 2001). Dietary RDP had no effect in DPPH performances observed in plasma, milk, ruminal fluid and mammary tissue, which were in agreement with the result of Schogor et al. (2013), who observed a consistent percentage of DPPH in cows fed polyphenol-rich diet from flax meal dosed of 5-15% compared with cows fed 0% flax meal. More recently, Tian et al. (2019) reported that inclusion of anthocyanin as flavonoid source in diets led to increase antioxidant status of dairy goat, which likely accounted for consistent in DPPH inhibition observed in milk and plasma. DPPH is a stable free radical to bind a hydrogen or electron radical shifting to a stable diamagnetic molecule, that could be comprehended by presence of lower EC50 indicating higher antioxidant activity (Smet et al., 2008). Our result is suggested that the polyphenols derived from RDP diet had a fundamental role to perform a chain-breaking mechanism. Firstly, bioactive polyphenols donate electrons or hydrogens as or scavenger to convert recently free radicals into stable peroxides. Secondly, bioactive polyphenols run decomposing lipid peroxides into the last stable outcomes (Gurnani et al., 2016).

Table 9.1 Mean value of TBARS (MDA equivalents, nmol/ml) in rumen fluid, mammary tissue, milk, plasma and erythrocyte Saanen does fed the TMR (CON) and TMR with PP (RDP).

Item	Diet		Time (Weeks)						SEM	P-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Rumen fluid	24.17^{p}	11.24^{q}	19.42	15.97	19.71	15.34	17.61	18.18	0.140	0.039	0.285	0.296
Mammary tissue	6.59^{p}	5.47^{q}	6.17^{a}	6.15^{a}	6.05^{b}	5.97^{b}	5.89^{b}	5.95	0.017	0.012	0.032	0.005
Milk	24.39^{p}	22.10^{q}	23.70^{a}	23.45^{b}	23.15^{b}	23.15^{b}	23.13^{b}	22.89^{c}	0.064	0.021	0.018	0.004
Plasma	18.53^{p}	13.83^{q}	16.29	15.44	16.68	16.34	16.19	16.15	0.053	0.040	0.362	0.567

Different superscript lowercase letters in each row within a treatment variable indicate significant differences. SEM, standard error of measurement.

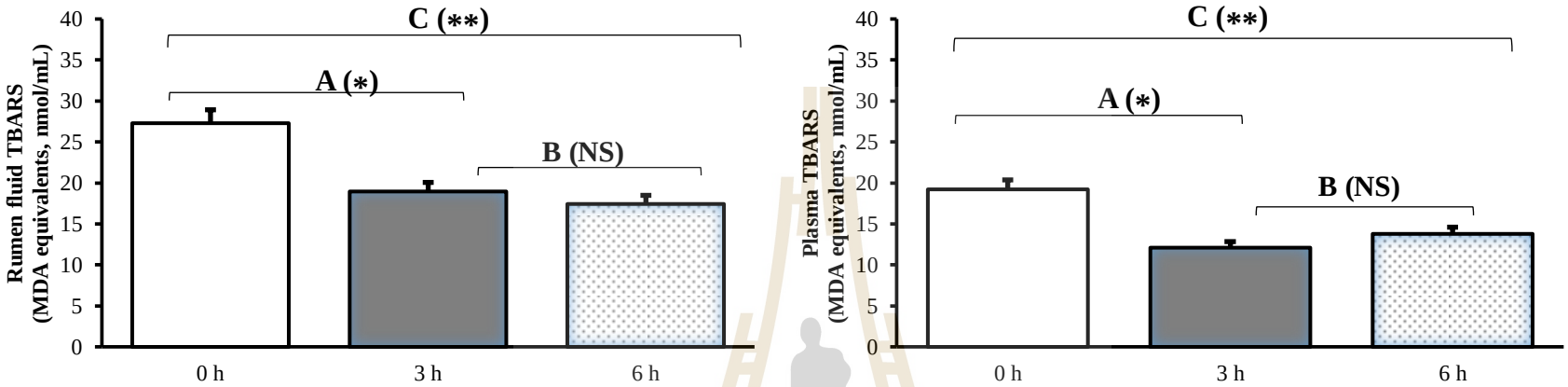

Figure 9.1 TBARS of rumen fluids and plasma observed after 0, 3 and 6 hours of feeding time. Contrast statements, as follows: A (0 h vs. 3 h), B (3 h vs. 6 h) and C (0 and 6 h vs. 3 h) at NS ($P>0.05$), * ($P<0.05$) and ** ($P<0.001$).

Table 9.2 Mean value of SOD, GPx and CAT profiles in rumen fluid, mammary tissue, milk, plasma and erythrocyte Saanen does fed the TMR (CON) and TMR with PP (RDP).

Item	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Rumen fluid												
SOD (µmol/min per mg protein)	12.04^{p}	13.68^{q}	12.90	12.72	12.93	12.90	12.86	12.86	0.035	0.021	0.681	0.081
GPx (nmol/min per mg protein)	11.71	11.34	11.52	11.30	11.91	11.37	11.53	11.53	0.034	0.075	0.910	0.978
CAT (µmol/min per mg protein)	10.87	10.50	10.87	10.52	10.51	10.83	10.68	10.68	0.011	0.063	0.407	0.729
Mammary tissue												
SOD (µmol/min per mg protein)	20.56^{p}	26.42^{q}	24.39^{a}	23.75^{b}	23.57^{b}	23.49^{b}	23.49^{b}	22.25^{c}	0.065	0.008	0.041	0.043
GPx (nmol/min per mg protein)	22.04	21.84	21.92	21.92	21.97	21.94	21.94	21.94	0.060	0.229	0.196	0.362
CAT (µmol/min per mg protein)	27.18	27.08	23.93	28.16	28.22	28.22	27.13	27.13	0.074	0.501	0.871	0.139
Milk												
SOD (µmol/min per mg protein)	138.40^{p}	144.75^{q}	144.60^{a}	141.96^{b}	141.50^{b}	141.60^{b}	141.57^{b}	138.25^{c}	0.388	0.047	<0.001	0.010
GPx (nmol/min per mg protein)	116.21	116.36	107.82	94.22	115.12	128.90	129.54	122.09	0.318	0.928	0.897	0.061
CAT (µmol/min per mg protein)	102.07	102.20	94.70	82.76	113.78	107.23	101.11	113.22	0.280	0.928	0.897	0.061
Plasma												
SOD (µmol/min per mg protein)	0.22^{p}	0.26^{q}	0.23	0.25	0.26	0.23	0.25	0.25	0.072	0.015	0.401	0.424
GPx (nmol/min per mg protein)	0.39	0.39	0.40	0.38	0.38	0.39	0.39	0.39	0.102	0.096	0.493	0.159
CAT (µmol/min per mg protein)	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.067	0.102	0.335	0.412

Table 9.2 Continue.

