EFFECTS OF ANTHOCYANIN-RICH PURPLE CORN (*Zea mays* L.) STOVER SILAGE ON ANTIOXIDANT ACTIVITIES IN DAIRY GOATS

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โดยได้แบ่งการทดลองเป็น 3 การทดลอง ดังนี้

การทดลองที่ 1 ได้ทำการหมักต้นข้าวโพด โดยแบ่งออกเป็น 2 ทรีทเมนต์ ดังนี้ ทรีทเมนต์ที่ 1 ต้นข้าวโพดข้าวเหนียวพันธุ์ลูกผสม (กลุ่มควบคุม) และทรีทเมนต์ที่ 2 ต้นข้าวโพดม่วงพันธุ์ลูกผสม (กลุ่มทรีทเมนต์) ซึ่งผลการทดลองแสดงให้เห็นถึงกลุ่มทรีทเมนต์มีระดับของวัตถุแห้ง และโปรตีนหยาบสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) แต่อย่างไรก็ตามกลุ่มทรีทเมนต์มีปริมาณระดับของพาราโกนิดิน-3-กลูโคไซด์ (P3G) ที่ต่ำกว่ากลุ่มควบคุม แต่มีระดับของพีโอนิดิน (Peo) และพีราโกนิดิน (Pel) สูงกว่าเมื่อเปรียบเทียบกับกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ดีลพินิดิน (Del) และมัลวิดิน (Mal) ไม่ตรวจพบในกลุ่มควบคุมในช่วงระหว่างการหมัก แต่ในกลุ่มทรีทเมนต์ไม่สามารถตรวจพบได้หลังจากวันที่ 7 ของการหมัก นอกจากนี้ระดับของแอนโทรไซยานินทั้งหมดในกลุ่มทรีทเมนต์ลดลงอย่างมาก ($P<0.05$) จากช่วงแรกถึงวันที่ 7 ของการทำการหมัก และหลังจากนั้นระดับของแอนโทรไซยานินคงที่ ($P<0.05$) เมื่อทำการเปรียบเทียบกลุ่มทรีทเมนต์ พบว่ากลุ่มควบคุมมีค่าระดับความเป็นกรดด่าง และค่าแอมโมเนีย-ไนโตรเจนสูงกว่ากลุ่มทรีทเมนต์อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) และเมื่อทำการเปรียบเทียบสารสกัดจากกลุ่มควบคุม พบว่าสารสกัดจากกลุ่มทรีทเมนต์มีการขับออกของระดับของ 2,2-ไดพีนี 1-1-ไพคิลไฮดราซีล (DPPH) ที่สูงกว่ากลุ่มควบคุม และมีระดับค่าของ IC50 ที่ต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) นอกจากนี้การผลิตแก๊สในกลุ่มทรีทเมนต์ลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ที่ 3 ชั่วโมง และมีระดับของส่วนที่ละลายทันที (a) และสัดส่วนของกรดอะซิติกต่อกรดโพรพิโอนิกที่ต่ำกว่า ที่ 12 ชั่วโมง แต่ไม่พบความแตกต่างของค่าสังเกตอื่นๆ เมื่อเปรียบเทียบกับกลุ่มควบคุม

การทดลองที่ 2 แพะนมพันธุ์ซาแนนที่มีสุขภาพดีที่ผ่านการตั้งท้องมาแล้ว จำนวน 8 ตัว มีน้ำหนักตัวเฉลี่ย±ส่วนเบี่ยงเบนมาตรฐาน อยู่ที่ 41.50±1.84 กิโลกรัม โดยได้ทำการจัดแผนการทดลองแบบ 4 × 4 ลาตินสแควร์ โดยได้แบ่งออกเป็น 4 ทรีทเมนต์ โดยทรีทเมนต์ที่ 1 คือ กลุ่มควบคุมแบบลบ โดยแพะได้รับฟางข้าว (RSNC); ทรีทเมนต์ที่ 2 คือกลุ่มควบคุมแบบบวกที่ 1 โดยแพะได้รับต้นข้าวโพดข้าวเหนียวพันธุ์ลูกผสมหมัก (SSPC); ทรีทเมนต์ที่ 3 โดยแพะได้รับต้นข้าวโพดม่วงพันธุ์ลูกผสมหมัก (APSS) และทรีทเมนต์ที่ 4 คือกลุ่มควบคุมแบบบวกที่ 2 แพะได้รับต้นข้าวโพดข้าวเหนียวพันธุ์ลูกผสมหมัก (SSPP) ที่ได้รับการเสริมสารสีจากต้นข้าวโพดม่วงปริมาณ 1 กรัมต่อวัน โดยผลการทดลองพบว่าปริมาณการกินได้ของวัตถุแห้ง ไม่พบความแตกต่างระหว่าง

ทรีทเมนต์อย่างมีนัยสำคัญทางสถิติ (P<0.05)นอกจากนี้แพะที่ได้รับกลุ่มควบคุมแบบลบ (RSNC) มีระดับปริมาณการย่อยได้ปรากฏ ปริมาณการดูดซึมไนโตรเจน และปริมาณการกักเก็บไนโตรเจนต่ำกว่าเมื่อเปรียบเทียบกับกลุ่มทรีทเมนต์อื่นๆ อย่างมีนัยสำคัญทางสถิติ (P<0.05) นอกจากนี้ไม่พบความแตกต่างของค่าความเป็นกรดด่าง และค่าแอมโมเนียไนโตรเจนระหว่างทรีทเมนต์ แต่ทว่าในกลุ่มทรีทเมนต์ APSS และ SSPP พบว่ามีระดับของกรดโพรพิโอนิก (PA) กรดบิวทิรก (BA) และปริมาณของกรดไขมันระเหยง่ายทั้งหมดที่สูงกว่า และมีสัดส่วนของกรดอะซิติกต่อกรดโพรพิโอนิกต่ำกว่าเมื่อเทียบกับกลุ่มควบคุมแบบลบ ในกลุ่มทรีทเมนต์ APSS และ SSPP พบว่ามีปริมาณระดับของการขับออก DPPH และมีปริมาณซุปเปอร์ออกไซด์ ดิสมูเทส (SOD) ในพลาสมาที่สูงกว่าอย่างมีนัยสำคัญทางสถิติ (P<0.05) แพะนมที่ได้รับทรีทเมนต์ APSS และ SSPP พบว่ามีปริมาณระดับของอีริวทรอย์ 2 ไลค์ 2 (NFE2L2) ที่สูงกว่า นอกจากนี้ยังพบว่าระดับของทูเมอร์ นีโครซีส แฟกเตอร์ (TNF) ในต่อมน้ำนมมีระดับที่ต่ำกว่า ถึงแม้ว่าทุกทรีทเมนต์ไม่ส่งผลต่อปริมาณ mRNA ของ SOD3 และ CAT ในต่อมน้ำนม แต่อย่างไรก็ตามแพะนมที่ได้รับทรีทเมนต์ APSS มีแนวโน้มที่ระดับของการแสดงออกของยีน SOD2, GPX1 และ GPX2 เพิ่มมากขึ้น

ในการทดลองที่ 3 แพะนมพันธุ์ซาแนนที่ผ่านการตั้งท้องในระยะให้น้ำนม จำนวนทั้งหมด 16 ตัว โดยมีน้ำหนักตัวเฉลี่ย±ส่วนเบี่ยงเบนมาตรฐาน คือ 41.80±2.92 กิโลกรัม โดยมีสุขภาพดีและมีเต้านมที่ได้สมมาตร ซึ่งได้แบ่งออกเป็น 2 กลุ่ม โดยแบ่งตามจำนวนวันในการให้ผลิตน้ำนมเฉลี่ยที่ 75 วัน (DIM) โดยใช้แผนการทดลองแบบบล็อกสมบูรณ์ (RCBD) และแบ่งออกเป็น 2 ทรีทเมนต์ ดังนี้ ทรีทเมนต์ที่ 1 คือ กลุ่มควบคุม โดยแพะได้นับ SSS (CSSS) และทรีทเมนต์ที่ 2 โดยแพะได้รับต้นข้าวโพดม่วงพันธุ์ลูกผสมหมัก (TPSS) ผลการทดลองแสดงให้เห็นถึงแพะในกลุ่ม TPSS พบว่ามีระดับของ SOD ในพลาสมาและในน้ำนมที่สูงกว่าอย่างมีนัยสำคัญทางสถิติ (P<0.05) เมื่อเปรียบเทียบกับกลุ่มควบคุม นอกจากนี้แพะในกลุ่ม TPSS มีปริมาณองค์ประกอบของน้ำตาลแล็กโตสในน้ำนมมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (P<0.05) แพะในกลุ่ม TPSS ไม่มีผลต่อ cyanidin-3-glucoside (C3G), Del, cyanidin (Cya), Pel และปริมาณ anthocyanins ทั้งหมดในน้ำนม (P>0.05) แต่ทว่าแพะในกลุ่ม TPSS มีระดับของ Peo และมัลวิดิน 3-โอ-กลูโคไซด์ (M3G) ในน้ำนม ที่สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (P<0.05)

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XINGZHOU TIAN : EFFECTS OF ANTHOCYANIN-RICH PURPLE CORN (*Zea mays* L.) STOVER SILAGE ON ANTIOXIDANT ACTIVITIES IN DAIRY GOATS. THESIS ADVISOR :
ASSOC. PROF. PRAMOTE PAENGKOUM, Ph.D., 159 PP.

PURPLE CORN STOVER SILAGE/ANTIOXIDANT/DAIRY GOATS

This report was divided into 3 experiments.

In experiment 1, the corn stovers were ensiled with two treatments: (1) hybrid sticky waxy corn stover and (2) hybrid purple waxy corn stover. The results indicated that anthocyanin-rich purple corn stover silage (PSS) had exhibited higher ($P<0.05$) levels of dry matter (DM) and crude protein (CP) relative to the sticky corn stover silage (SSS). Although PSS displayed a lower ($P<0.05$) level of pelargonidin-3-glucoside (P3G), it had higher ($P<0.05$) levels of peonidin (Peo), malvidin3-O-glucoside (M3G), cyanidin (Cya), pelargonidin (Pel), and total anthocyanins compared to the control. Specifically, total anthocyanins in anthocyanin-rich PSS decreased rapidly ($P<0.05$) prior to 7 days of ensilage, and then remained at relatively stable ($P>0.05$) constants. Compared to the PSS, SSS displayed higher ($P<0.05$) pH value and ammonia nitrogen (NH_3-N) content. Compared with the SSS extract, PSS extract showed a higher ($P<0.05$) level of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Moreover, PSS reduced ($P<0.05$) gas production at 3 h, immediately soluble fraction, and ratio of acetic acid (AA) to propionic acid (PA) at 12 h.

In experiment 2, eight healthy multiparous Saanen dairy goats were assigned to a double 4 × 4 Latin square design. There were four treatments: (1) negative control,

fed rice straw (RSNC); (2) positive control 1, fed SSS (SSPC); (3) goats fed PSS (APSS); and (4) positive control 2, fed SSPC with 1 g/d commercial purple corn pigment (SSPP). The results showed that goats receiving RSNC had lower ($P<0.05$) levels of nutrients apparent digestibility, nitrogen (N) retention, and volatile fatty acid (VFA) production compared to the other treatments. Dietary supplementation of the APSS and SSPP showed higher ($P<0.05$) levels of DPPH scavenging activity and superoxide dismutase (SOD) in plasma. Goats fed APSS and SSPP displayed higher ($P<0.05$) levels of the abundance of nuclear factor, erythroid 2 like 2 (NFE2L2), and lower ($P<0.05$) levels of tumor necrosis factor (TNF) in the mammary gland compared with those fed RSNC. Moreover, goats receiving APSS tended to increase ($P<0.05$) the levels of SOD2, GPX1, and GPX2 mRNA expression relative to the RSNC.

In experiment 3, sixteen multiparous lactating Saanen dairy goats were divided into two treatments in a randomized completed block design: (1) control, fed SSS (CSSS) and (2) goats fed PSS (TPSS). The results indicated that TPSS had exhibited a higher ($P<0.05$) level of SOD in plasma and milk as well as it led to a significant ($P<0.05$) elevation in milk lactose composition compared to the control. The TPSS treatment had no effect ($P>0.05$) on the milk cyanidin-3-glucoside (C3G), delphinidin (Del), Cya, Pel, and total anthocyanins. However, the TPSS treatment resulted in higher ($P<0.05$) levels of Peo and M3G in milk relative to the control.

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LIST OF ABBREVIATIONS

a	=	Gas production from the immediately soluble fraction
a+b	=	Potential extent of gas production
AA	=	Acetic acid
ADF	=	Acid detergent fiber
ANOVA	=	Analysis of Variance
AOAC	=	Association of Offcial Analytical Chemists
b	=	Gas production from the insoluble fraction
BA	=	Butyric acid
BW	=	Body weight
c	=	Gas production rate constant
C3G	=	Cyanidin-3-glucoside
CAT	=	Catalase
cDNA	=	Complementary DNA
CP	=	Crude protein
Cya	=	Cyanidin
Del	=	Delphinidin
DIM	=	Day in milk
DM	=	Dry matter
DMI	=	Dry matter intake

LIST OF ABBREVIATIONS (Continued)

DPPH	=	2,2-dipheny l-1-picrylhydrazyl
ECM	=	Energy-corrected milk
ED	=	Effective degradability
F	=	Forward
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GE	=	Gross energy
GP	=	Gas production
GPX	=	Glutathione peroxidase
HG	=	Housekeeper gene
HSF1	=	Heat shock transcription factor 1
hspA1A	=	Heat shock 70kDa protein 1A
IC50	=	Half maximal inhibitory concentration
LA	=	Lactic acid
M3G	=	Malvidin-3-O-glucoside
Mal	=	Malvidin
ME	=	Metabolizable energy
MEC	=	Milk energy content
MEO	=	Milk energy output
NCBI	=	National Center for Biotechnology Information
NDF	=	Neutral detergent fiber
NFE2L2	=	Nuclear factor, erythroid 2 like 2
NH_3-N	=	Ammonia nitrogen

LIST OF ABBREVIATIONS (Continued)

NRC	=	National Research Council
OM	=	Organic matter
OMD	=	Organic matter digestibility
P3G	=	Pelargonidin-3-glucoside
PA	=	Propionic acid
Pel	=	Pelargonidin
Peo	=	Peonidin
PSS	=	Purple corn stover silage
R	=	Reverse
RCBD	=	Randomized completed block design
Real-time PCR	=	Real-time polymerase chain reaction
SD	=	Standard deviation
SEM	=	Standard error of the mean
SNF	=	Solid not fat
SOD	=	Superoxide dismutase
SSS	=	Sticky corn stover silage
TAC	=	Total antioxidant capacity
TG	=	Target gene
TMR	=	Total mixed ration
TNF	=	Tumor necrosis factor
TS	=	Total solid
TVFA	=	Total volatile fatty acid

LIST OF ABBREVIATIONS (Continued)

VFA	=	Volatile fatty acid
WSC	=	Water soluble carbohydrates

CHAPTER I

INTRODUCTION

1.1 Introduction

Currently, the new natural antioxidants are quite popular due to their safety for the consumers. Anthocyanins, as a group of phenolic compounds widely existing in the plant kingdom (Bridle and Timberlake, 1997), which exhibit strong antioxidant property (Castañedaovando et al., 2009; Canuto et al., 2016). Consequently, they play an important role in preventing oxidative injury through their abilities to scavenge free radicals (FRs) that cause cellular damage (De Beer et al., 2017). Numerous studies have been reported to the flavonoid compounds (including anthocyanins) can be reduced oxidative stress (OS) in the ruminants (Hosoda et al., 2012; Suman et al., 2015; Stoldt et al., 2016).

Dairy small ruminants are inclined to OS status due to their intensive metabolic demands for maintenance and production (Bernabucci et al., 2005; Castillo et al., 2006). One study did show that high producing dairy ruminant can be optimized to a certain extent by the addition of diets with optimal levels of antioxidant capability micronutrients (Sordillo and Aitken, 2009).

Various advantages of the silage which including wide range of source of raw materials, reduce a lot of insect pests, store in a long time, maintain nutritional content of feedstuffs and have higher palatability and digestibility (Nichols et al., 1998). Purple corn is an abundance and economic source of anthocyanins colorant and

functional ingredient, which has been cultivated in South America, Peru, Bolivia and Asia (Jing and Giusti, 2005). Moreover, corn is regarded as an ideal feedstuff owing to its high yielding and easier silage properties so that being increasingly favored by farmers in tropical countries (Clawson, 1985). Of interest, previous studies demonstrated that purple corn by-products, such as the husk, cob, silk, and tassels also had abundant anthocyanins (Dykes and Rooney, 2007; Khampas et al., 2013). Thus, purple corn by-products seem to be a suitable functional ingredient for ruminants. However, those by-products are usually buried or burned after the corn kernels are harvested, which pollute the environments, it is not in line with the goal of sustainable development.