Item	Diet		Time (Weeks)						SEM	*P*-value		
	CTH	DPB	1	2	3	4	5	6		Diet	Time	Diet × Time
Erythrocyte												
SOD (µmol/min per mg protein)	10.66[p]	13.41[q]	11.66	12.30	12.16	11.70	12.20	12.20	0.033	0.010	0.243	0.478
GPx (nmol/min per mg protein)	11.86	11.86	11.88	11.88	11.82	11.86	11.86	11.86	0.032	0.069	0.524	0.134
CAT (µmol/min per mg protein)	11.62	11.62	11.61	11.61	11.64	11.62	11.62	11.62	0.032	0.064	0.595	0.383

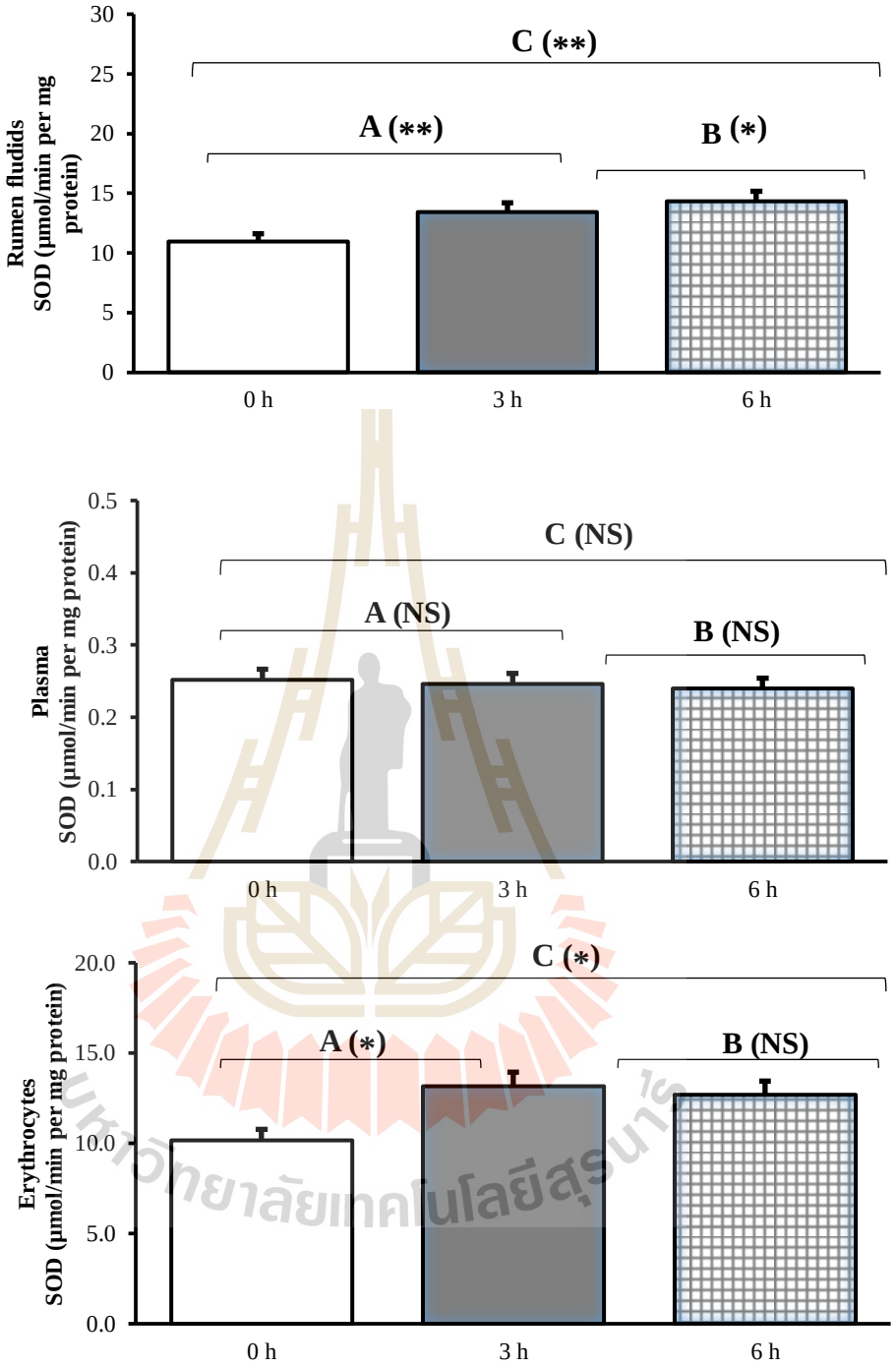

Figure 9.2 SOD of rumen fluids, plasma and erythrocytes observed after 0, 3 and 6 hours of feeding time. Contrast statements, as follows: A (0 h vs. 3 h), B (3 h vs. 6 h) and C (0 and 6 h vs. 3 h) at NS ($P>0.05$), * ($P<0.05$) and ** ($P<0.001$).