Thailand is a tropical country with hot temperature and a significantly different climate from others countries and areas, ruminant roughage must be supported which are not available in the dry season (Hare et al., 2009). The process of making silage has become common; silage has been an increasingly important source of ruminant forage feedstuff (Lounglawan et al., 2011; Lukkananukool et al., 2013). In addition, purple corn is a type of field crop that is widely grown in Thailand (Phinjaturus et al., 2016). Recently, farmers and researchers in Thailand are looking for new ways to improve ruminant health from the perspective of animal nutrition in terms of antioxidant activity. Therefore, the objective of this study was to investigate anthocyanin-rich purple corn stover silage (PSS) on antioxidant activities and milk production in Saanen dairy goats.

1.2 Research Objectives

1.2.1 To investigate anthocyanin-rich PSS on anthocyanin stability, and on *in vitro* fermentation.

1.2.2 To ascertain anthocyanin-rich PSS on antioxidant activities and milk production in dairy goats.

1.3 Research hypotheses

1.3.1 Anthocyanin compounds in anthocyanin-rich PSS can be stable in silage.

1.3.2 Anthocyanin-rich PSS has the ability to improve antioxidant activities in dairy goats.

1.3.3 Anthocyanin-rich PSS has no negative effect on rumen fermentation and milk production in dairy goats.

1.4 Scope and limitation of the study

1.4.1 Anthocyanin-rich purple corn planted in Suranaree University of Technology (SUT) farm.

1.4.2 Saanen dairy goats used in this study.

1.4.3 Evaluation of anthocyanin-rich PSS on antioxidant activities and milk production in dairy goats.

1.5 Expected results

1.5.1 To provide anthocyanin-rich PSS has the ability to improve dairy goats' health by enhancing antioxidant activity enzymes and modulating the gene expression of inflammatory and antioxidant in mammary gland of dairy goats.

1.5.2 To gain anthocyanin-rich PSS as a source of excellence roughage for ruminant, providing a suitable feed for commercial farming.

1.5.3 Further study can use the results in this thesis to determine individual anthocyanin composition on mammary epithelial cell culture in ruminants.

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CHAPTER II

LITERATURE REVIEWS

2.1 Anthocyanins in anthocyanin-rich purple corn

As shown in Table 2.1, anthocyanin-rich purple corn displays a higher level of anthocyanin content than other sources, probably making it become one of the most exciting new super feedstuffs at present (Munoz-Espada et al., 2004; Hosseinian and Beta, 2007; Lieberman, 2007).

Table 2.1 Anthocyanin content from different sources.

Sources	**Anthocyanin content (mg/100 g)**
Blackcurrant	190~270
Aronia	1480
Eggplant (*Aubergine*)	750
Marion blackberry	317
Black raspberry	589
Raspberry	365
Wild blueberry	558
Cherry	122
Redcurrant	80~420
Concord grape	326
Norton grape	888
Queen Garnet plum	277
Blood orange	200
Purple corn (*Zea mays* L.)	1642

Table 2.2 Relative quantitative anthocyanin structures in purple corn materials.

Compound	Anthocyanidin (%)	Materials	References
Cyanidin-3-glucoside	38.34		
Pelargonidin-3-glucoside	5.79		
Peonidin-3-glucoside	11.03		
Acylated cyanidin-3-glucoside	25.76		Pedreschi and
Acylated pelargonidin-3-glucoside	7.03	Andean	Cisneros-Zevallos
Acylated peonidin-3-glucoside	12.05	purple corn	(2007)
Cyanidin derivatives	64.10		
Peonidin derivatives	23.08		
Pelargonidin derivatives	12.82		
Compound	**Anthocyanidin (%)**	**Materials**	**References**
Catechincyanidin-3,5-diglucoside	7.8		
Cyanidin-3-glucoside	37.7		
Pelargonidin-3-glucoside	5.4		
Peonidin-3-glucoside	3.8		
Cyanidin-3-(6’’-malonylglucoside)	26.8		
Pelargonidin-3-(6’’-malonylglucoside)	1.7		
Peonidin-3-(6’’-malonylglucoside)	7.5	Bolivian	Cuevas et al.
Cyanidin-3-dimalonylglucoside	3.6	purple corn	(2011)
Pelargonidin-3-dimalonylglucoside	0.7		
Peonidin-3-dimalonylglucoside	1.1		
Unknown	3.9		
Cyanidin derivatives	75.9		
Peonidin derivatives	12.4		
Pelargonidin derivatives	7.8		

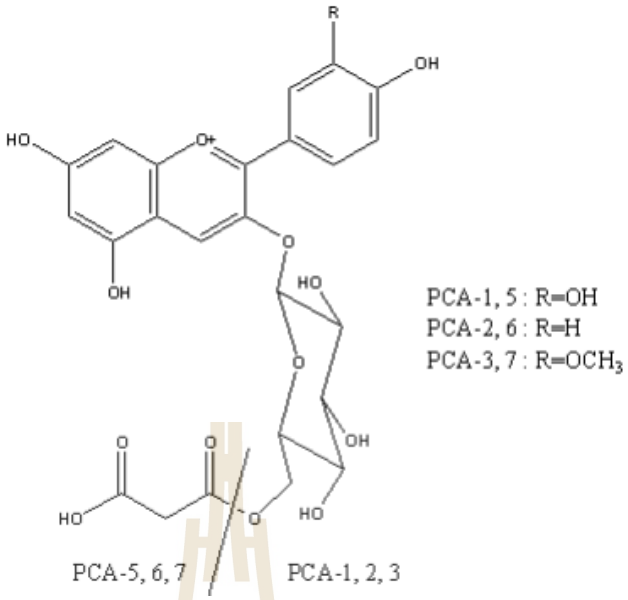

Figure 2.1 Structures of purple corn anthocyanins (adapted from Aoki et al., 2002). PCA-1 = cyanidin3-O-β-D-glucoside; PCA-2 = pelargonidin3-O-β-D-glucoside; PCA-3 = peonidin3-O-β-D-glucoside; PCA-5 = cyanidin3-O-β-D-(6-malonyl-glucoside); PCA-6 = pelargonidin3-O-β-D-(6-malonyl-glucoside); PCA-7 = peonidin3-O-β-D-(6-malonyl-glucoside).

Anthocyanidins (or aglycons) are the basic structures of the anthocyanins (Figure 2.1). The structure of anthocyanidin consist of an aromatic ring [A] bond to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring [B] (Konczak and Zhang, 2004; Sancho and Pastore, 2012). There are more than 500 different anthocyanins and 23 anthocyanidins to be found in plants, whereas only six are the most common in the plants, namely, pelargonidin (Pel), peonidin (Peo), cyanidin (Cya), malvidin (Mal), petunidin (Pet), and delphinidin (Del) (Table 2.2; Clifford, 2000).

2.2 Metabolism and digestion of anthocyanins in ruminants

No information reported the metabolism and digestion of anthocyanins in ruminants. However, Hosoda et al. (2009) revealed that the incubation of anthocyanin-rich corn with ruminal fluid did not cause degradation of total anthocyanins. Similarly, Song et al. (2012) showed that the anthocyanins in colored barley had higher stability in the ruminal fluid by *in vitro* technique. However, Leatherwood (2013) indicated that anthocyanins from purple-fleshed sweet potatoes extract are susceptible to rumen fluid. Thus, these literatures provide a basis for us that anthocyanins in plants are not broken down in the rumen; In contrast, anthocyanins pigment or powder or the plants extract can be degraded in the ruminal fluid.

Anthocyanins are flavonoids, so the possible absorption pathways of anthocyanins in plants in the ruminant (Figure 2.2): (1) anthocyanins can be by-pass the rumen to reach the small intestine, a small part of are absorbed into the blood, with the blood transported to the liver; (2) another small part of the decomposition and absorption in the colon microbial to reach the liver; (3) part of the anthocyanins can form a cycle in the liver with bile salt secrete into the small intestine; (4) anthocyanins can be absorbed into the tissue and metabolites are excreted by the kidneys with urine; and (5) the unabsorbed part are discharged with the feces (Gonthier et al., 2003).

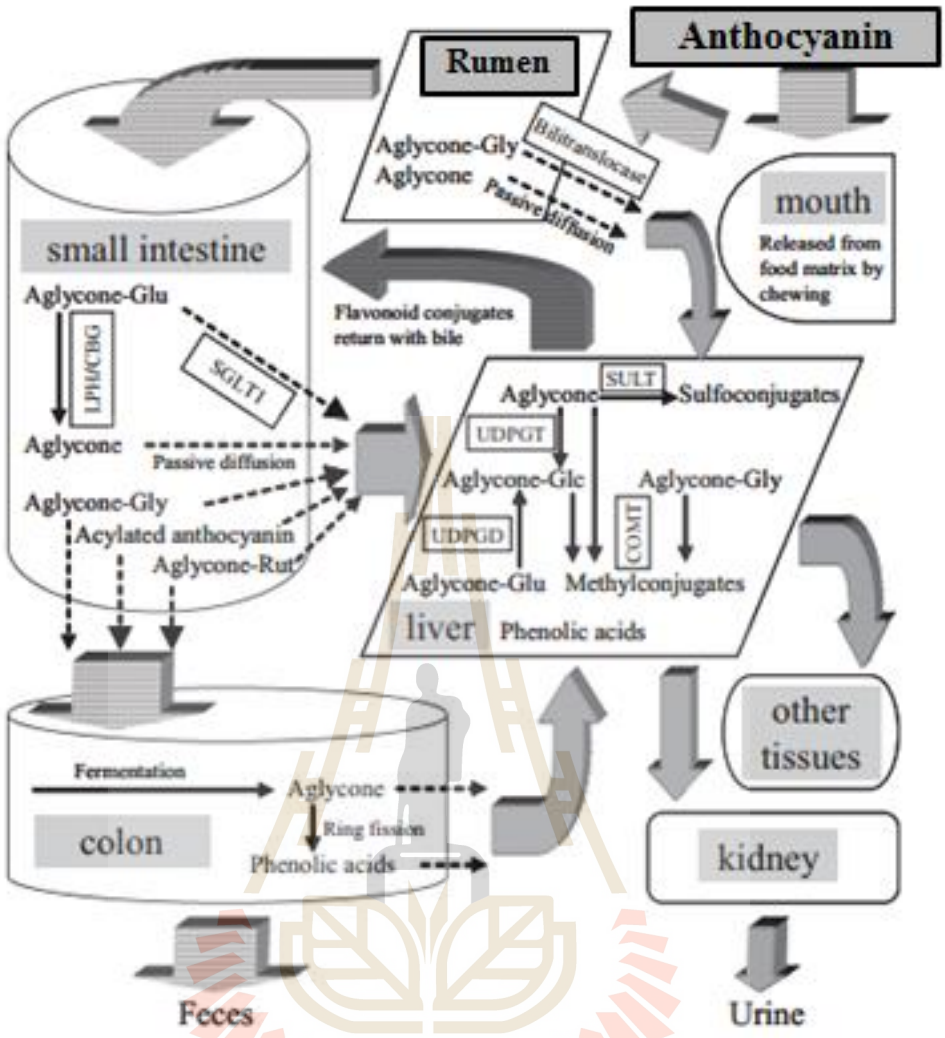

Figure 2.2 Possible pathways of anthocyanins absorption, metabolism, distribution, and excretion in ruminant (modified from He and Giustia, 2010).

2.3 The relationship between anthocyanins and free radicals

In normal condition, Superoxide ($O_2^{\cdot-}$) is converted in hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD); and then converted in water (H_2O) by the activity of glutathione peroxidase (GPX) and catalase (CAT; Figure 2.3). However, high metabolic load, dietary imbalances could be stimulated the activity of the NADPH oxidase, thereby resulting in OS status when excessive FR is not removed via

the antioxidant system, affecting on the glucose metabolism and competing with NADPH dependent metabolic pathways, such as energy metabolism, immunological functions, antioxidant capacity and calcium homeostasis. Accordingly, there are two main ways to alleviate OS status: (1) the neutralization of FRs by antioxidants; (2) the reduction of oxidized molecules by enzymatic system. The possible antioxidant mechanism for anthocyanins as follows: (1) the phenolic hydroxyl group possesses the function of directly scavenging oxygen FRs through its own structure; and (2) improve the body's antioxidant-related enzyme activity to achieve the purpose of improving the body's antioxidant capacity (Sakano et al., 2005; Soobrattee et al., 2005).

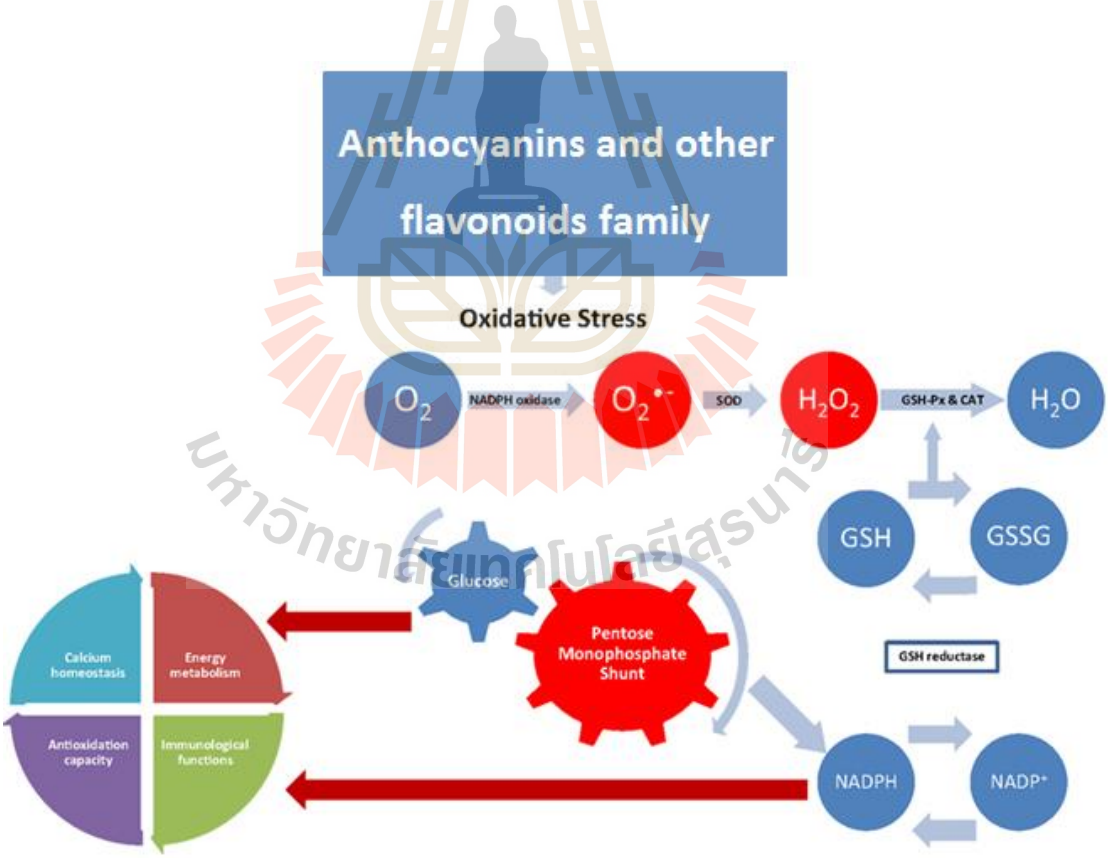

Figure 2.3 The relationship between anthocyanins and free radicals during normal metabolism by the activity of the NADPH oxidase (modified from Celi et al., 2014).

2.4 The mechanism of anthocyanin affect gene expression

Inflammation is a key process underlying various OS metabolic-linked diseases. FRs can inhibit nuclear factor kappa beta (NFκB), which bind to the promoter regions of genes coding for pro-inflammatory proteins including various cytokines and adhesion molecules, the predominant role players during inflammation. Anthocyanins protect against inflammation for animals via two main ways (Figure 2.4): (1) anthocyanins can be associated with alter inflammatory cytokine expression; and (2) anthocyanins have the ability to restrain the expression of adhesion molecules on endothelial cells (Kruger et al., 2014).

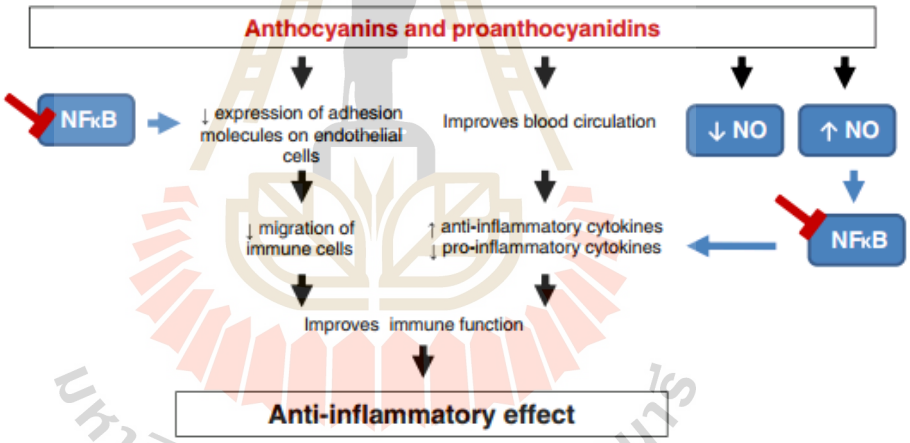

Figure 2.4 Anthocyanins' anti-inflammatory effect results in improved immune cell functions (adapted from Kruger et al., 2014). NFκB = nuclear factor kappa beta; NO = nitric oxide.