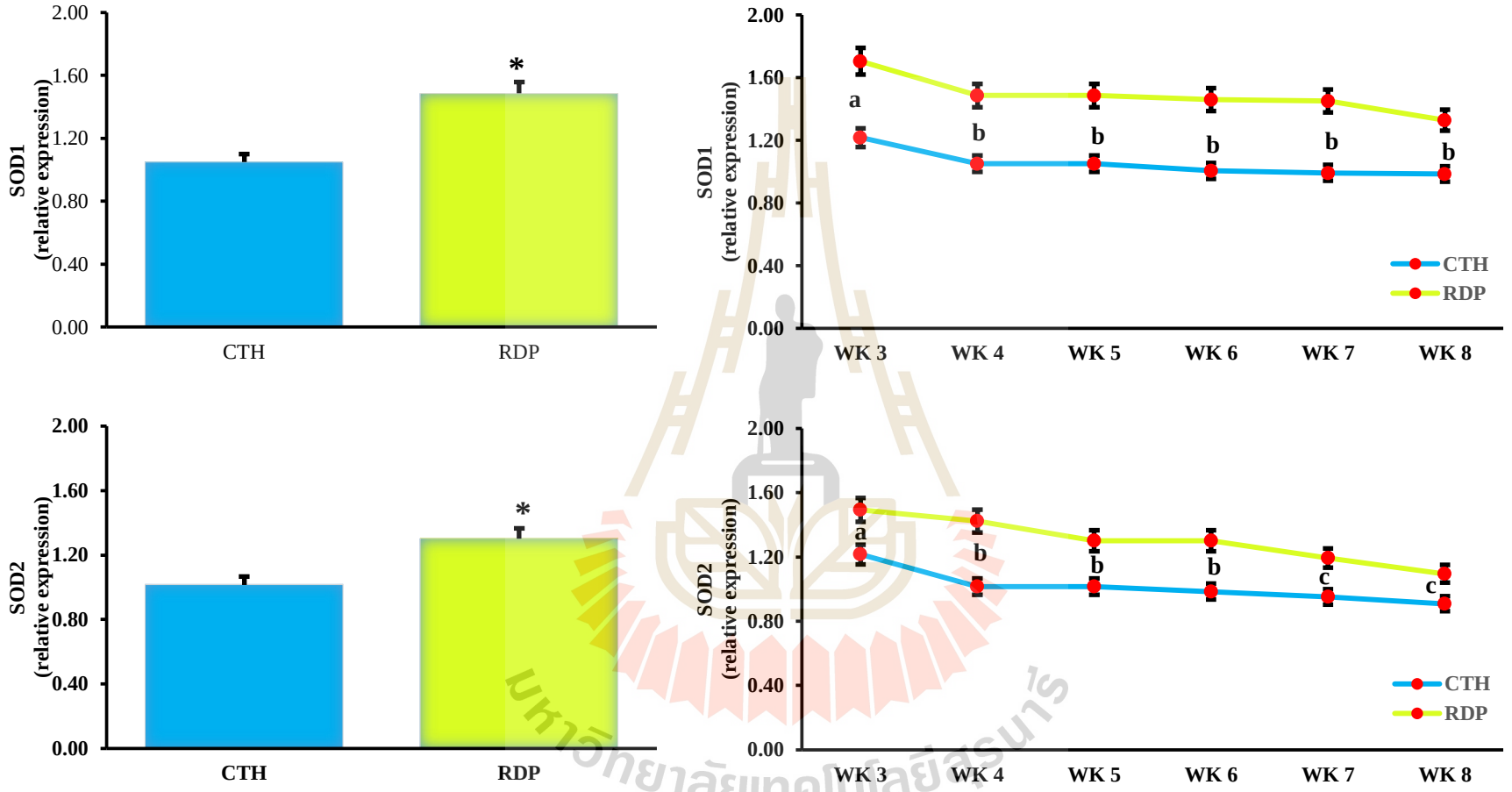

Figure 9.3 Relative mRNA abundance of SOD 1 and 2 genes in the mammary tissue. Values are means, with their standard errors represented by vertical bars. Gene expression of SOD1 and SOD2 was upregulated ($P<0.01$) in the mammary gland of goats receiving polyphenols in diet (RDP) relative to the no polyphenols in diet (CON). Different superscript lowercase letters in each dot within a treatment variable indicate significant differences ($P<0.05$).

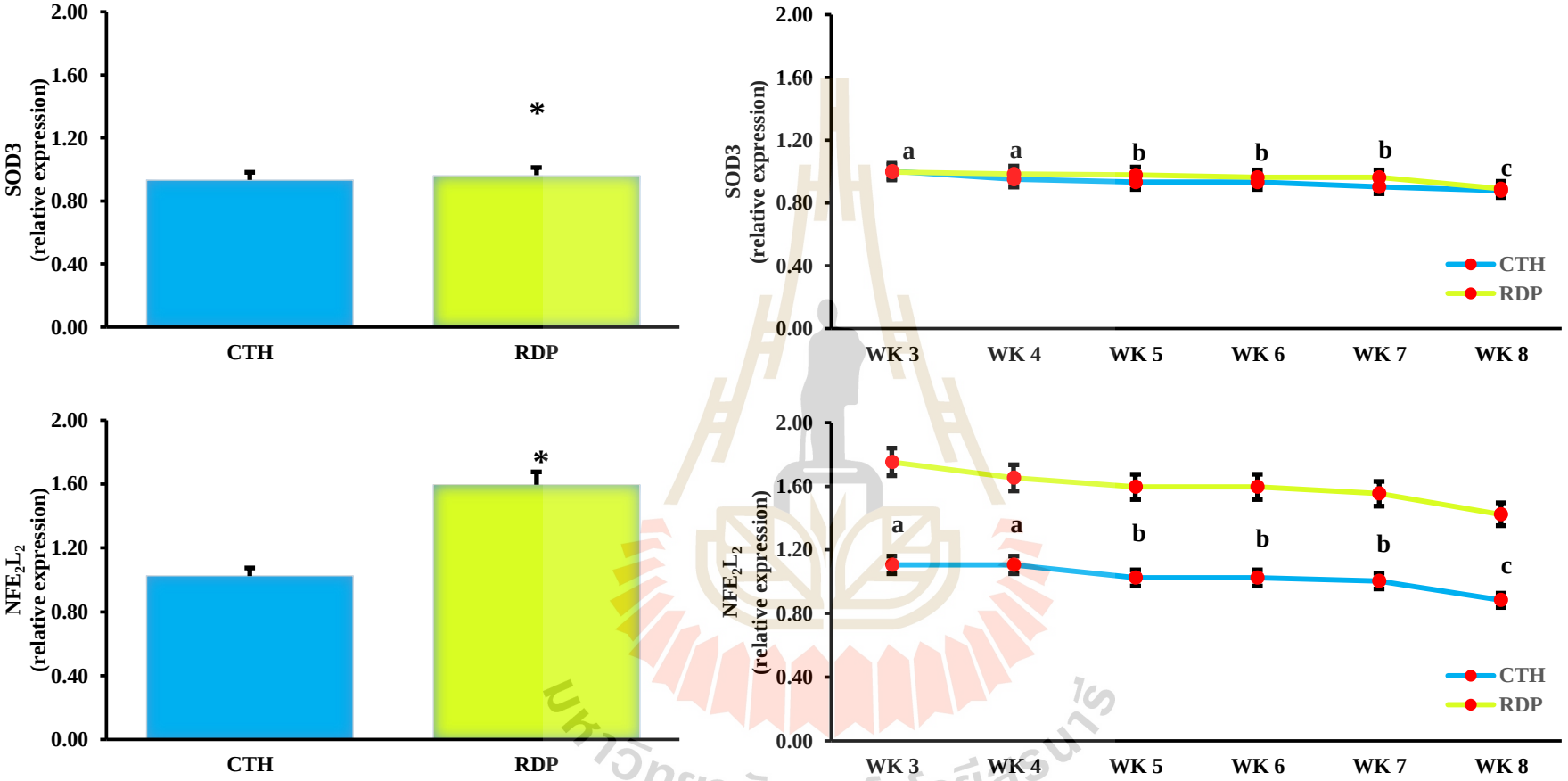

Figure 9.4 Relative mRNA abundance of SOD 3 and NFE_2L_2 genes in the mammary tissue. Values are means, with their standard errors represented by vertical bars. Gene expression of SOD3 and NFE_2L_2 was upregulated ($P<0.01$) in the mammary gland of goats receiving polyphenols in diet (RDP) relative to the no polyphenols in diet (CON). Different superscript lowercase letters in each dot within a treatment variable indicate significant differences ($P<0.05$).

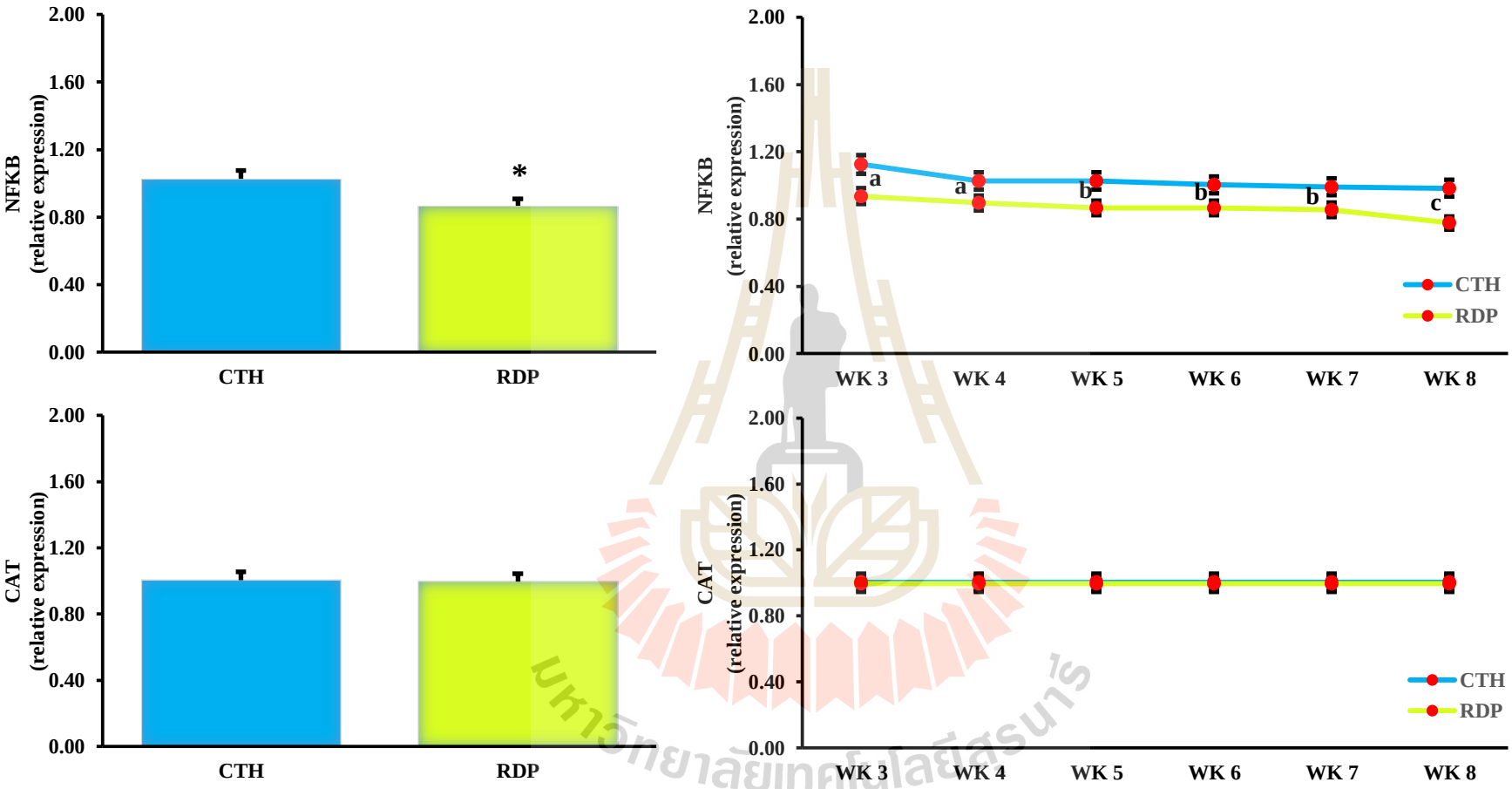

Figure 9.5 Relative mRNA abundance of NFKB and CAT genes in the mammary tissue. Values are means, with their standard errors represented by vertical bars. Gene expression of NFKB was downregulated (P<0.05) and CAT was unchanged (P>0.05) in the mammary gland of goats receiving polyphenols in diet (RDP) relative to the no polyphenols in diet (CON). Different superscript lowercase letters in each dot within a treatment variable indicate significant differences (P<0.05).