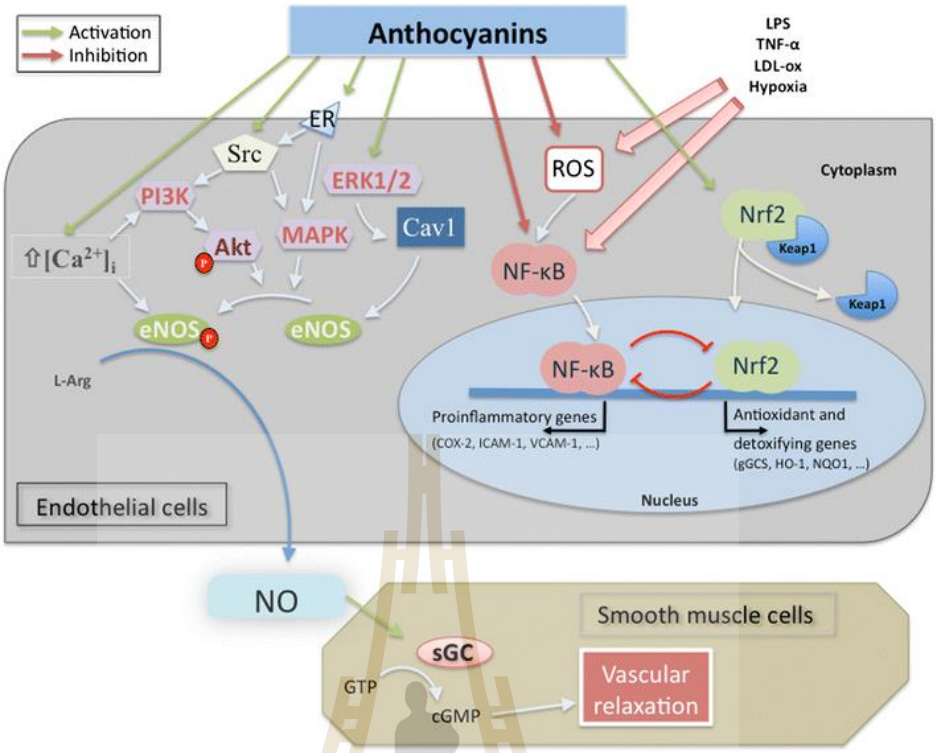

Figure 2.5 Effects of anthocyanin in vascular endothelium (adapted from Speciale et al., 2014).

ROS = reactive oxygen species; PI3K = phosphatidylinositol 3-kinase; MAPK = mitogen-activated protein kinases; ERK1/2 = extracellular regulated kinase 1 and 2, *Akt* protein kinase B; LDL-*ox* = oxidized low density lipoprotein; TNF-a = tumor necrosis factor a; eNOS = endothelial NO synthase, NO = nitric oxide, ER = estrogen receptor; KEAP1 = kelch-like-ECH-associated protein 1; Nrf2 = nuclear factor erythroid-2 (NF-E2)-related factor 2.

Anthocyanins are able to modulate inflammatory pathway by inhibiting reactive oxygen species (ROS) and the redox-sensitive transcription factor NFκB. Indirect mechanisms involved in ROS scavenging ability of anthocyanins could be

also linked to acute activation of antioxidant and detoxifying enzymes modulated by nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) transcription factor (Speciale et al., 2014). These targets include the following (Figure 2.5): (1) activation of phase II antioxidant detoxifying enzymes; (2) activation of peroxisome proliferator-activated receptors (PPAR)γ, thereby down-regulation of pro-inflammatory enzymes, such as COX-2 and iNOS; (3) inhibition of tyrosine kinases and modulation of several cell survival/cell-cycle genes; and (4) regulation of calcium homeostasis. Ye et al. (2016) showed that under homeostatic conditions, Nrf2 signaling is repressed by Kelch-like ECH-associated protein 1 (Keap1). However, Nrf2 is activated and triggered to translocate into the nucleus, where it elicits a series of anti-oxidative responses, thereby activating multiple inflammatory and antioxidant genes involved in cellular defense when animals receiving the antioxidants (Goldring et al., 2006).

2.5 Effect of anthocyanins on ruminants health

2.5.1 Factors affecting the stability of anthocyanin

The structure of sugar in anthocyanin might be used as substrate for lactic acid fermentation during the ensilage period (Figure 2.6). Anthocyanins can be long-term stability in the low pH environment. One study did show that the degradation rate of anthocyanins increased with increasing of pH values under the same external conditions (Kırca et al., 2007). There are three kinds of chemical equilibrium in anthocyanins in acid aqueous solution, which including acid-base equilibrium, hydration equilibrium and ring-chain tautomeric equilibrium (Brouillard, 1982). Four types of structures in anthocyanins, which including quinonoidal anhydrobase (A, blue), flavylium cation (AH^+, red), carbinol pseudobase (B,

colorless), and chalcone (C, colorless or light yellow), making the color changes and stability/unstability. When pH is high, the acid-base balance has become the main reaction of anthocyanins proton transfer reaction from (AH^+) to (A) or ionization (A^- or A^{2-}); A^- or A^{2-} compared to the AH^+ structure is unstable, and thus more easily degraded to other products. In contrast, when pH is low, the above three chemical equilibrium exist simultaneously and anthocyanin structure can be maintained.

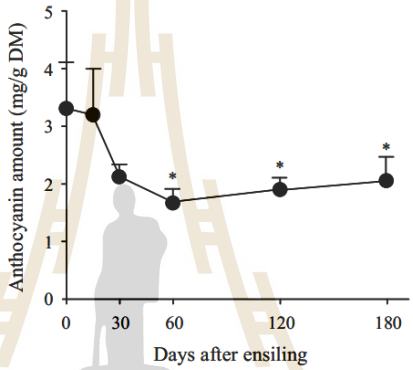

Figure 2.6 Change in anthocyanin content of anthocyanin-rich corn during ensilage (adapted from Hosoda et al., 2009) Data are displayed as means with standard deviations. * Asterisks show statistical difference from the level at day 0 ($P<0.05$).

Anthocyanins may only have potential value in improving ruminant health rather than providing individual bodies with access to nutrients because anthocyanins in anthocyanin-rich plants are not broken down in the rumen (Figure 2.7).

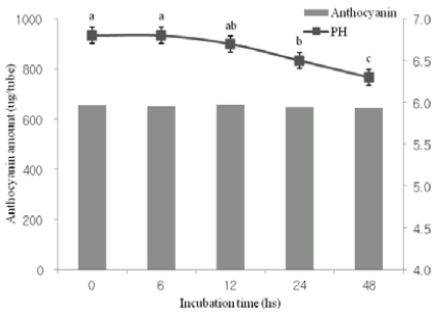

Figure 2.7 Changes in anthocyanin of colored barley content during *in vitro* technique (adapted from Song et al., 2012). [a,b,c] Treatments with different letters are different at $P<0.05$.

2.5.2 Effect of anthocyanins on rumen fermentation parameters

Chemical composition seemed to be became main factor that affecting rumen fermentation parameters because anthocyanins in plants are not broken down in the rumen. As shown in Table 2.3. Anthocyanin-rich purple corn silage had no effect on the ruminal pH and VFA concentration, whereas can significant increase NH_3-N values, this is may be due to anthocyanin-rich purple corn silage showed the relatively high crude protein (CP), resulting in enhancing ruminal NH_3-N concentration (Hosoda et al., 2012). Burgess et al. (1973) also demonstrated that corn silage had unaffected on the propionate and acetate to propionate ratio content, whereas it could significant increase acetate and butyrate values. This difference may reflect the high ADF content of the forage corn silage, thus causing an increase in the molar percentage of acetate/butyrate in the rumen.

Table 2.3 Effect of anthocyanins on rumen fermentation parameter.

Item	Control	AR[1]	Animals	SEM	References
pH	6.57	6.48		0.05	
Ammonia-N (mg/dL)	12.8[b]	14.6[a]		0.8	
VFA concentration (mmoL)			Lactating dairy cows		Hosoda et al. (2012)
Acetate	67.8	68.1		1.5	
Propionate	22.7	23.2		0.7	
Butyrate	12.4	13.4		0.4	
Total	107.5	109.2		2.7	
Item	**Oats silage**	**Corn silage**	**Animals**		**References**
VFA concentration (mmoL)					
Acetate	61.6[b]	65.3[a]	Lactating dairy cows	0.58	Burgess et al. (1973)
Propionate	20.3	20.7		0.58	
Butyrate	12.3[b]	16.0[a]		0.51	
Acetate to propionate ratio	3.22	2.98		0.32	

[1] AR = Anthocyanin-rich purple corn silage

In the same row, values with different letter superscripts mean significant difference ($P<0.05$). The same as Table 2.4 and Table 2.5.

2.5.3 Effect of anthocyanins on antioxidant activity

FRs are necessary for the living state of cells and organisms because they can protect animals normal function well at a normal level (Nagy, 2001; Dröge, 2002). However, the redox homeostasis could be imbalance when excessive FRs in the

body, leading to many degenerative diseases, such as cancer, cardiovascular disease, immune-system decline, and cataracts (Allen and Tresini, 2000). Anthocyanins had the potential to mitigate OS status and good for ruminants' health due to the inclusion of anthocyanins can be increased antioxidant capacities, especially resulted in an enhancement in plasma SOD activity (Table 2.4).

Table 2.4 Effect of anthocyanins on antioxidant activity**.**

Item[1]	**Control**	**Pigment**	**Animals**	**SEM**	**References**
Plasma TAC (μmol/L)	453.8	458.8		6.8	
Plasma GPX (μmol/L)	5.1	5.9	sheep	0.2	Hosoda et al.
Plasma SOD (U/Ml)	164.2[b]	184.4[a]		4.0	(2012c)
Urinary 8-OHdG (μg/MBS/day)	2.8	2.3		0.2	
Items	**Control**	**AR**	**Animals**		**References**
Total antioxidant status (mmol/L)	1.06	1.04	Lactating	0.01	Hosoda et al. (2012b)
SOD (U/mL)	2.4[b]	3.9[a]	dairy cows	0.5	

[1] AR = Anthocyanin-rich purple corn silage; TAC = total antioxidant capacity; GPX = glutathione peroxidase; SOD = superoxide dismutase.

2.5.4 Effect of anthocyanins on milk yield and composition

Hosoda et al., (2012b) showed that lactating dairy cows fed anthocyanin-rich purple corn silage tended to decrease the milk yield because the bitter taste of anthocyanins might be effect on the dry matter intake (DMI), thereby affecting the milk yield and milk composition.

Table 2.5 Effect of anthocyanins on milk yield and composition.

Item[1]	Control	AR	Animals	SEM	References
Milk yield (kg/day)	28.5	27.1		0.3	
FCM yield (kg/day)	27.0	26.1		0.2	
Composition (%)					
Fat	3.66	3.76	Lactating	0.05	Hosoda et
Protein	3.36	3.38	dairy cows	0.02	al. (2012b)
Lactose	4.64	4.58		0.01	
Total solids	12.67	12.73		0.06	
Solids-not-fat	9.00	8.96		0.01	

[1] AR = Anthocyanin-rich purple corn; FCM = fat corrected milk.

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CHAPTER III

CHEMICAL COMPOSITION, ANTHOCYANIN STABILITY, ANTIOXIDANT ACTIVITY, AND *IN VITRO* RUMEN FERMENTATION OF ANTHOCYANIN-RICH (*Zea mays* L.) STOVER

3.1 Abstract

The objective of this study was to observe the chemical composition, anthocyanin stability, and antioxidant activity during the storage period and *in vitro* rumen fermentation of anthocyanin-rich purple corn (*Zea mays* L.) stover (PS) and sticky corn stover (SS). The samples were ensiled with two treatments: (1) hybrid sticky waxy corn stover (control) and (2) hybrid purple waxy corn stover (treatment). Samples were stored in mini-silos for periods of 0, 7, 14, 21, 42, 63, 84, and 105 d. The results showed that anthocyanin-rich purple corn stover silage (PSS) showed higher ($P<0.05$) levels of dry matter (DM) and crude protein (CP) relative to the sticky corn stover silage (SSS). Although anthocyanin-rich PSS displayed a lower ($P<0.05$) level of pelargonidin-3-glucoside (P3G), it had higher ($P<0.05$) levels of peonidin (Peo) and pelargonidin (Pel) compared to the control. Delphinidin (Del) and malvidin (Mal) did not detect in SSS during the ensilage period; in PSS, Del was no longer detected after 7 days of ensilage. Specifically, total anthocyanins in anthocyanin-rich PSS decreased rapidly ($P<0.05$) prior to 7 days of ensilage, and then remained at

relatively stable (P>0.05) constants. Compared to the anthocyanin-rich PSS, SSS displayed significantly higher (P<0.05) pH value and ammonia nitrogen (NH_3-N) content. Compared with the SSS extract, anthocyanin-rich PSS extract showed a higher (P<0.05) level of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and displayed a lower (P<0.05) half maximal inhibitory concentration (IC50) value. Moreover, anthocyanin-rich PSS reduced (P<0.05) gas production (GP) at 3 h, and displayed lower levels of immediately soluble fraction (a) and ratio of acetic acid (AA) to PA at 12 h, but the other parameters were unaffected (P>0.05) relative to the control. Taken together, the results indicated that: (1) anthocyanins could be stable in silage; (2) anthocyanin-rich PSS showed better silage fermentative quality and stronger antioxidant activity; and (3) anthocyanin-rich PSS had no negative effect on rumen fermentation parameters.

Key words: anthocyanin-rich purple corn stover silage, anthocyanin stability, silage fermentative quality, antioxidant activity, rumen fermentation

3.2 Introduction

Anthocyanins, a group of flavonoid compounds that are naturally occurring plant pigments responsible for color change from red through purple to blue, are present in a wide variety of plants, such as blackcurrant, wild blueberry, concord grape, and purple corn (Zhao et al., 2009; Silva et al., 2016; Zhang et al., 2016). Typically, anthocyanins have been reported to exhibit potent antioxidant activity (Owoade et al., 2015). Thus, they may remove excessive free radicals to alleviate oxidative stress in ruminants when the animal's antioxidant system balance is disrupted during metabolic disorder (Jomova and Valko, 2011; Georgiev et al., 2014).

Thailand is a special tropical country with a different climate than other countries and areas due to its a long dry season from November to March (Hashimoto et al., 2004). Thus, roughage must be provided to ruminants since it is not available during that period. Purple corn is a rich and economical source of anthocyanin colorants for human consumption (Jing and Giusti, 2007; Hosoda et al., 2012c). One study did show that purple corn by-products also had abundant anthocyanins (Cevallos-Casals and Cisneros-Zevallos, 2004). In addition, purple corn is a type of field crop that is widely grown in Thailand (Harakotr et al., 2014; Phinjaturus et al., 2016). There has been little information reported about anthocyanin in purple corn stover silage (PSS) on forage yield, silage fermentative quality, anthocyanin stability, and antioxidant activity or on rumen fermentation in ruminants. Accordingly, in order to provide a preliminarily understanding of the potential of anthocyanin-rich PSS as a roughage for ruminants, we investigated its chemical composition, silage fermentative parameters, anthocyanin composition, antioxidant activity during different lengths of ensilage and *in vitro* incubation with ruminal fluid.

3.3 Materials and Methods

3.3.1 Plant management and silage making

A field experiment was conducted from June 16, 2016 to September 1, 2016, during the rainy season at the Suranaree University of Technology (SUT) farm, Nakhon Ratchasima, Thailand (14°53'37.9"N, 102°01'22.0"E). F1 hybrid purple waxy corn and F1 hybrid sticky waxy corn were cultivated in 0.5 m distance under the same fertilizer conditions (N-P_2O_5-K_2O, 50-50-50 kg/ha; Hydro Thai Limited, Bang Kruai, Thailand) using a completely randomized design (CRD) with three duplicates per

treatment. Corn grain was harvested at the yellow ripe stage, and then corn stover (without the corn grains) was cut into pieces approximately 6-8 cm in length above the soil surface by a cutting machine (SCB-2800, Fermier Engineers Private Limited, Tamil Nadu, India). The fresh materials were immediately transferred to the laboratory and chopped into pieces 2-3 cm in length by an electric automation grinder (Model 5222, Mitsubishi, Tokyo, Japan). Next, the materials were placed into 0.5 L mini-silos, which were kept in the dark at 15-25°C ambient temperature for a period of 0, 7, 14, 21, 42, 63, 84, and 105 d, respectively.