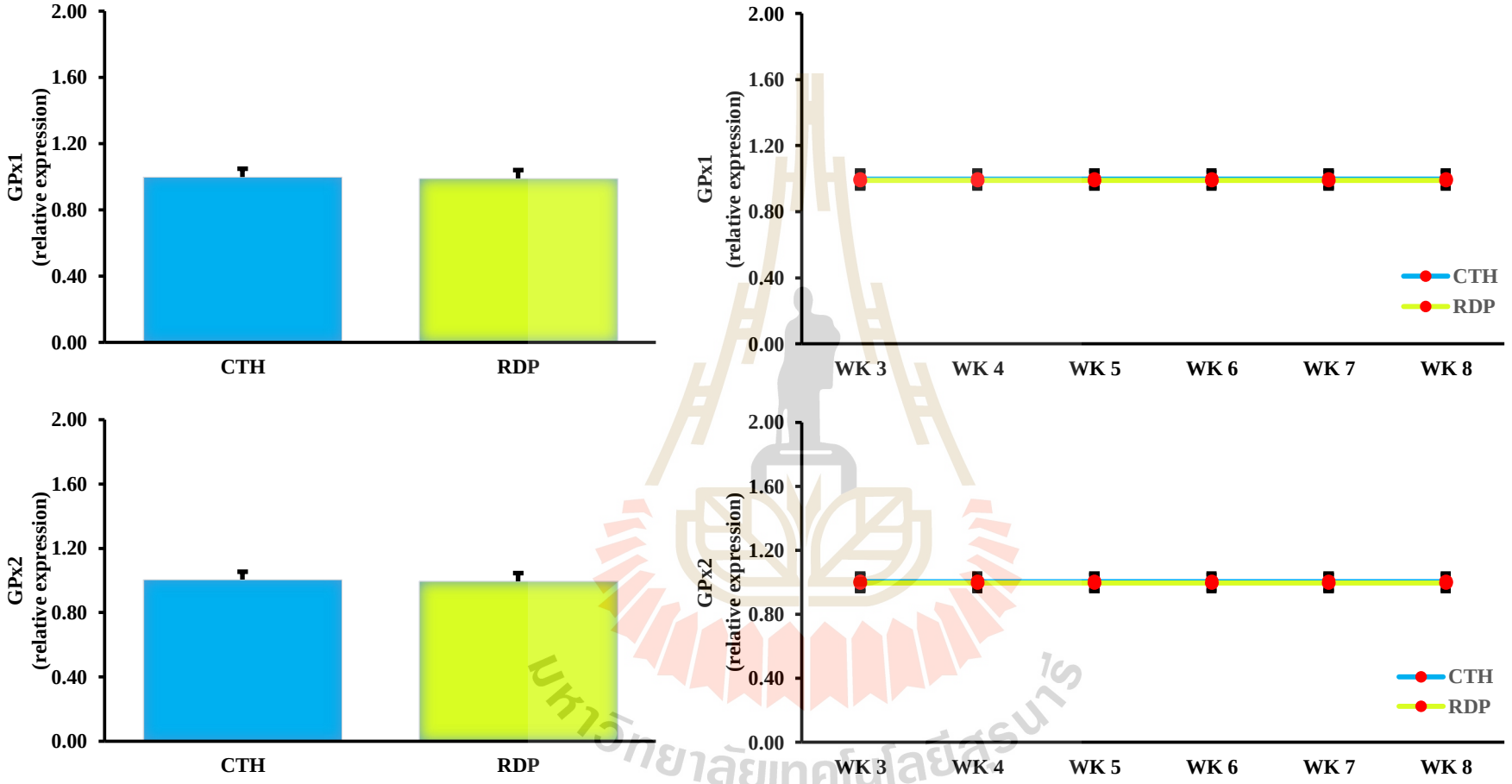

Figure 9.6 Relative mRNA abundance of GPx 1 and 2 genes in the mammary tissue. Values are means, with their standard errors represented by vertical bars. Gene expression of GPx1 and GPx2 were unchanged (P>0.05) in the mammary gland of goats receiving polyphenols in diet (RDP) relative to the no polyphenols in diet (CON).

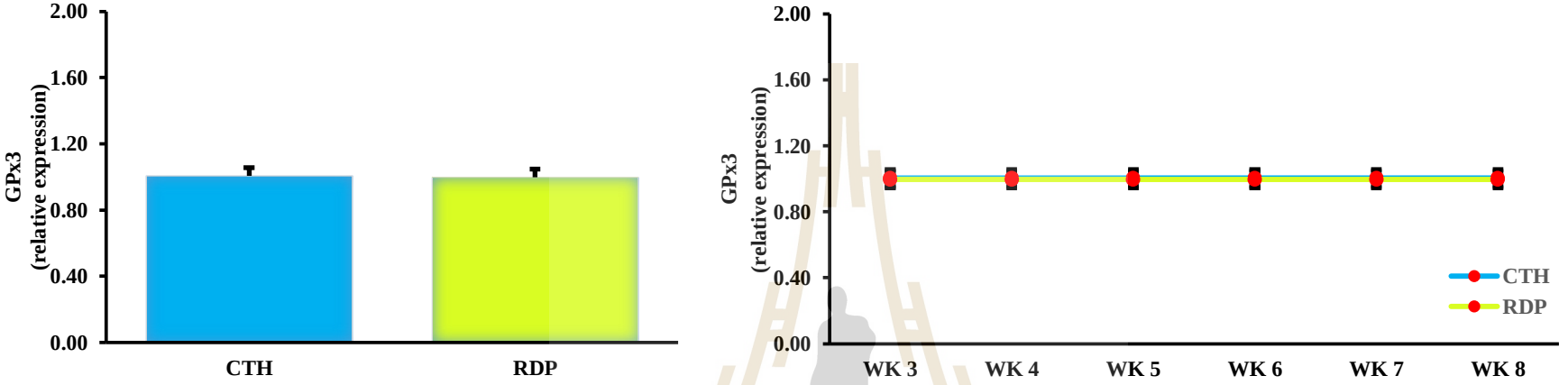

Figure 9.7 Relative mRNA abundance of GPx 3 gene in the mammary tissue. Values are means, with their standard errors represented by vertical bars. Gene expression of GPx3 was unchanged (P>0.05) in the mammary gland of goats receiving polyphenols in diet (RDP) relative to the no polyphenols in diet (CON).

Quantity of malondialdehyde (MDA) production has been broadly assessed as mutagenic product of lipid peroxidation by omega-3 and omega-6 fatty acids due to its facile reaction with thiobarbituric acid (Ayala et al., 2014). In present study, TBARS assay was used to monitor the MDA performance and sunflower oils as omega-6 fatty acid source was success to make all goats under status of abundant peroxidation. As expected, bioactive polyphenols in RDP decreased TBARS of goats observed in ruminal fluid, plasma, mammary tissue and milk. The reduction of TBARS in ruminal fluid and plasma was varied within the feeding time. Dietary antioxidant supplementation effects on TBARS concentration have been consistent. For instance, bioactive polyphenols had successfully limited TBARS concentration in rumen fluids after the cows fed by supplementation of flax meal in diets (Schogor et al., 2013). The four hours after intake of the diets, antioxidant activity in ruminal fluid was a gradually increase, as result of activated defence by bioactive polyphenols to protect readily lipids towards oxidation in rumen. In other reports, in studies dairy goats fed by grain and sodium butyrate, a minor oxidative stress occurred in rumen resulted improvement of ruminal epithelial cells and defence systems (Ma et al., 2018). Since the lipid covered by bioactive polyphenols, the antioxidant richer in rumen may be subsequently digested and metabolised as similar as dietary lipid (Chilliard et al., 2007), which all residual fractions will mobilize into the milk through blood stream passed by mammary gland. It is suggested that activated mode of polyphenols needs a suitable time, at least 3-4 h post-feeding and our results were consistent with those discovered previously in dairy goats. Therefore, a lower TBARS in plasma, mammary gland and milk was originated from a TBARS content in rumen, which likely indicated optimization of activated polyphenols to maintain the health gut.

SOD is the endogenously enzyme in first line defence system against reactive oxygen species. In term of rendering the potentially harmful superoxide anion less hazardous, SOD has an important role to catalyses the dismutation of two molecules of superoxide anion radical to hydrogen peroxide and molecular oxygen, whereas further transformation of hydrogen peroxide to water is handled by GPx and CAT (Ayala et al., 2014). In present study, bioactive polyphenols in RDP increased SOD concentrations observed in mammary tissue, milk, rumen fluid, plasma and erythrocyte, which were similar result with previous studies (Aliahmat et al., 2012; Tian et al., 2018; Tian et al., 2019). According to McSweeney et al. (2001), bioactive compounds of polyphenols especially flavonoids could be degraded in rumen and absorbed across the rumen wall or subsequently transferred to intestine metabolism. Those absorbed then were carried by blood stream to the liver before secreted to milk via mammary gland. Aliahmat et al. (2012) confirmed that a significant increase SOD activity in liver and erythrocyte was detected in mice receiving *Piper betle* that contained polyphenol compounds such as flavonoids, essential oils and other phenolics. However, degraded antioxidant substate from diet in rumen may be interfered by other substrates of formulated ration in diets such non-enzymatic substrate (Hosoda et al., 2012). For this reason, there are a couple of results have been observed in previous study regard of plant polyphenols in antioxidant perspective. For example, in studies of cytoprotective effects of tea polyphenols in cultured bovine mammary epithelial cells exposed to hydrogen peroxide, an increased about 50% for SOD activity was obtained as deteriorating cellular oxidative stress-related injury (Zhao et al., 2014). In contrast, Côrtes et al. (2012) reported no significant difference in mammary gland of cows fed by flax hull richer in polyphenols and infused with flax oil in the abomasum compared with cows fed by

respective control diet. Given by those examples, it seemed likely that the activity of SOD in rumen fluid, blood, mammary tissue and milk of dairy animals has been exhibited to escalate due to antioxidant supplementation (RDP). However, in combining form with other antioxidant sources likely oil supplementation, the effect was related to fat and/or oil type incorporated into the diet, because oxidised fat may interrupt the way of SOD in prevention of oxidative stress.

Increase of SOD in rumen fluids, erythrocytes and plasma observed in varied feeding times was in agreement with TBARS mode. In present study, achieving antioxidant value from RDP diet at least 6 h post-feeding for increasing SOD at rumen fluids and erythrocytes. However, plasma SOD differed with those sites, where plasma may have different transport mechanism or erythrocytes obtained such a deterioration (Köse et al., 2002). MDA or TBARS affected oxidative status may involve later destruction in membrane structure and function, resulting in alleviation in membrane integrity. While, PP containing polyphenols may be assessed by erythrocytes as foreign molecules, thereby they are reported to have capacity to induce erythrocytic membrane (Revin et al., 2019a; Revin et al., 2019b).

Despite of increased SOD activity by dietary RDP, activity of GPx and CAT in observed in mammary tissue, milk, rumen fluid, plasma and erythrocyte of present study showed in similar performance. These results were corroborated with previous studies (Tian et al., 2018; Tian et al., 2019) that flavonoids gave a consistent effect on mitigating oxidative stress through performing massively donation into the first line defence system. As consequence, there were not remained superoxide anion radical in the dismutation tending to lack of GPx and CAT activities (Mittler, 2002).

In present study, the bioactive polyphenols in RDP influenced mammary NFE_2L_2, SOD1, SOD2 and SOD3 mRNA abundances, and did not affect CAT, GPx1, GPx2 and GPx3 genes. NFE_2L_2, also known as nuclear factor E2-related factor-2 is a transcription factor that owns a well-documented detoxification benefit to alleviate oxidative stress (Nguyen et al., 2009). Previous studies that used dairy animals had highlighted in enhancement of NFE_2L_2 mRNA abundance as affected by inclusion of polyphenols in the diets (Schogor et al., 2013; Ma et al., 2018; Tian et al., 2018). NFE_2L_2 generated an elaborated expression of the antioxidant response element in the promoter region of a number of genes (Zhu et al., 2005). For this reason, SOD1, SOD2, SOD3 and NFKB to be invited on modulating antioxidant response, thereby caused lack of RDP effect on CAT, GPx1, GPx2 and GPx3 genes. Furthermore, in the current study shown a reduction of somatic counting cells in milk produced by goats receiving the RDP diet. It could suggest that bioactive polyphenols exhibiting either the anti-inflammatory role through attenuating oxidative DNA damage in mammary tissue. According to Ighodaro and Akinloye (2018), SOD3 is indispensable enzyme to act antioxidant defence against vascular and cardiovascular diseases, namely neurological diseases, lung disease, atherosclerosis, diabetes, hypertension and inflammatory conditions. In other reports, SOD3 could be influenced by antioxidant vitamin C, butylated hydroxyanisole and other antioxidant agents such as plant polyphenols (Singh and Bhat, 2012). Accordingly, NFKB gene was down-regulated as affected the dietary RDP, which was in agreement with prior study (Subbaramaiah et al., 2013). This is clear due to NFKB known as reversed gene of NFE_2L_2. Therefore, in current study suggested that bioactive polyphenols could improve defence mechanisms through eliminating reaction of inflammatory mediators such as NFKB.

9.6 Conclusion

The inclusion of PP from initially valuable plant which is richer in secondary metabolites likely PAs, FOs and EOs in the diet of dairy goats resulted in significant influences of activity of antioxidant enzymes and oxidative stress indicators in the ruminal fluid, mammary tissue, milk and plasma. Moreover, the mRNA abundance of the antioxidant enzymes and oxidative stress-related genes namely SOD1, SOD2, SOD3 and NFE_2L_2 in mammary tissue was related to exhibit an escalation in similar way. In general, the inclusion of PP in the diet suggests that this polyphenol-rich supplement can improve the oxidative status of Saanen goats in early lactation. Although, stability motion of PP in later lactation stage is still questionable. Further studies are required to clarify the role of PP on the oxidative status of goat during the mid and late lactations. Notably, a host of PP effects on the oxidative status of goats could prompt to a prophylactic strategy against dairy diseases affecting the health status.

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CHAPTER X

OVERALL CONCLUSION AND IMPLICATION

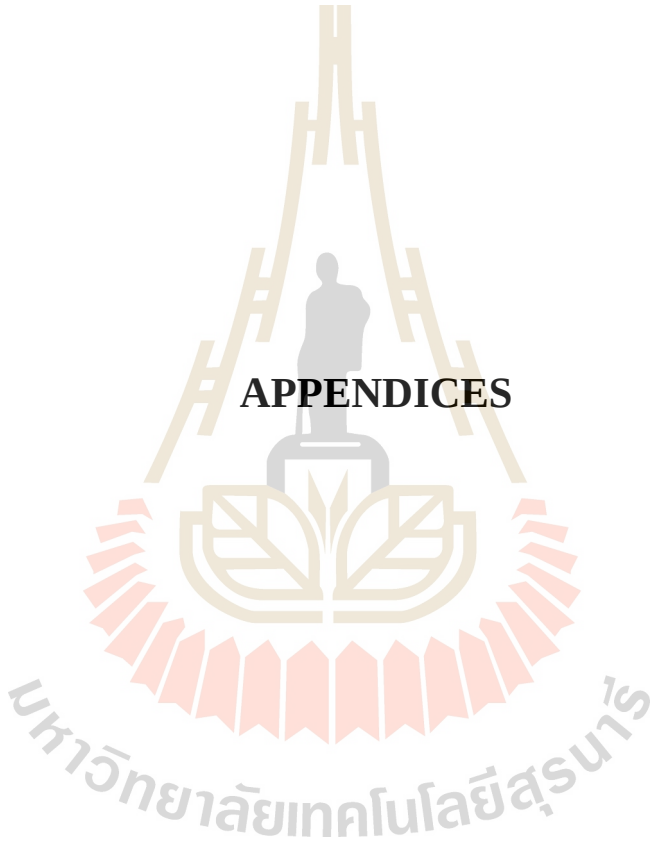
10.1 Conclusion

In general, this study presents systematic review including meta-analysis and factual elaboration in comprehensive observations. The initial plant, *Piper betle* L. leaves exhibit a well-documented worth benefit due to richer in PAs, FOs and EOs, even carrying ascorbic acids. Those secondary metabolites had been tested and observed resulting in satisfied outcome, where provision of PP dosed of 12.8 g/kg in the diet (<2% DM, as recently expectation by experts) of dairy goats addresses the positive impacts on animal performance, animal health and environmentally-friendly concerns. These achievements suggest to remark that PP seemed to have strong antimicrobial and antioxidant properties.