3.3.2 Chemical analysis

After ensilage, 20 g of fresh silage samples were placed into a 150-mL beaker, covered with 100-mL of distilled water, and blended for 30 minutes at room temperature before being filtered through filter paper (Whatman™ No. 1441-125, GE Healthcare Life Sciences, Marlborough, MA, USA), then pH determination was done immediately by a portable pH meter. Meanwhile, the filtrate solution was stored at -20°C until measured. Ammonia nitrogen (NH3-N) was analyzed by the steam distillation method of Bremner and Keeney (1965). Lactic acid (LA) was assayed using a high performance liquid chromatography (HPLC; 1260 Infinity II LC, Agilent Technologies, Santa Clara, CA, USA). Moreover, the anthocyanin content in silage was extracted using 1% hydrochloric acid (HCl) dissolved in 95% methanol solution at 50°C for 24 h, and then the supernatant was collected and transferred into a 50-mL volumetric flask for the determination of anthocyanin composition by a HPLC according to Hosoda et al. (2009) and Yang et al. (2009). The remaining samples were dried at 65°C in a vacuum oven for 72 h, then ground and passed through a 1-mm sieve. Dry matter (DM), crude protein (CP), and ash were measured according to the feed proximate analysis of the Association of Offcial Analytical Chemists (AOAC,

2005). Neutral detergent fiber (NDF) was detected with sodium sulfite and a heat stable amylase, whereas acid detergent fiber (ADF) was measured by sequential analysis of the residual NDF by the method of Van Soest et al. (1991). Each sample was run in triplicate. Organic matter (OM) was calculated using the following formula: OM = 100-ash. Gross energy (GE) was analyzed using a Parr 6200 calorimeter (Moline, Illinois, USA). Water soluble carbohydrates (WSC) were assayed by a microplate reader (Spectracount^{TM}, Packard Canberra, Meriden, CT, USA) after reaction with anthrone reagent (Sigma-Aldrich, Pcode: 101694154) as described by McDonald and Henderson (1964).

3.3.3 DPPH scavenging activity

Antioxidant activity for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the corn stover silage extract was determined spectrophotometrically according to Thaipong et al. (2006) and Zhang et al. (2018) using a stable free radical DPPH (Sigma-Aldrich, Pcode: 101845869) with a minor modification. Briefly, an aliquot of 2.00 mL of the appropriate dilution (1/5, 1/4, 1/3, 1/2, 1) of two corn stover silage extracts at 21 days was added to 2.00 mL of 0.1 mmol/L DPPH solution and then shaken vigorously. The OD value was detected at 517 nm using a microplate reader after incubation for 30 min in a 30°C water bath in the dark. DPPH scavenging activity (%$DPPH_{SC}$) was calculated by the following formula:

$$\%DPPH_{SC} = (Ac - As) \times 100 / Ac$$

where Ac is the absorbance of the control and As is the absorbance of the sample. The half maximal inhibitory concentration (IC50) value was calculated by

GraphPad Prism 5 software, which denotes the concentration of a substance required for 50% inhibition *in vitro*.

3.3.4 *In vitro* rumen fermentation

Three healthy multiparous Saanen dairy goats (body weight (BW), 42.50 ± 0.50 kg; mean ± standard deviation (SD)) were used as ruminal fluid donors. The experimental animals were housed in clean individual pens with free access to water and were fed diets with a concentrate/roughage ratio of 50:50 (10.13% CP and 66.33% total digestible nutrient) according to the National Research Council (NRC, 1981). Ration was offered in equal amounts twice daily at 08:00 and 17:00. Ruminal fluid was obtained from goats before morning feeding via the mouth using a vacuum pump into an Erlenmeyer flask. The ruminal fluid was immediately passed through four layers of cheesecloth and mixed in equal volume, and then transported to the laboratory. The culture fluid was prepared by mixing the ruminal fluid and a phosphate-bicarbonate buffer with carbon dioxide (CO2) gas in a 39°C water bath as per Menke and Steingass (1988). A total of 100-mL glass gas-tight syringes (Kabuskiki Kaisha, Japan) were used as fermentation vessels; they were filled with 30-mL of mixture solution with 0.50 g of substrate in each syringe for 3, 6, 9, 12, 24, 48, 72, and 96 h incubation to estimate gas production (GP). The fermentation was stopped by submerging the syringe into ice-cold water, and then the pH of the rumen fluid was immediately measured using the portable pH meter. In the meantime, 20-mL of fermentation liquid and 5 mL of HCl (6 mol/L) were kept in a container after being mixing together, then stored in a refrigerator at -20°C until the samples were analyzed for NH_3-N and individual volatile fatty acid (VFA) content. Acetic acid (AA), propionic acid (PA), and butyric acid (BA) were determined using HPLC. The total VFA (TVFA) value was calculated from the following equation: TVFA = AA + PA +

BA. Each sample had three replications and two control replications. Moreover, organic matter digestibility (OMD), metabolizable energy (ME), and effective degradability (ED) were calculated using the following formulas by Menke et al. (1979), Menke and Steingass (1988), and Eliman and Ørskov (1984), respectively:

(1) OMD (%) = 0.986 × GP (24 h) + 0.0606 × CP + 11.03;

(2) ME (MJ/kg) = −0.20 + 0.1410 × OMD;

(3) ED (%) = a + b × c / (k + c).

where k is ruminal outflow rate and the value sets as 0.031 h.

GP was calculated according to the following equation by Ørskov and McDonald(1979).

$$y = a + b\,(1-e^{-ct})$$

where y denotes the volume of gas produced at time t, a describes the immediately soluble fraction (mL), b is the insoluble fraction (mL), c is the rate constant for the insoluble fraction b (%/h), t expresses incubation time (h), and a+b represents the potential extent of gas production (mL).

3.4 Statistical analysis

All statistical analyses were performed using the GLM procedure of SAS System version 9.1.3 (SAS Inst. Inc., Cary, NC, USA). The replicate was considered the experimental unit in all of the statistical analyses. The treatment means during the storage days or dilution times or incubation time were assessed using Analysis of Variance (ANOVA) followed by the Duncan's new multiple range test. The effects of

the storage days or dilution times or incubation time were compared with trend analysis using orthogonal polynomial contrasts test. IC50 value was calculated from linear regression analysis by GraphPad Prism 5 software. Student's *t*-test was applied for the differences in the rumen fermentation parameters. Differences were considered statistically significant at $P<0.05$.

3.5 Results

3.5.1 Chemical composition

There were linear ($P<0.05$) effect of ensilage time on DM, GE, NDF, ADF, and WSC between two treatments (Table 3.1). Anthocyanin-rich PSS showed higher ($P<0.05$) levels of DM and CP, compared to that of sticky corn stover silage (SSS). Differently, SSS displayed higher ($P<0.05$) concentration of WSC in comparison of anthocyanin-rich PSS. No notable differences ($P>0.05$) were observed in ash, OM, and NDF levels of SSS and PSS. The level of ADF tended to significantly ($P<0.05$) increase prior to 14 days of ensilage, and then remained at a relatively stable value ($P>0.05$) among treatments. Additionally, although there was no difference ($P>0.05$) in GE between the two samples, ANOVA analysis showed that GE content in PSS was significantly ($P<0.05$) different during the ensilage period.

Table 3.1 Changes in chemical composition according to ensiling period of SSS and PSS (DM basis).

Item[1]	Time (day)	DM (%)[2]	Ash (%)	OM (%)	GE (Kcal/g)	CP (%)	NDF (%)	ADF (%)	WSC (%)
SSS	0	23.30^{b}	6.37	93.63	3.93	6.57	57.74	19.89^{b}	6.40^{a}
	7	25.36^{ab}	6.59	93.41	3.76	6.59	59.44	23.87^{ab}	3.17^{b}
	14	26.53^{ab}	6.72	93.28	4.04	6.87	60.48	25.54^{a}	2.55^{c}
	21	26.74^{ab}	6.49	93.51	4.06	6.83	64.40	26.48^{a}	2.49^{c}
	42	25.88^{ab}	6.50	93.50	4.00	6.96	62.24	25.27^{a}	2.34^{cd}
	63	27.03^{ab}	6.83	93.17	4.00	6.98	62.18	24.30^{a}	2.00^{d}
	84	27.71^{a}	6.73	93.27	4.04	7.01	64.78	25.36^{a}	1.88^{d}
	105	28.40^{a}	6.92	93.08	4.04	7.05	64.44	26.23^{a}	1.94^{d}
	SEM[3]	1.18	0.40	0.40	0.09	0.14	2.78	1.25	0.15
	Mean	26.37^{B}	6.64	93.36	3.98	6.86^{B}	61.96	24.62	2.85^{A}
PSS	0	26.40	6.91	93.09	3.93^{b}	7.24	56.24	24.04^{c}	4.65^{a}
	7	28.61	6.98	93.02	3.95^{b}	7.24	60.50	24.27^{bc}	2.72^{b}
	14	28.46	7.37	92.63	3.96^{ab}	7.29	63.13	26.04^{a}	2.42^{bc}
	21	28.44	6.96	93.04	3.99^{a}	7.38	63.79	25.06^{abc}	2.28^{bc}
	42	28.69	6.39	93.61	4.00^{a}	7.37	63.68	25.85^{ab}	2.01^{c}
	63	28.93	6.37	93.63	4.00^{a}	7.40	64.07	24.43^{abc}	2.15^{c}
	84	28.96	6.35	93.65	4.00^{a}	7.40	64.00	25.75^{ab}	2.00^{c}
	105	28.95	6.71	93.29	3.99^{a}	7.37	63.19	25.58^{abc}	2.37^{bc}
	SEM	1.48	0.36	0.36	0.01	0.11	2.51	0.46	0.13
	Mean	28.43^{A}	6.75	93.25	3.98	7.34^{A}	62.33	25.13	2.57^{B}
P-value[4]	L	0.054	0.649	0.649	0.028	0.296	0.003	0.0004	<0.0001
	Q	0.305	0.371	0.371	0.237	0.961	0.832	0.151	<0.0001
	C	0.787	0.545	0.545	0.077	0.679	0.823	0.675	0.015

Values represented the means of 3 replicates (n = 3).

Treatment with different superscript letters within the same column are significantly different (P<0.05). Lowercase letters represent comparison within treatment; capital letters mean comparison of two treatments.

[1] SSS = sticky corn stover silage; PSS = anthocyanin-rich purple corn stover silage.

[2] DM = dry matter; OM = organic matter; GE = gross energy; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; WSC = water soluble carbohydrates.

[3] SEM = standard error of the mean.

[4] L = linear; Q = quadratic; C = cubic. The same as below.

3.5.2 Anthocyanin composition

As shown in Table 3.2. There were linear ($P<0.05$) effect of storage days on Cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (P3G), peonidin (Peo), malvidin-3-O-glucoside (M3G), cyanidin (Cya), pelargonidin (Pel), and malvidin (Mal) between two treatments. The only exception was the level of total anthocyanins during ensilage period that showed cubic ($P<0.019$) effect. C3G remained relatively constant and no significant ($P>0.05$) difference was found among treatments. Delphinidin (Del) and Mal were unable to be detected in SSS during the entire ensilage period; in PSS, Del was not detected after 7 days of ensilage. Moreover, the concentrations of P3G, Peo, M3G, Cya, and Pel decreased ($P<0.05$) to stable values after 21 days of ensilage. Anthocyanin-rich PSS showed a lower ($P<0.05$) level of P3G, whereas it had significantly higher ($P<0.05$) total anthocyanin content, Peo, M3G, Cya, and Pel levels than that of SSS. Specifically, total anthocyanins in two treatments decreased rapidly ($P<0.05$) prior to 7 days of ensilage, and then remained at relatively stable ($P>0.05$) constants.

Table 3.2 Changes in anthocyanin composition according to ensiling period of SSS and PSS (DM basis).

Item	Time (day)	Anthocyanin composition (mg/g)[1]								Total
		C3G	Del	P3G	Peo	M3G	Cya	Pel	Mal	
SSS	0	0.028	-	0.084^{a}	0.034^{a}	0.040^{a}	0.111^{a}	0.091^{a}	-	0.387^{a}
	7	0.028	-	0.078^{ab}	0.031^{ab}	0.040^{a}	0.103^{ab}	0.083^{ab}	-	0.362^{ab}
	14	0.025	-	0.074^{ab}	0.028^{b}	0.035^{ab}	0.098^{ab}	0.079^{b}	-	0.339^{ab}
	21	0.025	-	0.072^{b}	0.030^{ab}	0.040^{a}	0.100^{ab}	0.080^{ab}	-	0.347^{ab}
	42	0.027	-	0.075^{ab}	0.028^{b}	0.034^{b}	0.104^{ab}	0.081^{ab}	-	0.350^{ab}
	63	0.028	-	0.071^{b}	0.029^{b}	0.034^{b}	0.098^{ab}	0.078^{b}	-	0.337^{ab}
	84	0.024	-	0.070^{b}	0.028^{b}	0.032^{b}	0.091^{b}	0.077^{b}	-	0.323^{b}
	105	0.025	-	0.068^{b}	0.029^{b}	0.032^{b}	0.091^{ab}	0.075^{b}	-	0.319^{b}
	SEM1	0.001	-	0.003	0.001	0.002	0.005	0.004	-	0.016
	Mean	0.026	-	0.074^{A}	0.030^{B}	0.036^{B}	0.099^{B}	0.080^{B}	-	0.345^{B}
PSS	0	0.029	0.515	0.075	0.045^{a}	0.113^{a}	0.178^{a}	0.111^{a}	1.820^{a}	2.410^{a}
	7	0.025	-	0.070	0.037^{b}	0.065^{b}	0.115^{b}	0.101^{ab}	0.319^{b}	0.733^{b}
	14	0.025	-	0.067	0.037^{b}	0.059^{b}	0.111^{b}	0.090^{bc}	0.324^{b}	0.713^{b}
	21	0.025	-	0.069	0.038^{b}	0.057^{bc}	0.114^{b}	0.092^{bc}	0.326^{b}	0.720^{b}
	42	0.025	-	0.068	0.032^{b}	0.041^{d}	0.109^{b}	0.082^{c}	0.349^{b}	0.706^{b}
	63	0.024	-	0.066	0.031^{b}	0.037^{d}	0.094^{b}	0.076^{c}	0.351^{b}	0.679^{b}
	84	0.024	-	0.064	0.032^{b}	0.038^{d}	0.094^{b}	0.075^{c}	0.345^{b}	0.672^{b}
	105	0.025	-	0.067	0.032^{b}	0.046^{cd}	0.098^{b}	0.077^{c}	0.323^{b}	0.669^{b}
	SEM2	0.001	-	0.004	0.002	0.004	0.007	0.005	0.044	0.065
	Mean	0.025	-	0.068^{B}	0.036^{A}	0.057^{A}	0.114^{A}	0.088^{A}	0.520	0.913^{A}
P-value	L	0.002	-	0.019	0.030	0.007	0.0004	0.002	<0.0001	0.208
	Q	0.458	-	0.345	0.075	0.080	0.007	0.156	<0.0001	0.311
	C	0.882	-	0.909	0.848	0.706	0.473	0.670	<0.0001	0.019

Values represented the means of 3 replicates (n = 3).

[1] C3G = cyanidin-3-glucoside; Del = delphinidin; P3G = pelargonidin-3-glucoside; Peo = peonidin; M3G = malvidin-3-O-glucoside; Cya = cyanidin; Pel = pelargonidin; Mal = malvidin; - = not detected.

3.5.3 Silage fermentative quality

As expected, the pH value of the two silages dropped rapidly ($P<0.05$) during the first seven days of ensilage, and then the levels remained between 3.5 and 4.0 throughout the remainder of the experiment (Figure 3.1). In addition, PSS showed a slightly lower pH value compared to that of the control silage, but no significant difference ($P>0.05$) was found between two treatments. The level of NH_3-N in PSS was significantly lower ($P<0.05$) than that of the control (Figure 3.2). LA concentrations in both of the silages increased ($P<0.05$) during the first seven days of ensilage, and then remained relatively constant ($P>0.05$) after 14 days of storage (Figure 3.3). In particular, PSS had a significantly higher ($P<0.05$) LA level than that of SSS.

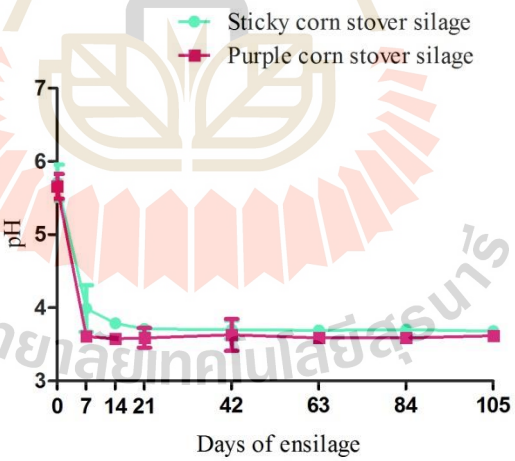

Figure 3.1 Changes in pH value during fermentation of silage Data are displayed as means with SD. Values represented the means of 3 replicates (n = 3).

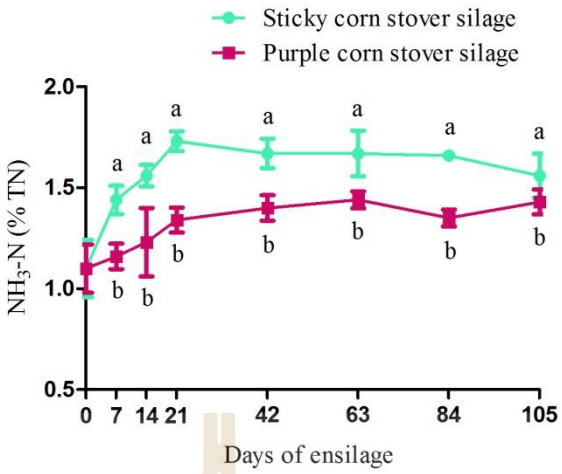

Figure 3.2 Changes in NH_3-N content during fermentation of silage Values with different letter superscripts mean significant difference ($P<0.05$). TN = total nitrogen. Data are displayed as means with SD. Values represented the means of 3 replicates (n = 3).

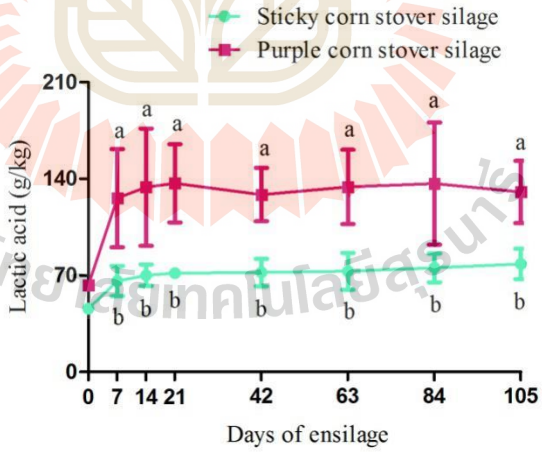

Figure 3.3 Changes in LA content during fermentation of silage Values with different letter superscripts mean significant difference ($P<0.05$). Data are displayed as means with SD. Values represented the means of 3 replicates (n = 3).

3.5.4 DPPH scavenging activity

As shown in Table 3.4. DPPH scavenging activity of SSS and PSS extracts increased with increasing concentration in dilute solution, reaching a maximum at 73.5% and 81.1%, respectively, and the values remained relatively constant (P>0.05) after 1/3 dilution. As expected, DPPH scavenging activity mean in the anthocyanin-rich PSS extract was greater (P<0.05) than that of the control. Accordingly, PSS extract showed a lower (P<0.05) level of IC50 compared to the SSS extract (0.65 µg/mL vs. 2.80 µg /mL).

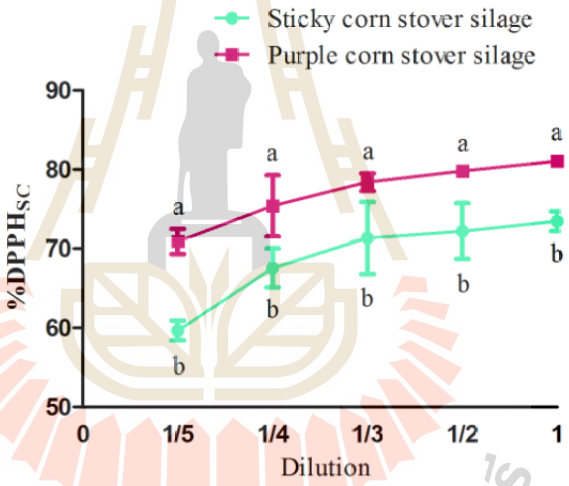

Figure 3.4 DPPH scavenging activity of corn stover silage extract Values with different letter superscripts mean significant difference (P<0.05). Data are displayed as means with SD. Values represented the means of 3 replicates (n = 3).

3.5.5 *In vitro* rumen fermentation

For GP, with the exception that SSS was significantly higher (P<0.05) than that of PSS at 3 h, no significant difference (P>0.05) was found for the other

lengths of incubation (Figure 3.5). Similarly, SSS showed a significantly higher ($P<0.05$) content of the a value, and no significant differences ($P>0.05$) were observed in the b, c, a+b, OMD, ME, and ED (Table 3.3). As shown in Table 3.4, there were no significant differences ($P>0.05$) in the pH value, NH_3-N concentration, and individual VFA content between PSS and SSS. However, SSS showed a significantly higher ($P<0.05$) ratio of AA to PA at 12 h of incubation time compared to anthocyanin-rich PSS.

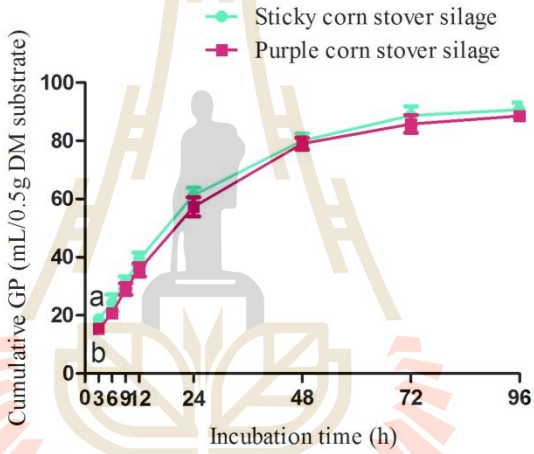

Figure 3.5 Cumulative gas volume estimated throughout 96 h of corn stover silages Values with different letter superscripts mean significant difference ($P<0.05$). Data are displayed as means with SD. Values represented the means of 3 replicates (n = 3).

Table 3.3 Comparison of GP kinetics, OMD, ME, and ED of SSS and PSS.

Item[1]	SSS	PSS	SEM	P-value
a (mL)	7.26	3.71	0.0190	<0.0001
b (mL)	85.2	86.9	0.530	0.145
a+b (mL)	92.4	90.6	0.534	0.139
c (% h)	0.04	0.04	0.0002	0.6838
OMD (%)	71.9	68.0	1.68	0.176
ME (MJ/kg)	9.94	9.39	0.237	0.176
ED (%)	54.9	52.3	1.07	0.0966

Values represented the means of 3 replicates (n = 3).

[1] a = gas production from the immediately soluble fraction; b = gas production from the insoluble fraction; c = gas production rate constant; a+b = potential extent of gas production; OMD = organic matter digestibility; ME = metabolizable energy; ED = effective degradability.

Table 3.4 Comparison of ruminal fluid pH, NH_3-N, and VFA values of SSS and PSS.

Item[1]	Time (h)	SSS	PSS	SEM	P-value
pH	12	6.82	6.87	0.0217	0.208
	24	6.65	6.67	0.0222	0.444
	48	6.56	6.56	0.00666	1.00
	Mean	6.66	6.70	0.0423	0.477
NH_3-N (mg/dL)	12	2.00	1.95	0.0267	0.323
	24	2.01	1.91	0.0738	0.422
	48	2.89	2.87	0.0504	0.789
	Mean	2.34	2.20	0.166	0.573
AA (mmol/L)	12	78.2	73.1	3.45	0.419
	24	83.3	81.5	1.23	0.357
	48	96.0	92.4	1.32	0.122
	Mean	86.8	82.3	2.92	0.309
PA (mmol/L)	12	70.4	69.5	0.920	0.577
	24	79.7	75.6	1.13	0.0647
	48	94.5	88.3	2.65	0.175
	Mean	82.9	77.8	3.23	0.297
BA (mmol/L)	12	14.7	13.9	0.198	0.0899
	24	16.3	15.8	0.298	0.318
	48	20.8	19.0	0.455	0.110
	Mean	17.6	16.3	0.841	0.300
TVFA (mmol/L)	12	163	156	3.13	0.264
	24	179	173	2.45	0.142
	48	211	200	3.66	0.0891
	Mean	187	176	6.81	0.290
Ratio of acetate to propionate	12	1.11	1.05	0.00361	0.0033
	24	1.05	1.08	0.0592	0.572
	48	1.02	1.05	0.0310	0.522
	Mean	1.05	1.06	0.0203	0.797

Values represented the means of 3 replicates (n = 3).

[1] NH_3-N = ammonia nitrogen; AA = acetic acid; PA = propionic acid; BA = butyric acid; TVFA = total volatile fatty acid.

3.6 Discussion

3.6.1 Silage fermentative quality and anthocyanin stability

DM and WSC content in plants are of great importance for silage (Wilkins, 1982). In this study, we found that DM tended to increase during the entire ensilage period although it did not differ. Possibly because the extravasate was lost from the plant material during storage ensilage, thereby increasing DM level (Maeda et al., 2011). Conversely, a previous study showed that WSC was broken down by bacterial metabolism during the whole ensilage period, thus reducing its value (Sanderson, 1993). Fiber content was a major component of the plant cell wall and was insoluble in water, making it difficult to be exploited by microorganisms during the storage period (Mertens, 2003). Hence, the concentrations of NDF between SSS and PSS remained fairly constant throughout the experiment. On the contrary, ensilage treatment could accelerate the growth of lactic acid bacteria (LAB), but the ensilage treatment lacked of proteolytic enzyme and relevant ash/OM degrading enzyme; thus, ash and OM values also did not differ among treatments and have been quite stable during the entire ensilage period. Moreover, the levels of GE and ADF tended to increase during the ensilage period, which was reported to be related to the strength of silage fermentation, in which the loss of moisture is induced, leading to high DM, with consequent concentration of the GE and ADF (McDonald and Edwards, 1976; Meineri and Peiretti, 2005). Additionally, we also found the concentration of CP in PSS was significantly higher than that of SSS. The reason may be that in purple plant response to environmental stresses through anthocyanin metabolism, plant nutrients can be improved under anthocyanin protection (Chalker-Scott, 1999). These results were consistent with a previous report by Hosoda et al. (2012a) who reported that

anthocyanin-rich purple corn silage showed a significantly higher CP value compared to the control.

Anthocyanins are sensitive to high pH, light, and temperature during storage period (Markakis and Jurd, 1974; Francis and Markakis, 1989; Laleh et al., 2006; Li et al., 2011). However, light and temperature factors could be considered to be negligent in this study because all the samples were compressed under anaerobic conditions, placed in mini-silos, and kept a dark environment at 15-25°C ambient temperature. Reyes and Cisneros-Zevallos (2007) showed that the degradation rate of anthocyanins were positively correlation with the pH value under the same external conditions. Consequently, the silage had a lower pH value after seven days of ensilage, which provided the necessary condition for the stability of anthocyanins. Specifically, anthocyanins consisted of anthocyanidin and sugar(s), which the sugar structure was used as a substrate during the ensilage period (Hosoda et al., 2009). Therefore, the decreased range of anthocyanins in PSS was greater compared to SSS, possibly indicating that more sugar in PSS took part in the silage fermentation for easier ensilage. These observations were in agreement with Song et al. (2012) who demonstrated that colored barley showed higher total anthocyanins content and maintained a level of 42% in storing silage. Moreover, Cya seemed to be the main anthocyanin composition in two types of silage, which is consistent with the findings of previous studies (Pedreschi and Cisneros-Zevallos, 2007; Cuevas Montilla et al., 2011).

The pH value declined rapidly at the fermentation phase when the silage became anaerobic, which was usually in the range of 3.6-4.0 for the excellent silage (Guan et al., 2002). This was because LAB developed, leading to it became the

predominant population during anaerobic phase, resulting in large amounts of organic acid (Kang et al., 2014). Particularly, anthocyanin-rich PSS displayed a lower pH value, perhaps due to the structure of purple corn anthocyanin was bonded to sugar (Aoki et al., 2002), resulting in easy fermentation during storage in anthocyanin-rich PSS relative to the control. Generally, poor silage preservation had a high level of NH_3-N. This was due to breakdown of the protein in forage (proteolysis) occurring as a result of the activity of plant enzymes prior to the establishment of anaerobic conditions (Acosta et al., 1991; Hu et al., 2015). However, wetter silage also had higher concentration of NH_3-N because of the potential for clostridial fermentation (Kung and Shaver, 2001). Thus, SSS had a relatively high level of NH_3-N, perhaps owing to it had lower DM content in comparison to PSS. Additionally, since the sugar in anthocyanin was used as a substrate for lactic fermentation, which may have produce high LA content (Hosoda et al., 2009). These findings were in agreement with Song et al. (2012) who showed that colored barley silage had a higher level of LA compared to that of normal barley silage.

3.6.2 DPPH scavenging activity

Anthocyanins, as a bioactive secondary plant metabolite, have been shown to have high antioxidant activity (Akula and Ravishankar, 2011). DPPH was a free radical, which was able to produce a violet solution in an organic solvent and was stable at room temperature. The value of DPPH tended to decline under antioxidant molecule circumstances (Mensor et al., 2001). Accordingly, this was a handy and rapid way of being able to assay antioxidant activity by measuring DPPH scavenging activity (Cheng et al., 2006). In this experiment, anthocyanin-rich PSS extract had a stronger level of the DPPH scavenging activity than the control extract. One of the

possible explanations was that anthocyanins could provide electrons to DPPH, so the solution was reduced to its non-free radical form to strengthen antioxidant activity (Jordão and Correia, 2016). The amount of DPPH scavenging activity was in accordance with Hayashi et al., (2003). Indeed, anthocyanins are powerful antioxidants due to their special chemical structural formula, preventing free radicals from being oxidized nearby cells (Castañeda-Ovando et al., 2009). In addition, the IC50 value was negatively associated with the DPPH scavenging activity (Yang and Zhai, 2010). As expected, PSS extract had lower IC50 level in this study, which was consistent with a previous report by Pedreschi and Cisneros-Zevallos (2006) who demonstrated that purple corn extract had a higher antioxidant activity, primarily depending on anthocyanins involved in enzyme inactivation and scavenging of electrophiles.

3.6.3 *In vitro* rumen fermentation

Previous studies indicated that the incubation of anthocyanin-rich corn or colored barley with ruminal fluid had no effect on the degradation of anthocyanin (Hosoda et al., 2009; Song et al., 2012). Their results may be a definite explanation that anthocyanins in plants are not broken down in the rumen. In the current study, anthocyanin-rich PSS had a lower level of GP at 3 h as well as the a parameter, perhaps because it had the relatively high content of fiber content and relatively low level of WSC, resulting in slower degradation rate (Brebu and Vasile, 2010). Consistent with our results, Mangan (1988) who reported that the flavonoid family can be prevented bloating by reducing the degradability of ruminal fluid nutrients. A similar conclusion was also given for the lower amount of GP in anthocyanin-rich grape pulp (Spanghero et al., 2009). Therefore, anthocyanins may only have potential value in improving ruminant health rather than providing individual bodies with access

to nutrients. Correddu et al. (2015) demonstrated that the rumen metabolism of lactating dairy-ewe was markedly influenced by dietary supplementation with anthocyanin-rich grape. Similarly, the feeding of anthocyanin-rich purple corn silage may increase superoxide dismutase (SOD) activity in the plasma, but had no effect on rumen fermentation parameters in lactating dairy cows (Hosoda et al., 2012a). In short, it was safe to assume that anthocyanins had no negative impact on rumen fermentation, but they had the potential to inhibit GP for prevention of ruminant bloating.

In this report, anthocyanins might be able to affect carbohydrate metabolism to provide more energy for ruminants by inhibiting AA production, increasing the proportion of PA. Hosoda et al. (2012a) reported that the feeding of anthocyanin-rich purple corn silage resulted in a lower AA to PA ratio in lactating dairy cows. However, Hosoda et al. (2012b) showed that the feeding of purple rice silage had higher ruminal fluid pH value and lower VFA because of poor nutrients and silage fermentative quality. As a consequence, nutrient composition could be one of the main factors affecting rumen fermentation, since anthocyanins may escape from the rumen to reach the small intestine for digestion and absorption. However, based on the present study, some chemical composition parameters in both silages were so significantly different that it was difficult to identify clearly whether the difference in rumen fermentation was responsible for the amount of anthocyanin or chemical composition difference. An interesting perspective on anthocyanins showed that they have the potential to be absorbed into milk, thereby improving consumers' health. In this regard, additional experimental data based on *in vivo* studies are needed to verify the effect of antioxidant activity for dairy ruminants.

3.7 Conclusion

The present study indicated that although the anthocyanins content in anthocyanin-rich PS declined during the ensilage period, it could be maintained in a stable condition. The anthocyanin-rich PSS showed excellent silage fermentative quality, higher level of anthocyanins, and stronger antioxidant activity compared to the SSS. Additionally, anthocyanin-rich PSS had no negative effect on rumen fermentation parameters. Thus, anthocyanin-rich PSS has the potential to become an ideal roughage for ruminants. Further studies are needed to determine the mechanism of feeding anthocyanin-rich PSS on rumen microorganisms, plasma antioxidant activities, expression of oxidative stress-related genes, and milk production in dairy ruminants.