10.2 Implication

Study in animal metabolic and rumen-derived product as treated by herb plant to provide and attenuate the exigency consumers is considerable challenging and unique. In recent database, deposition of natural compound derived from PP in milk is unpredictable because the limitation of applied study. Further study regard of phytochemical's depositions in milk as rumen-derived product is wisely recommended.

APPENDICES



APPENDIX A

Full electronic search strategy on using keywords

Table A1 Full electronic search strategy for ISI Web of Knowledge.

Search	Searchquery	Items found
#1	Biohydrogenation [Topic]	1380
#2	Conjugated linoleic acid [Topic]	8791
#3	Rumen [Topic]	20671
#4	Tannin [Topic]	22637
#5	Condensed tannin [Topic]	4997
#6	Hydrolysable tannin [Topic]	2641
#7	Meat [Title/Abstract]	26614
#8	Milk [Title/Abstract]	53500
#9	*In vivo* [Title/Abstract]	143213
#10	*In vitro* [Title/Abstract]	204802
#11	#1 OR #2	9298
#12	#1 AND #3	928
#13	#1 AND #3 AND #4	77
#14	#2 AND #3 AND #4	52
#15	#2 AND #3 AND #4 AND #5 OR #6	2672
#16	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #9 OR #10	204832
#17	#2 AND #3 AND #4 AND #5 OR #6 AND #8 AND #9 OR #10	204832
#18	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	204832
#19	#1 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	204840
#20	#1 AND #2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	204822

Table A2 Full electronic search strategy for Mendeley.

Search	Search query	Items found
#1	Biohydrogenation	1180
#2	Conjugated linoleic acid	6672
#3	Rumen	33781
#4	Tannin	14954
#5	Condensed tannin	2444
#6	Hydrolysable tannin	464
#7	Meat	144574
#8	Milk	283876
#9	*In vivo*	1215559
#10	*In vitro*	1627361
#11	#1 OR #2	6862
#12	#1 AND #3	740
#13	#1 AND #3 AND #4	26
#14	#2 AND #3 AND #4	12
#15	#2 AND #3 AND #4 AND #5 OR #6	6
#16	#2, #3, #4, #5, #6, #7, #9, #10	13
#17	#2, #3, #4, #5, #6, #8, #9, #10	13
#18	#2, #3, #4, #5, #6, #7, #8, #9, #10	6
#19	#1, #3, #4, #5, #6, #7, #8, #9, #10	3
#20	#1, #2, #3, #4, #5, #6, #7, #8, #9, #10	4

Table A3 Full electronic search strategy for Scopus.

Search	Searchquery	Items found
#1	Biohydrogenation [All fields]	4559
#2	Conjugated linoleic acid [All fields]	28624
#3	Rumen [All fields]	64893
#4	Tannin [All fields]	88392
#5	Condensed tannin [All fields]	18919
#6	Hydrolysable tannin [All fields]	9372
#7	Meat [Article title, abstract, keywords]	111074
#8	Milk [Article title, abstract, keywords]	177011
#9	*In vivo* [Article title, abstract, keywords]	938584
#10	*In vitro* [Article title, abstract, keywords]	1319131
#11	#1 OR #2	30182
#12	#1 AND #3	3409
#13	#1 AND #3 AND #4	501
#14	#2 AND #3 AND #4	460
#15	#2 AND #3 AND #4 AND #5 OR #6	305
#16	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #9 OR #10	13
#17	#2 AND #3 AND #4 AND #5 OR #6 AND #8 AND #9 OR #10	20
#18	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	8
#19	#1 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	9
#20	#1 AND #2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	5

Table A4 Full electronic search strategy for PubMed.

Search	Searchquery	Items found
#1	Biohydrogenation [Text word]	576
#2	Conjugated linoleic acid [Text word]	2781
#3	Rumen [Text word]	9528
#4	Tannin [Text word]	2883
#5	Condensed tannin [Text word]	429
#6	Hydrolysable tannin [Text word]	67
#7	Meat [Title/Abstract]	39226
#8	Milk [Title/Abstract]	84859
#9	*In vivo* [Title/Abstract]	709160
#10	*In vitro* [Title/Abstract]	911115
#11	#1 OR #2	3172
#12	#1 AND #3	399
#13	#1 AND #3 AND #4	10
#14	#2 AND #3 AND #4	3
#15	#2 AND #3 AND #4 AND #5 OR #6	69
#16	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #9 OR #10	934132
#17	#2 AND #3 AND #4 AND #5 OR #6 AND #8 AND #9 OR #10	911115
#18	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	911115
#19	#1 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	911115
#20	#1 AND #2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	911115

Table A5 Full electronic search strategy for Google Scholar.

Search	Searchquery	Items found
#1	Biohydrogenation	13100
#2	Conjugated linoleic acid	76000
#3	Rumen	219000
#4	Tannin	93600
#5	Condensed tannin	29500
#6	Hydrolysable tannin	17000
#7	Meat	2570000
#8	Milk	1650000
#9	*In vivo*	738000
#10	*In vitro*	722000
#11	#1 OR #2	8660
#12	#1 AND #3	12100
#13	#1 AND #3 AND #4	1750
#14	#2 AND #3 AND #4	2040
#15	#2 AND #3 AND #4 AND #5 OR #6	1110
#16	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #9 OR #10	768
#17	#2 AND #3 AND #4 AND #5 OR #6 AND #8 AND #9 OR #10	847
#18	#2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	740
#19	#1 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	841
#20	#1 AND #2 AND #3 AND #4 AND #5 OR #6 AND #7 AND #8 AND #9 OR #10	496

APPENDIX B

Results of the standard meta-analysis, sensitivity analysis and risk of bias for other parameters relate to biohydrogenation

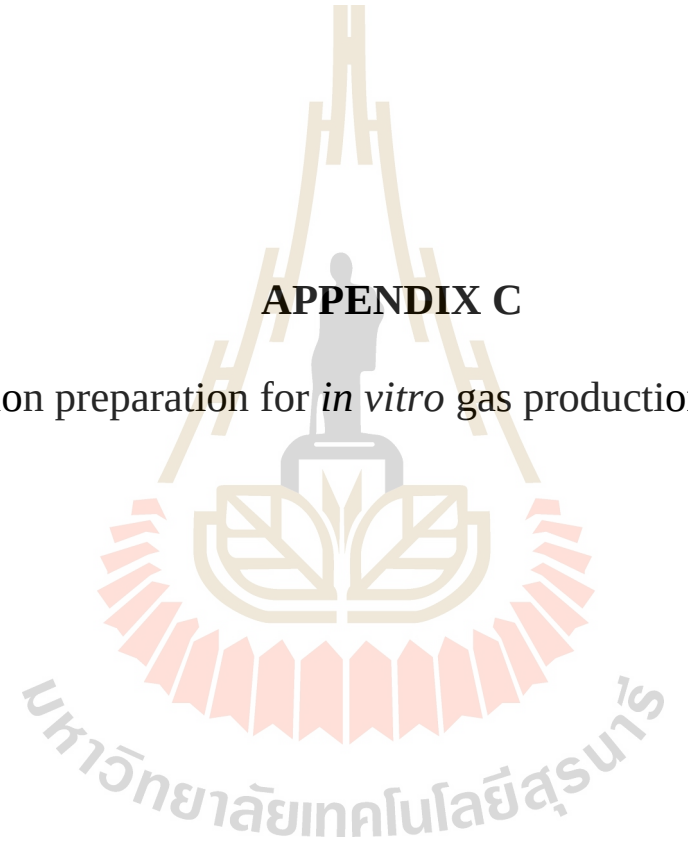
Figure B1 Results of the standard meta-analysis, sensitivity analysis and risk of bias for other parameters.