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CHAPTER IV

EFFECTS OF ANTHOCYANIN-RICH PURPLE CORN (*Zea mays* L.) STOVER SILAGE ON DIGESTIBILITY, ANTIOXIDANT ACTIVITIES, AND MAMMARY GLAND TISSUE INFLAMMATORY AND ANTIOXIDANT GENE EXPRESSION IN DAIRY GOATS

4.1 Abstract

Eight healthy multiparous Saanen dairy goats (body weight (BW), 41.50 ± 1.84 kg; mean ± standard deviation (SD)) were assigned to a double 4 × 4 Latin square design. There were four treatments: (1) negative control, fed rice straw (RSNC); (2) positive control 1, fed sticky corn stover silage (SSS; SSPC); (3) goats fed anthocyanin-rich purple corn (*Zea mays* L.) stover silage (PSS; APSS); and (4) positive control 2, fed SSPC with 1 g/d commercial purple corn pigment (SSPP). The results showed that dry matter intake (DMI) did not differ (P>0.05) among the treatments. Goats receiving RSNC had lower (P<0.05) levels of nutrient apparent digestibility, nitrogen (N) absorption, N retention, propionic acid (PA), butyric acid (BA), and total volatile fatty acid (TVFA), ratio of acetic acid (AA) to PA compared to the other treatments. Dietary supplementation of the APSS and SSPP showed higher (P<0.05) levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and superoxide dismutase (SOD) in plasma. Goats fed APSS and SSPP displayed higher

(P<0.05) levels of the abundance of nuclear factor, erythroid 2 like 2 (NFE2L2), and lower (P<0.05) level of tumor necrosis factor (TNF) in the mammary gland compared with those fed RSNC. Although the mRNA abundance of SOD3 and catalase (CAT) in mammary gland were unaffected (P>0.05) among the treatments, goats receiving APSS tended to increase (P<0.05) the levels of SOD2, GPX1, and GPX2 mRNA expression. There were significant (P<0.05) positive correlations between DPPH scavenging activity, total antioxidant capacity (TAC), SOD, and CAT enzymes in plasma and the abundance of NFE2L2 in mammary gland. Similarly, stronger (P<0.05) positive correlations were noted between some inflammatory genes expression and several antioxidant genes expression. Therefore, the current study indicated that the consumption of anthocyanin-rich PSS by dairy goats had no negative effect on DMI, apparent digestibility, nitrogen utilization and rumen fermentation parameters, whereas it had the potential to alleviate the oxidative stress condition by improving antioxidant capacity in plasma and modulating inflammatory and antioxidant mRNA gene expression in mammary gland.

Key words: anthocyanin-rich purple corn stover silage, digestibility, antioxidant activity, gene expression, dairy goats

4.2 Introduction

Free radicals (FRs) and other reactive oxygen species (ROS) are constantly formed in the animals' body (Aruoma, 1998). In general, animals are protected against FRs or ROS by a wide range of their natural antioxidant enzymes (Miller et al., 1993). However, animals from tropical and subtropical countries/areas are prone to oxidative stress (OS) status due to they have a long high temperature period (Salles et al., 2010).

A recent study has already shown that supplementation of antioxidants in ruminant diet could be alleviated OS status (Rizzo et al., 2013). Moreover, additional literatures have been reported to the anthocyanins have a stronger antioxidant capacity than other antioxidants (Rice-evans et al., 1995; Bagchi et al., 1997). Furthermore, anthocyanins had the ability to modulate related antioxidant mRNA gene expression (Elliott et al., 1992). For example, Han et al. (2006) indicated that the inclusion of anthocyanin-rich purple potato flake extract in rats had the strong activity of scavenging FR and showed higher levels of hepatic superoxide dismutase (SOD) and glutathione peroxidase (GPX) mRNA expression.

Purple corn has a larger amount of anthocyanins compare to other materials source, which is a kind of field crop vastly grown in Thailand (Phinjaturus et al., 2016). Our previous work has shown that anthocyanin-rich purple corn stover silage (PSS) had an abundant of anthocyanin composition and displayed a stronger 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity relative to the sticky corn stover silage (SSS), making it become one of the most exciting new super roughage for ruminants. To date, information about anthocyanins to prevent OS status in dairy goats is scarce. Accordingly, the present study was to investigate the effect of anthocyanin-rich PSS on plasma antioxidant capacity, and mRNA abundance of the related to inflammatory and antioxidant genes in the mammary gland of Saanen dairy goats.

4.3 Materials and Methods

4.3.1 Animal, diets and experimental design

The experiment was conducted at Suranaree University of Technology (SUT) farm, Nakhon Ratchasima, Thailand (14°53'37.9"N, 102°01'22.0"E). Eight

healthy multiparous Saanen dairy goats (body weight (BW), 41.50 ± 1.84 kg; mean ± standard deviation (SD)) were assigned to a double 4×4 Latin square design. Periods were 21-d in length with 19-d for adapting goats to diets and 2-d for sampling. The four treatments were: (1) negative control, fed rice straw (RSNC); (2) positive control 1, fed SSS (SSPC); (3) goats fed anthocyanin-rich PSS (APSS); and (4) positive control 2, fed SSPC with 1 g/d commercial purple corn pigment (SSPP). Purple corn pigment is the commercial product (Nanjing Herd Source Bio-technology Co., Ltd., Nanjing, China). 1 g/d purple corn pigment was used in this study according to Hosoda et al. (2012b). The goats were housed in clean individual pens with free access to water (H_2O) and were fed diet with a ratio of roughage: concentrate at 50:50. Ration was offered in equal amounts twice daily at 08:00 and 17:00 for ad libitum intake. The diets (Table 4.1) were formulated to meet requirements for dairy goat having 40 kg of BW according to the NRC (1981). Moreover, nutrient composition and anthocyanin composition in two types of corn stover silage was shown in Table 4.2.

Table 4.1 Ingredient and nutrient composition of experimental diets.

Item	Treatment			
	RSNC[1]	**SSPC**	**APSS**	**SSPP**
Ingredient (% DM)				
Rice straw	50.0			
Sticky corn silage		50.0		50.0
Purple corn silage			50.0	
Soybean meal	9.5	7.7	7.2	7.7
Rice bran	10.0	10.0	10.0	10.0
Cassava chip	10.9	24.2	24.7	24.2
Corn	15.0	6.5	6.5	6.5
Salt	0.4	0.4	0.4	0.4
Limestone	0.2	0.2	0.2	0.2
Vitamin premix[2]	0.5	0.5	0.5	0.5
Mineral permix[3]	0.5	0.5	0.5	0.5
Palm olein	3.0			
Total	100.0	100.0	100.0	100.0
Purple corn pigment (g/d)[4]				1.0
Chemical composition				
DM (%)[5]	93.84	58.79	57.93	58.74
OM (% DM)	90.08	92.74	93.20	92.84
CP (% DM)	10.06	10.07	10.06	10.10
GE (kJ/g DM)	16803.05	16739.33	16879.16	16795.85
NDF (% DM)	47.44	43.23	43.37	43.37
ADF (% DM)	27.64	25.26	24.51	25.16
Hemicellulose (% DM)	19.79	17.97	18.86	18.21
Ash (% DM)	9.92	7.26	6.80	7.16

[1] RSNC = negative control of goats fed rice straw; SSPC = positive control of goats fed sticky corn stover silage; APSS = goats fed anthocyanin-rich purple corn stover silage; SSPP = positive control of goats fed sticky corn stover silage with 1 g/d commercial purple corn pigment.

[2] Vitamin premix contained (per kg of premix): vitamin A 12000000 IU; vitamin D_3 2400000; vitamin E 750 mg; vitamin B_1 980 mg; vitamin B_2 960 mg; vitamin B_6 654 mg; vitamin B_{12} 1658 µg; vitamin B_9 133 mg; calcium pantothenate 2940 mg; nicotinamide 8910 mg; K 637 mg; vitamin B_4 446 g; Na 289.4 g; citrate 850.5 mg.

[3] Mineral premix contained (per kg of premix): Fe 12 g; Cu 2 g; Mn 7 g; Mg 5 g; Zn 15 g; Co 2 g; I 2 g; Ca 195.27 g; P 144.33 g.

[4] Purchased from Nanjing Herd Source Bio-technology Co., Ltd., Nanjing, China.

[5] DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Table 4.2 Nutrient composition and anthocyanin composition in two types of corn stover silage.

Item[1]	Type of corn stover silage[2]	
	SSS	**PSS**
Nutrient composition		
DM (%)	23.16	21.58
OM (% DM)	92.94	93.81
CP (% DM)	6.56	7.11
GE (kJ/g DM)	16949.06	17044.35
NDF (% DM)	62.42	61.27
ADF (% DM)	39.36	38.12
Hemicellulose (% DM)	23.06	23.16
Ash (% DM)	7.06	6.19
Anthocyanin composition		
C3G (mg/100g DM)	3.06	2.92
Del (mg/100g DM)	8.36	8.82
P3G (mg/100g DM)	-	-
Peo (mg/100g DM)	3.27	4.62
M3G (mg/100g DM)	3.85	7.14
Cya (mg/100g DM)	11.25	14.01
Pel (mg/100g DM)	9.01	10.17
Mal (mg/100g DM)	-	39.77
Total (mg/100g DM)	38.80	87.46

[1] DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy; NDF = neutral detergent fiber; ADF = acid detergent fiber; C3G = cyanidin-3-glucoside; Del = delphinidin; P3G = pelargonidin-3-glucoside; Peo = peonidin; M3G = malvidin-3-O-glucoside; Cya = cyanidin; Pel = pelargonidin; Mal = malvidin; - = not detected.
[2] SSS = sticky corn stover silage; PSS = anthocyanin-rich purple corn stover silage.

4.3.2 Sample collection

Data were obtained for continuously monitored DMI in each day. Feed ingredients (approximately 100 g) were sampled weekly and composited by period. At the conclusion of each period, feed ingredients and feed refusal composites were dried in the oven at 65°C for 72 h, and then ground and passed through a 1-mm sieve and kept at 4°C until further analysis.

Fecal (around 100 g) and urine (approximately 30 mL) samples were collected on days 15 through 21 (2 days of adaptation period and 5 days of metabolic trial period). At the conclusion of each period, fecal samples were dried in the oven at 65°C for 72 h and ground to pass through a 1-mm sieve after grinding and kept 4°C until further analysis. Fecal and urine samples were also composited by each period. Daily collection of urine of each animal was acidified with 20% sulphuric acid (H_2SO_4) to keep the pH < 3, and then stored at -20°C until the analysis of chemical composition.

On day 20 of each period, blood (6 mL) and ruminal fluid (around 30 mL) samples were collected at 0, 2, and 4 h from jugular vein by vacuette® tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) with K_3-EDTA and by a vacuum pump through the mouth, respectively. For blood, after centrifugation at 4000 r/min for 15 min at 4°C (Sorvall™ Legend™ XT/XF Centrifuge Series, Thermo Fisher Scientific Pte Ltd., Waltham, USA), the plasma was transferred to 1.5 ml tubes and stored at -80°C until further analysis. Alternatively, ruminal fluid pH value was measured immediately using a portable pH meter, and then its portion was passed through 4 layers of cheesecloth to remove feed particles. Meanwhile, 20 mL ruminal fluid sample and 5

mL HCl (6 mol/L) were stored in a container after mixing together, and then moved to -20°C until analysis the rumen fermentation parameters.

Mammary gland tissue biopsies were conducted on day 21 of each period by the methods of Farr et al. (1996) and Zhang et al. (2015). The goat was administered with a subcutaneous injection of a Penomycin (Procaine and Sodium penicillins with Streptomycin; Manufacturing Co., Ltd., Sukhumvit Road, Samutprakam) and the sample was taken using a semi-automatic biopsy needle (16 G × 90 mm, SAG-16090, TSK Corporation, Japan). The sample was immediately transferred to the 1.5 mL tube and snap frozen in liquid nitrogen and stored at -80°C until the assay was performed.

4.3.3 Chemical analyses

Dry matter (DM), crude protein (CP)/nitrogen (N) and ash were analyzed according to the feed proximate analysis of the Association of Official Analytical Chemists (AOAC, 2005). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured by the method of Van Soest et al. (1991). Organic matter (OM) and hemicellulose were calculated using the following equations, respectively: OM = 100-ash; hemicellulose = NDF-ADF. Gross energy (GE) was analyzed using a Parr 6200 calorimeter (Moline, Illinois, USA). The anthocyanin composition in the silage extract was analyzed by a high performance liquid chromatography (HPLC; 1260 Infinity II LC, Agilent Technologies, Santa Clara, CA, USA) according to Hosoda et al. (2009).

4.3.4 Rumen fermentation parameters

The ammonia nitrogen (NH_3-N) was assayed as per the method of Bremner and Keeney (1965). Acetic acid (AA), propionic acid (PA), and butyric acid

(BA) were determined using the HPLC. Total volatile fatty acid (TVFA) = AA + PA + BA.

4.3.5 Analysis of antioxidant activity enzymes in plasma

The DPPH scavenging activity in the plasma was assayed spectrophotometrically according to Wei and Chiang (2009), using a stable free radical DPPH (Sigma-Aldrich, Pcode: 101845869) with a slight modification. Briefly, 50 µL of each sample was added to 1 mL of methanolic solution of DPPH reagent (25 µmol/L) in a 1.5 mL tube. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min, and then centrifuged at 4000 r/min for 10 min at 4°C. 200 µL of the supernatant was transferred to a 96-well plate immediately and the absorbance was detected at 517 nm via a microplate reader (Epoch, BioTek, Luzern, Switzerland). DPPH scavenging activity was calculated by the following formula: DPPH scavenging activity = (Ac-As) × 100/Ac, where Ac is the absorbance of the control, and As is the absorbance of the sample.

The TAC in the plasma was determined using a commercially available kit (Catalog Number MAK187-1KT, Sigma-Aldrich, Product of USA), according to manufacturers' protocol. In the kit, either the concentration of the combination of both small molecule and protein antioxidants, or the concentration of only small molecule antioxidants can be determined. Cu^{2+} ion is converted to Cu^{+} by both small molecules and proteins. However, the use of the Protein Mask prevents Cu^{2+} reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu^{+} ion chelates with a colorimetric probe, giving a broad absorbance peak at 570 nm, which is proportional to the total antioxidant capacity. The kit gives antioxidant capacity in

Trolox (a water-soluble vitamin E analog, serves as an antioxidant standard) equivalents (ranging from 4-20 nmole/well).

The activity of SOD enzyme was measured according to a commercial assay kit (PCode 101869510, 19160-1KT-F, Sigma-Aldrich, Product of Japan). The Kit-WST allows very convenient SOD assaying by utilizing a water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with reactive oxygen species ($O_2^{\cdot-}$) are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Absorbance was measured at 440 nm using a microplate reader (Epoch, BioTek, Luzern, Switzerland). Calculation the SOD activity (inhibition rate %) was done using the following equation. SOD activity (inhibition rate %) = {[(Ablank 1 – Ablank 3) – (Asample – Ablank 2)]/ (Ablank 1 – Ablank 3)} × 100.

Glutathione Peroxidase (GPX) enzyme activity was determined by an indirect method, using the Sigma Glutathione Peroxidase Cellular Activity Assay kit (Catalog number: CGP1-1KT, Sigma-Aldrich, Product of Israel). The kit is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced (NADPH). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPX activity, since GPX is the rate limiting factor of the coupled reactions. The GPX positive control was purchased glutathione peroxidase from erythrocytes (Catalog number: G6137-100UN, Sigma-Aldrich, Product of USA). 1 unit of GPX will cause the formation of 1.0 µmol of

$NADP^+$ from NADPH per minute at pH 8.0 at 25°C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide.

The catalase (CAT) enzyme activity was determined by a colorimetric assay, using a commercial kit (Pcode 1002565186, CAT100-1KT, Sigma-Aldrich, Product of Israel). This assay method is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalatic pathway) and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining by a colorimetric method. The colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) that absorbs at 520 nm. One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25°C at a substrate concentration of 50 mM hydrogen peroxide.

4.3.6 Analysis of mammary gland tissue gene expression

Total RNA was extracted from the mammary gland using TRIzol Reagent method according to Labrecque et al. (2009), with a minor modification. Briefly, the sample was transferred into 2 mL tube, and added 1 mL TRIzol Reagent (Thermo Fisher Scientific, USA) and 0.1 g of the 0.5 mm dia. Zirconia/silica (Cat. No. 11079105z, BioSpec Products) and run in a homogenizer. The total RNA was dissolved with DEPC water (Ambion by Life Technologies, made in USA) and was DNase digested the RNase-Free DNase I. After that, purity of the extracted RNA was analyzed by assaying the 260/280 absorbance ratio, using a NanoVue

spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, United States). Integrity of the extracted RNA was assessed by verifying the presence of 18S and 28S RNA bands, using an ImageQuant LAS 500 imager (GE Healthcare Bio-Sciences, Pittsburgh, United States) electrophoresis on a 1% agarose gel. Complementary DNA (cDNA) synthesis was performed as described by ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, USA).

All samples were assayed using quantitative real-time Polymerase Chain Reaction (real-time PCR) amplification by a Roche LightCycler® 480 real-time PCR System (Roche Diagnostics GmbH, Penzberg, Germany). Nine target genes (TG) were: nuclear factor, erythroid 2 like 2 (NFE2L2), heat shock transcription factor 1 (HSF1), heat shock 70kDa protein 1A (hspA1A), tumor necrosis factor (TNF), SOD2, SOD3, GPX1, GPX2, and CAT; and the housekeeper gene (HG): glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Nucleotide sequences obtained from the National Center for Biotechnology Information (NCBI) and the primers set for all genes were designed using GenScript real-time PCR (TaqMan) Primer Design tool and were synthesized by the Bio Basic Inc. Company (Table 4.3). The real-time PCR amplifications were performed 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 (500 nM) and reverse (500 nM) primers, respectively. After that, the plates (LightCycler® 480 multiwell plate 96, white; Roche Diagnostics GmbH, Mannheim, Germany) were centrifuged at 4°C, 1500 rpm for 3 min (Universal 320, Hettich Zentrifugen, Germany). Cycling conditions were 10 min at 95°C for pre-incubation, followed by forty cycles of 30 s at 95°C for amplification, and 55°C to 57.5°C for 1 min (annealing

temperature optimized depending on primers), and cooling at 40°C for 30 s. Amplifications were performed in triplicate for each gene.

Table 4.3 Primer sequences used for real-time PCR amplifications in this study.

Gene[1]	Primer sequences (5' –3')[2]	Accession number	Product size (nt)	Annealing temperature (°C)
NFE2L2	(F) GGCCCATTGACCTCTCTGAT	NM_001314327.1	131	57
	(R) TGATGCCATGCTTGGACTTG			
HSF1	(F) GCCATGAAGCATGAGAACGA	NM_001314344.2	111	55.5
	(R) CAGCGAGATGAGGAACTGGA			
hspA1A	(F) GGACATCAGCCAGAACAAGC	JN656104.1	137	57
	(R) TGGACGTGTTGAAGTCGATG			
TNF	(F) CGTATGCCAATGCCCTCAAG	NM_001286442.1	82	57
	(R) ATGAGGTAAAGCCCGTCAGT			
SOD2	(F) CTGTTGGTGTCCAAGGTTCC	XM_018053428.1	153	56
	(R) AATAAGCATGCTCCCACACG			
SOD3	(F) ATCCACGTGCACCAGTTTG	NM_001285675.1	74	56
	(R) ACGGACATCGGGTTGTAGTG			
GPX1	(F) CCTGGTGGTACTCGGCTT	XM_005695962.3	91	57.5
	(R) TCGGACGTACTTCAGGCAAT			
GPX2	(F) CCGCACCTTCCAAACCATTA	XM_005685982.3	103	55.5
	(R) AAAGACACTCAGGGCAGGAG			
CAT	(F) CCTGTTCAGTGATCGAGGGA	XM_005690077.3	97	57
	(R) AACTGCCTCTCCATTGGCAT			
GAPDH	(F) CACAGTCAAGGCAGAGAACG	XM_005680968.3	109	56
	(R) GTACTCAGCACCAGCATCAC			

[1] NFE2L2 = nuclear factor, erythroid 2 like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70kDa protein 1A; TNF = tumor necrosis factor; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

[2] F = forward; R = reverse.

4.4 Statistical analysis

All results were analyzed as a 4 × 4 double Latin square design using the Analysis of Variance (ANOVA) procedure of Statistical Analysis System 9.1.3 (SAS Inst. Inc., Cary, NC). The relative mRNA abundance was calculated as $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = (Ct_{\text{target gene unknown sample}} - Ct_{\text{GAPDH unknown sample}}) - (Ct_{\text{target gene calibrator sample}} - Ct_{\text{GAPDH calibrator sample}})$. Averaged abundance of the gene in RSNC data was considered as the calibrator, and the data was analyzed by ANOVA procedure. Pearson correlation coefficients (r) were analyzed the relationship between antioxidants in plasma and the genes expression of inflammatory and antioxidant in mammary gland. Differences were considered statistically significant at $P<0.05$.

4.5 Discussion

4.5.1 DMI and digestibility

There was no significant difference ($P>0.05$) in the DMI (g/d), BW (%), and g/kg $BW^{0.75}$ (%) for four treatments in all experimental periods (Table 4.4). However, the apparent digestibility of DM, OM, CP, GE, NDF, and ADF in the goats receiving the SSPC, APSS, and SSPP were significantly higher ($P<0.05$) than that in the RSNC goats.

No significant differences ($P>0.05$) were observed in the N intake and N excretion from urine among the treatments (Table 4.5). N excretion from feces and total N excretion of the RSNC were significantly higher ($P<0.05$) than that of the SSPC and APSS treatments. In contrast, the N absorption and N retention in the RSNC were significantly lower ($P<0.05$) than those in the other treatments.

Table 4.4 DMI and apparent digestibility of dairy goats in different treatments.

Item[1]	Treatment[2]				SEM	P-value
	RSNC	SSPC	APSS	SSPP		
DMI (g/d)	908	933	941	931	12.9796	0.4231
BW (%)	2.19	2.25	2.27	2.24	0.0313	0.4428
g/kg $BW^{0.75}$ (%)	55.54	57.07	57.51	56.92	0.7946	0.4428
Apparent digestibility (%)						
DM	6.80[b]	63.92[a]	66.90[a]	64.78[a]	1.7657	0.0065
OM	1.19[b]	67.14[a]	9.85[a]	68.16[a]	1.5208	0.0066
CP	47.71[b]	59.65[a]	57.84[a]	56.32[a]	1.9374	0.0363
GE	59.20[b]	64.43[a]	67.43[a]	65.53[a]	1.5391	0.0201
NDF	32.45[b]	44.28[a]	43.35[a]	45.95[a]	3.2529	0.0418
ADF	31.95[b]	43.69[a]	42.58[a]	45.14[a]	2.4924	0.0207

Values represented the means of 8 replicates (n = 8).

Means with different letters within a row are significantly different (P<0.05).

[1] BW = body weight; DMI = dry matter intake; DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy; NDF = neutral detergent fiber; ADF = acid detergent fiber. SEM = standard error of the mean.

[2] RSNC = negative control of goats fed rice straw; SSPC = positive control of goats fed sticky corn stover silage; APSS = goats fed anthocyanin-rich purple corn stover silage; SSPP = positive control of goats fed sticky corn stover silage with 1g/d purple corn pigment. The same as below.

Table 4.5 Nitrogen utilization of dairy goats in different treatments.

Item	Treatment				SEM	P-value
	RSNC	SSPC	APSS	SSPP		
N intake (g/d)	14.62	15.04	15.15	15.04	0.2092	0.4034
N excretion from feces (g/d)	7.65[a]	6.07[b]	6.39[b]	6.57[ab]	0.2922	0.0464
N excretion from urine (g/d)	5.22	4.35	4.09	4.31	0.3263	0.2466
Total N excretion (g/d)	12.87[a]	10.42[b]	10.48[b]	10.88[b]	0.3125	0.0036
N absorption (g/d)	6.97[b]	8.97[a]	8.76[a]	8.47[a]	0.2922	0.0213
N absorption (%)	47.71[b]	59.65[a]	57.84[a]	56.32[a]	1.9374	0.0363
N retention (g/d)	1.75[b]	4.62[a]	4.67[a]	4.16[a]	0.3087	0.0009
N retention (%)	11.95[b]	30.72[a]	30.85[a]	27.67[a]	2.1708	0.0011

Values represented the means of 8 replicates (n = 8).

[2] RSNC = negative control of goats fed rice straw; SSPC = positive control of goats fed sticky corn stover silage; APSS = goats fed anthocyanin-rich purple corn stover silage; SSPP = positive control of goats fed sticky corn stover silage with 1g/d purple corn pigment. The same as below.

4.5.2 Rumen fermentation parameters

Treatments had no effect (P>0.05) on the pH, NH_3-N and AA values (Table 4.6). Goats receiving RSNC showed lower (P<0.05) levels of PA, BA, and TVFA than the other treatments. For VFA (% Molar), RSNC treatment displayed lower (P<0.05) levels of PA and BA, whereas it reduced (P<0.05) AA and ratio of AA to PA compared to the other groups.

Table 4.6 Rumen fermentation parameters of dairy goats in different treatments.

Item[1]	**Treatment**				**SEM**	**P-value**
	RSNC	**SSPC**	**APSS**	**SSPP**		
pH	6.82	6.88	6.80	6.78	0.0584	0.7056
NH_3-N (mg/dL)	11.94	12.33	11.65	12.20	0.7302	0.9277
AA (mmol/L)	40.13	40.87	41.40	42.35	1.5851	0.7912
PA (mmol/L)	14.44^{b}	16.99^{a}	17.74^{a}	19.34^{a}	0.8640	0.0039
BA (mmol/L)	5.11^{b}	8.64^{a}	8.52^{a}	9.18^{a}	0.4030	<0.0001
TVFA (mmol/L)	59.68^{b}	66.50^{ab}	67.66^{a}	70.87^{a}	2.5167	0.0283
VFA (% Molar)						
AA	67.21^{a}	61.60^{b}	61.18^{b}	59.71^{b}	0.8132	<0.0001
PA	24.22^{b}	25.50^{ab}	26.23^{ab}	27.27^{a}	0.7536	0.0527
BA	8.57^{b}	12.89^{a}	12.59^{a}	13.02^{a}	0.3613	<0.0001
Ratio of AA to PA	2.90^{a}	2.50^{b}	2.50^{b}	2.28^{b}	0.1241	0.0126

Values represented the means of 8 replicates (n = 8).

[1] NH_3-N = ammonia nitrogen; AA = acetic acid; PA = propionic acid; BA = butyric acid; TVFA = total volatile fatty acid.

4.5.3 Antioxidant activity in plasma

As shown in Table 4.7. There was no effect (P>0.05) of dietary treatments on the activity of plasma TAC, GPX, and CAT, whereas goats receiving APSS and SSPP led to a significant elevation (P<0.05) for the activity of DPPH scavenging activity and SOD in plasma.

Table 4.2 Antioxidant activity in plasma of dairy goats in different treatments.

Item[1]	Treatment				SEM	P-value
	RSNC	SSPC	APSS	SSPP		
DPPH scavenging activity (%)	19.92[b]	22.90[ab]	24.88[a]	24.81[a]	1.0340	0.0069
TAC (nmole/µl)	37.32	37.62	40.11	38.95	0.8399	0.1303
SOD (inhibition rate %)	87.12[b]	87.08[b]	90.48[a]	90.61[a]	0.9254	0.0094
GPX (mmol/min/ml)	35.02	34.84	35.61	36.04	0.4865	0.3264
CAT (µmoles/min/ml)	63.00	66.30	67.27	66.84	2.6556	0.6863

Values represented the means of 8 replicates (n = 8).

[1] DPPH = 2, 2-diphenyl-1-picrylhydrazyl; TAC = total antioxidant capacity; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase.

4.5.4 Relative mRNA abundance of inflammatory genes

The abundance of NFE2L2 mRNA was down-regulated (P<0.05) gene in the mammary gland of goats receiving RSNC relative to the other treatments (Figure 4.1). Goats fed the SSPC diet had higher (P<0.05) levels of HSF1, hspA1A, and TNF mRNA abundance in the mammary gland compared with those fed the RSNC and SSPP treatments. There was no difference (P>0.05) in the HSF1, hspA1A, and TNF mRNA abundance of goats receiving APSS relative to the RSNC.

Furthermore, the addition of SSPP in goats decreased (P<0.05) mammary gland TNF mRNA expression when compared with the RSNC treatment.

4.5.5 Relative mRNA abundance of antioxidant genes

As shown in Figure 4.2. Goats receiving SSPC displayed a higher (P<0.05) level of GPX1 mRNA abundance in the mammary gland compared to the RSNC treatment. APSS treatment showed the higher (P<0.05) levels of the mRNA abundance of SOD2, GPX1, and GPX2. GPX1 mRNA abundance was up-regulated (P<0.05) in the mammary gland of goats fed SSPP than of goats fed RSNC. Of interest, no differences (P>0.05) were observed for SOD3 and CAT mRNA abundance among all treatments.

4.5.6 Pearson correlation coefficients

Significant (P<0.05) positive correlations were observed between DPPH scavenging activity, TAC, SOD, and CAT enzymes in plasma and NFE2L2 (Table 4.6). There were significant (P<0.05) negative correlations between TAC, SOD, and GPX enzymes and the abundances of hspA1A and TNF. Moreover, significant (P<0.05) positive correlations were detected between several antioxidant enzymes (DPPH scavenging activity, TAC, CAT) and the mRNA expression of some antioxidant genes (SOD2, GPX1).

As shown in Table 4.7. There were significant (P<0.05) positive correlations between some inflammatory genes expression and several antioxidant genes expression, such as, NFE2L2 and SOD2, GPX1; HSF1 and SOD3, GPX1; hspA1A and GPX1, CAT; and TNF and CAT.

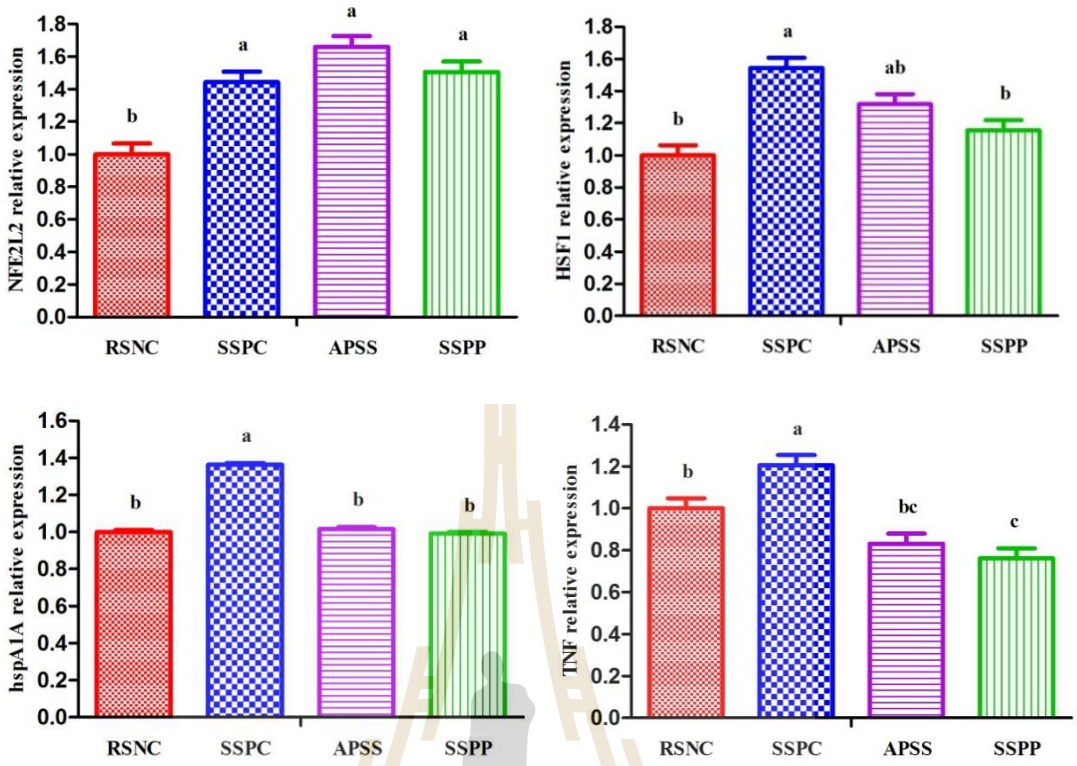

Figure 4.1 Relative mRNA abundance of inflammatory genes in mammary gland tissue of dairy goats in different treatments. RSNC = negative control of goats fed rice straw; SSPC = positive control of goats fed sticky corn stover silage; APSS = goats fed anthocyanin-rich purple corn stover silage; SSPP = positive control of goats fed sticky corn stover silage with 1g/d purple corn pigment. Data reported as least-squares means ± SEM. Values represented the means of 8 replicates (n = 8). Relative quantification (RQ) of mRNA abundance for each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method with the RSNC treatment as reference expression point. NFE2L2 = nuclear factor, erythroid 2 like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70kDa protein 1A; TNF = tumor necrosis factor. $^{a-c}$ means different letters within a row are significantly different ($P<0.05$)