Parameter	Standard meta-analysis						Sensitivity analysis			Trim and fill test		No of missing *n* in Rosenthal's Fail-safe N test	No of missing *n* in Gleser and Olkin's Fail-safe N test	No of missing *n* in Orwin's Fail-safe N test	No of missing *n* in Fisher's Fail-safe N test	P from Egger's test for funnel plot asymetry[§]
	n	SMD/Effect size	CI LL	CI UL	*P**	Heterogeneity[€]	*n*	Ln ratio[δ]	*P**	No of missing *n*	funnel plot side					
FA supplementation (g/100g FAME)																
C18:3 n-3	2	0.31	-0.04	0.66	0.288	Yes(99%)	35	35.48	0.097	0	Right	13715	0	0	8791	0.082
C18:2 n-6	2	0.11	-0.003	0.225	0.787	Yes(95%)	36	26.32	0.436	0	Right	6301	0	0	4770	0.169
C18:1 n-9	2	0.29	0.20	0.39	0.025	Yes(98%)	36	50.00	0.295	0	Right	15172	0	159	6163	0.812
Gas production (ml/g OM)	1	0.10	-0.57	0.76	0.477	Yes(94%)	4	0	0.289	0	Right	79	0	2	101	0.336
Total VFA (mmol/l)	5	0.02	0.003	0.05	0.221	Yes(96%)	15	12.50	0.177	0	Right	1123	0	0	2977	0.019
C2	5	0.04	-0.002	0.07	0.999	Yes(95%)	15	11.54	0.723	0	Right	593	0	0	1519	0.020
C3	5	0.13	0.08	0.17	0.071	Yes(96%)	15	55.00	0.070	0	Right	2218	0	0	4	0.027
C4	5	0.02	-0.01	0.05	0.064	Yes(99%)	15	8.70	<0.001	0	Right	3403	0	0	2922	0.265
C5	5	0.02	0.01	0.04	0.893	Yes(75%)	12	33.33	0.525	0	Right	501	0	0	1495	0.073
Iso-C4+Iso-C5	2	0.19	-0.13	0.51	0.107	Yes(99%)	9	20.00	0.245	0	Right	1591	0	0	2887	0.284
FA profile (g/100g FAME)																
C18:2 cis-9, trans-11 (CLA)	2	0.21	-0.23	0.65	0.012	Yes(100%)	38	6.38	0.930	0	Right	72890	0	0	8955	0.067
Trans-11 18:1	2	0.20	-0.11	0.51	0.802	Yes(95%)	36	20.51	0.448	0	Right	6399	0	0	4707	0.071
C18:0	2	0.46	-0.30	1.22	0.439	Yes(97%)	36	18.09	0.201	0	Right	8367	0	0	7499	0.065
Desaturation index	1	0.0002	0.0003	0.0600	0.944	Yes(98%)	27	0	0.624	0	Right	3264	0	0	4484	0.052
CLA index	1	0.06	0.31	0.60	0.995	Yes(96%)	27	0	0.739	0	Right	6525	0	82	7377	0.069
SFA	4	0.40	0.38	0.43	1.000	Yes(98%)	36	44.94	0.840	0	Right	16566	0	27	10334	<0.001
MUFA	4	0.44	0.39	0.49	0.229	Yes(97%)	36	30.34	0.145	0	Right	17397	0	152	11739	0.019
PUFA	4	0.38	0.32	0.44	0.183	Yes(99%)	36	40.22	0.117	0	Right	18361	0	24	10457	<0.001

n, number of data points included in the comparison; SMD, standardized mean difference; CI LL, confidence interval lower; CI UL, confidence interval upper; **P* value<0.05 indicates significance of the difference among *in vitro* and *in vivo* observations; [€]Heterogeneity and the I^2 Statistic; [δ]Ln ratio was calculated as Ln (*in vivo/in vitro* × 100%). [§]*P*<0.05 indicates significant publication bias.

APPENDIX C

Solution preparation for *in vitro* gas production technique



C1 Macromineral solution:

- Na_2HPO_4 = 5.7 g
- KH_2PO_4 = 6.2 g
- $MgSO_4$ = 0.6 g

Make up to 1 liter with distilled water

C2 Buffer solution:

- $NaHCO_3$ = 35 g
- $(NH_4)\ HCO_3$ = 4 g

Make up to 1 litre with distilled water

C3 Micromineral solution:

- $CaCl_2 2.H_2O$ = 13.2g
- $MnCl_2 4.H_2O$ = 10.0 g
- $CoCl_2 6.H_2O$ = 1.0 g
- $FeCl_2 6.H_2O$ = 0.8 g

Make up to 1 litre with distilled water

C4 Resazurin aqueous: (100mg/100ml)

C5 Preparation of artificial saliva:

Artificial saliva

- Distilled water = 475.0 ml
- Macromineral solution = 240.0 ml
- Buffer solution = 240.0 ml

- Micromineral solution = 0.12 ml
- Resazurin aqueous = 1.22 ml

Reducing solution

- Distilled water = 47.5 ml
- 1M NaOH = 2.0 ml
- $Na_2S9.H_2O$ = 336 mg

BIOGRAPHY

Mr. Rayudika Aprilia Patindra Purba, he was born at Indramayu region, Jawa Barat province, Indonesia, in 1993. He graduated and accepted Bachelor degree in 2016 from Jenderal Soedirman University, Indonesia. During undergraduate period, he had worked in laboratory and rural area to apply a novelty from lab to farm. He used to concern in poultry development. He was one of manager in sustainable farm located in Central Java. He obtains a host of rewards. For instance, he gets a Patent Technology as The Number S00201200250 Held by The General Directorate of Intellectual Property (HKI) Indonesia. In 2016, he moved to Thailand to accept the prestigious scholarship for accelerated program (Master and Doctorate), which was granted by SUT scholarship for ASEAN Phase II granted in Suranaree University of Technology. During doctoral programs, he has been active in International academic journals and conferences. He accepts the reward as the best (first) youth scientist for oral speaker on international conference of Sustainable Animal Agriculture for Developing Countries (SAADC) 2017. He has been one of active international peer-reviewers with Publons ID (verified). Apart from the academic, he would have passionate enthusiasm in social circumstances in national and international organisations.