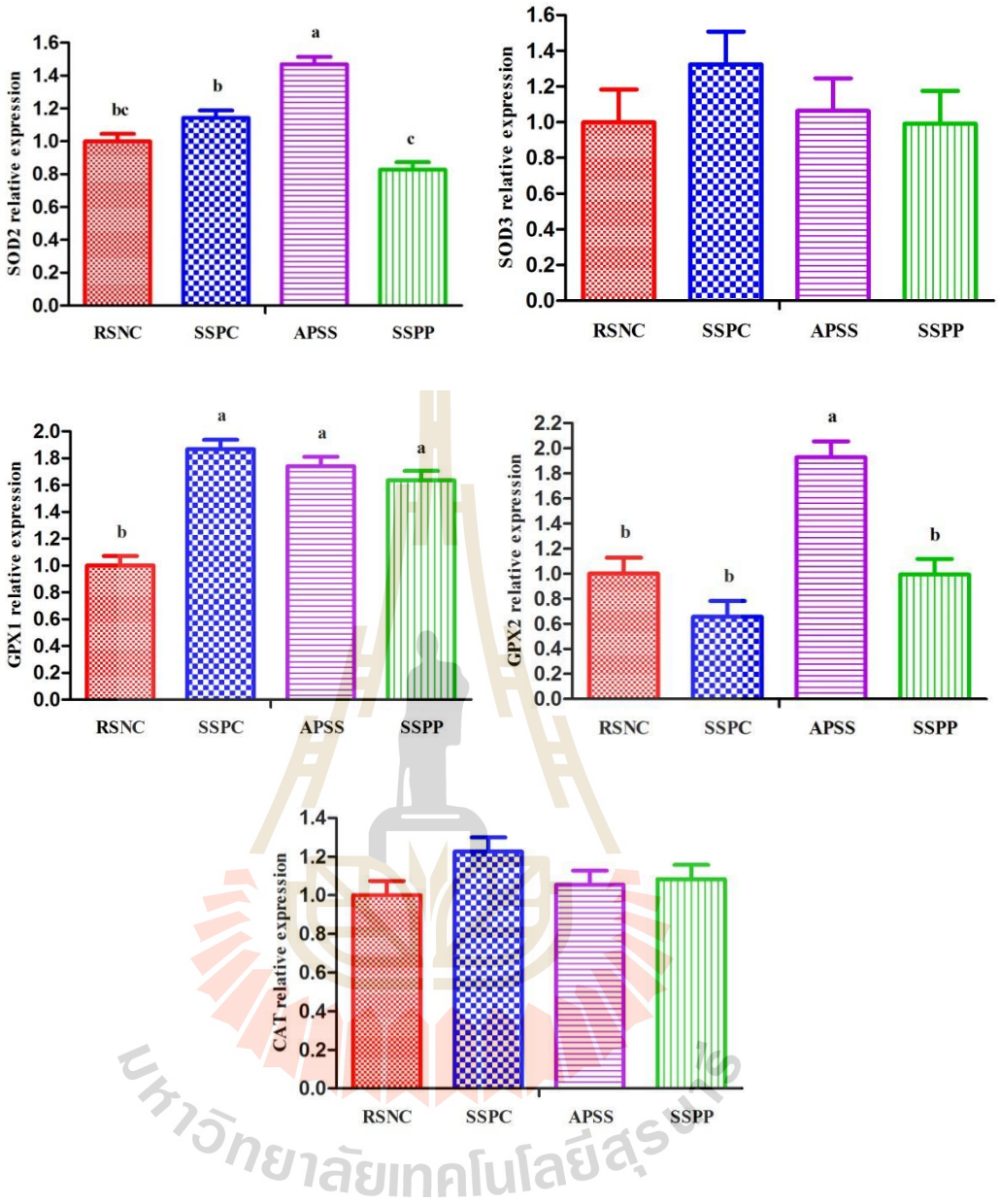

Figure 4.2 Relative mRNA abundance of antioxidant genes in mammary gland tissue of dairy goats ***in different treatments***. RSNC = negative control of goats fed rice straw; SSPC = positive control of goats fed sticky corn stover silage; APSS = goats fed anthocyanin-rich purple corn stover silage; SSPP = positive control of goats fed sticky corn stover silage with 1g/d purple corn pigment. Data reported as least-squares means ± SEM. Values represented the means of 8 replicates (n = 8). Relative

quantification (RQ) of mRNA abundance for each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method with the RSNC treatment as reference expression point. SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase. $^{a-c}$ means different letters within a row are significantly different ($P<0.05$).

Table 4.8 Pearson correlation coefficients between antioxidant enzymes in plasma and genes expression in mammary gland.

Item		NFE2L2	HSF1	hspA1A	TNF	SOD2	SOD3	GPX1	GPX2	CAT
DPPH	r	0.917	0.303	-0.093	-0.510	0.208	0.011	0.692	0.433	0.157
scavenging	P	<0.0001	0.292	0.764	0.090	0.422	0.972	0.003	0.159	0.608
activity										
TAC	r	0.785	0.073	-0.436	-0.694	0.503	-0.160	0.338	0.829	-0.168
	P	0.003	0.803	0.137	0.012	0.040	0.619	0.201	0.001	0.583
SOD	r	0.690	-0.139	-0.592	-0.853	0.096	-0.262	0.227	0.636	-0.221
	P	0.013	0.635	0.033	0.0004	0.714	0.411	0.397	0.026	0.468
GPX	r	0.509	-0.300	-0.668	-0.891	-0.223	-0.329	0.069	0.430	-0.265
	P	0.091	0.297	0.013	<0.0001	0.389	0.297	0.800	0.163	0.318
CAT	r	0.940	0.489	0.134	-0.314	0.310	0.128	0.826	0.354	0.285
	P	<0.0001	0.076	0.664	0.320	0.227	0.691	<0.0001	0.259	0.345

Table 4.9 Pearson correlation coefficients between inflammatory and antioxidant genes expression.

Item		SOD2	SOD3	GPX1	GPX2	CAT
NFE2L2	r	0.450	–0.089	0.760	0.484	0.142
	P	0.040	0.783	0.001	0.111	0.643
HSF1	r	0.362	0.588	0.698	–0.032	0.253
	P	0.154	0.044	0.003	0.922	0.404
hspA1A	r	0.119	0.491	0.524	–0.541	0.619
	P	0.649	0.105	0.037	0.069	0.024
TNF	r	0.084	0.486	0.202	–0.586	0.607
	P	0.749	0.109	0.454	0.055	0.028

4.6 Discussion

4.6.1 DMI and digestibility

In the present experiment, we found that although anthocyanin-rich PSS displayed a higher level of total anthocyanins relative to the SSS, goats consumed the feed in all treatments did not affect DMI, indicating that the feeding of the anthocyanins did not led to lower palatability for goats. It was likely that although anthocyanins had the potential to effect on the palatability, they had the ability to improve antioxidant activity, thereby without decreasing DMI. In addition, it is well known that the feed nutritional value usually dependents on its digestive characteristics and nutrient composition, especially for the CP and fiber fractions. In the current report, goats receiving APSS or SSPP showed remarkably higher apparent digestibility in all nutrients, better nitrogen balance and energy balance relative to the

negative control. However, Hosoda et al. (2012b) who demonstrated that apparent nutrient digestibilities were significantly lower in the purple rice silage treatment than in control groups due to purple rice silage had higher levels of fiber fractions in comparison to the control rice silage. This discrepancy may be explained that both the anthocyanin-rich PSS and SSS had the similar levels of chemical composition except the OM and ash content in this study. Alternatively, all treatments were equal in CP, thus fiber fractions were the major important factor affecting on the digestibility in this study. Rice straw had a high level of fiber and was difficult degraded by rumen microorganism, resulting in the lower level of digestibility in RSNC treatment compared to the other treatments.

4.6.2 Rumen fermentation parameters

Ruminal fluid pH value is too low (< 5.6) or too high (> 7.3) should be impacted on bacterial growth in ruminants (Keunen et al. 2002). In addition, ruminal fluid concentration of the NH_3-N is generally affected by CP content in the diet (Hristov et al. 2004). In the current study, no significant difference was observed for the pH value and NH_3-N concentration among the treatment. This was due to all diets were calculated to be the isonitrogenous and isoenergetic, thereby resulting in the pH and NH_3-N content did not differ. This result was according with Hosoda et al. (2012a), who demonstrated that lactating dairy cows receiving higher level CP of anthocyanin-rich corn silages did not effect on the ruminal fluid pH value, but showed a higher level of NH_3-N concentration compared with the control silage group. Our previous study showed that anthocyanins might be able to affect carbohydrate metabolism to provide more energy for ruminants by inhibiting AA production, increasing the proportion of PA. The results of the current study showed that the

addition of APSS or SSPP of the dairy goats showed higher levels of PA, BA, and TVFA, whereas they had lower ratio of AA to PA compared to the negative control, perhaps because RSNC treatment diet had high level of fiber so that it was difficult degrade in the rumen, thereby showing lower level of VFA production. Of interest, no significant difference was observed during SSPC, APSS, and, SSPP treatments in this study. This might be because although anthocyanin-rich PSS showed higher level of anthocyanins, they seemed to be not broken down in the rumen; in addition, two types of silage had the similar chemical composition, thereby resulting in did not affect the VFA contents. Additionally, the type of VFA formed in the rumen depends on the type of substrate fermented, rumen environment, and microbial population (Messana et al., 2013). The formation of AA is always accompanied by H_2 and CO_2 production, whereas PA production requires H_2 to be formed as PA (Luthfi et al., 2018). Thus the fermentation of cell wall fiber increased AA : PA ratio in the RSNC treatment. Paengkoum (2010) had a similar result, who indicated that there was no significant difference in the VFA proportions and AA to PA ratio when Thai native×Anglo-Nubian goats fed whole-plant corn silage as roughage diets. One study also did show that lactating dairy cows receiving the anthocyanin-rich corn silage did not affected ruminal fluid VFA production when compared with those fed control silage (Hosoda et al. 2012a).

4.6.3 Antioxidant activity in plasma

Literature has been reported to anthocyanins can be absorbed intact into blood (McGhie et al., 2003; Passamonti et al., 2003). Moreover, anthocyanins have been shown a strong antioxidant activity by providing electrons to excessive FRs (Dixon et al., 2005). DPPH is a kind of FR, which is reduced in aqueous solution

containing antioxidant molecule (Mensor et al., 2001). Thus, APSS and SSPP had rich in radical scavengers, anthocyanins in plasma have the ability to provide electrons to the DPPH, thereby increasing the level of DPPH scavenging activity. As discussed elegantly by Toaldo et al. (2016), who demonstrated that anthocyanin-rich grape juices can be increased blood antioxidant capacity in humans.

Superoxide anion radical ($O_2^{\cdot-}$) is generated during normal metabolism by the activity of the NADPH oxidase, it is converted in hydrogen peroxide (H_2O_2) by SOD enzyme, followed by converted in H_2O via GPX and CAT enzymes (Simkó, 2007). Thus, these enzymes can neutralize excessive FRs before they react with metal catalysts to form more reactive compounds. However, the balance might be disrupted when animals under the OS conditions. Roberts et al. (2006) showed that the rats under OS status is accompanied by up-regulation of NAD(P)H oxidase, pointing to increased ROS production capacity, and down-regulation the key enzymes in the antioxidant defense system. APSS and SSPP can significant increase plasma SOD activity, may suggesting that anthocyanins were absorbed into blood, resulting in providing electrons to the $O_2^{\cdot-}$, thereby alleviating the OS condition. Moreover, anthocyanin-rich plants might be caused elevations in messenger RNA expression, and resulting in effect of the activity of SOD in the blood (Yang et al., 2010). In line with the present finding, a significant increase SOD in the plasma has been reported of lactating dairy cows fed anthocyanin-rich purple corn silage relative to the control (Hosoda et al., 2012a). Of interest, goat receiving APSS and SSPP were unaffected on the activity of GPX and CAT in plasma, perhaps because higher level of SOD activity in plasma alleviated the burden of antioxidant defenses system, thereby other enzymes did not differ (Mittler, 2002).

4.6.4 Inflammatory gene expression

Anthocyanins have been reported to modulate inflammatory pathway by inhibiting ROS and nuclear factor-kappa B (NF-κB) transcription factor (Côrtes et al., 2006; Speciale et al., 2014). The NFE2L2 gene, also known as Nrf2, which is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins and protect against oxidative damage triggered (Kaspar et al., 2009). Indeed, the inducers can be led to the release of Nrf2 and allowed its nuclear translocation, activating the expression of a battery of cytoprotective genes (Espinosa-Diez et al., 2015). In this study, mammary gland NFE2L2 mRNA expression increased when supplementation of APSS and SSPP. The possible reason was that anthocyanins have the potential to inherent radical scavenging activity, preventing OS damage through inducing Nrf2 signaling pathways, a transcription factor implicated in the expression of several antioxidant/detoxificant enzymes (Martín et al., 2010). Indeed, anthocyanins as a source of Nrf2 activating agent, which can directly modify sensor cysteines present in Kelch-like ECH protein (Keap1), leading to Nrf2 proteins translocate into the nucleus and bind to the antioxidant response element, thereby modulating the expression of Nrf2 target genes that have anti-inflammatory and antioxidant functions (Aboonabi and Singh, 2015). These results concur with Kruger et al. (2014) who suggested that anthocyanins can be activated the expression of Nrf2, resulting in increasing the expressions of inflammatory and antioxidant genes.

HSF1 regulates the heat shock response (HSR) pathway in animals by acting as the major transcription factor for heat shock proteins (HSPs) (Calderwood et al., 2009). As a result, animals elicited counterattack mechanisms, which effected on the transcriptional pathways that activated chaperones for refolding misfolded proteins of

the HSPs of the HSF1 cascade (Akhtar et al., 2012). The present results revealed that the SSS supplementation up-regulated mammary gland HSF1 mRNA abundance, perhaps due to lower level of anthocyanins in SSS that not enough is being done to alleviate the OS status for dairy goats. Of interest, feeding dietary oil could be reduced mitochondrial ROS production and showed lower oxidative damage in chicken (Mujahid et al., 2009). Hence, high level of oil in RSNC treatment might be alleviated OS status for dairy goats, resulting in lower level of HSF1 gene abundance in the mammary gland relative to the SSPC.

As a member of the HSP 70 family and a chaperone protein, hspA1A, also termed Hsp72, which facilitates the proper folding of newly translated and misfolds proteins (Mayer and Bukau, 2005). The chaperones are released from HSF1 to perform their protein-folding roles and the exportation of HSF1 to the cytoplasm is inhibited, allowing HSF1 to trimerize and accumulate in the nucleus to stimulate transcription of target genes under OS status (Shamovsky and Nudler, 2008). Accordingly, the abundance of mammary gland hspA1A mRNA was increased in the SSS treatment, perhaps because higher level of hspA1A mRNA expression can protect again OS injury by activation of the HSF1 signaling pathway because anthocyanins in animals did not have enough to neutralize redundant FRs (Chauhan et al., 2014).

TNF, being an endogenous pyrogen, is a cell signaling protein (cytokine) involved in systemic inflammation and a major mediator of apoptosis, inflammation and immunity (Norman, 1998). Lipopolysaccharide molecule can be embedded in the outer membrane of the Gram-negative bacteria, which can be activated TNF (Cohen, 2002). Anthocyanins were found to be effective inhibitors of the Gram-negative bacteria (He and Giusti, 2010). As expected, APSS and SSPP treatments tended to

inhibit the expression of TNF in mammary gland, suggesting that inhibited animals' damage. Consistent with our results, anthocyanins have been reported to significant inhibit the TNF transcription via *in vivo* model of experimental angiogenesis (Atalay et al., 2003).

4.6.5 Antioxidant gene expression

In a recent article shown that dietary anthocyanin-rich plants could be increased the expression of SOD and GPX via activation of the transcription factor NFE2L2 gene (Rahman et al., 2006). Thus, APSS treatment increased the SOD2 mRNA abundance in mammary gland of dairy goats, which may be associated with higher level of NFE2L2 relative expression. However, APSS and SSPP had no effect on the SOD3 mRNA abundance. This might be because differences isoforms were discriminated at the transcript levels and differences in antioxidant enzymes concentration in mammary gland of ruminants (Côrtes et al., 2012). Moreover, SOD2 (in the mitochondria) and SOD3 (on cell surfaces and in the extracellular matrix) are located different area (Jung, 2014), which also might be an affect factor.

GPX plays an important role in protecting cardiac mitochondria from reoxygenation damage *in vivo* (Thu et al., 2010). Han et al. (2006) shown that rat fed anthocyanin-rich purple potato flakes shown in rats can be enhanced related hepatic SOD and GPX mRNA expression. Accordingly, APSS increased the levels of mRNA GPX1 and GPX2 expression in mammary gland, perhaps because anthocyanins in anthocyanin-rich plants can be absorbed into mammary tissue, leading to improving antioxidant capacity. Therefore, GPX2 was unaffected by inclusion of SSPP compared to the RSNC, suggested that these mRNA discrepancies might be the differences in bioavailability and absorption of the anthocyanins metabolites because anthocyanins

in plants seem to be not broken down in the rumen (Song et al., 2012), but the plants extract or anthocyanins powder is more susceptible in rumen fermentation (Leatherwood, 2013). However, this assume need to be further confirmed.

Dieterich et al. (2000) shown that an increase in endogenous CAT can be regarded as an effort made by the body to protect itself from an oxidative assault, whereas its expression seemed to be much lower than with other antioxidant enzymes. Similarly, no difference was detected for the mRNA abundance of CAT in mammary gland. This is because CAT is absent in the mitochondria, the reduction of H_2O_2 to H_2O is carried out by GPX (Ighodaro and Akinloye, 2017). In addition, anthocyanins increased the abundance of some SOD and GPX genes that alleviate OS status in dairy goats, and consequently did not effect on the CAT mRNA expression.

4.6.6 Pearson correlation coefficients

Anthocyanins can directly scavenging oxygen FRs by providing the electrons, thereby improving the body's antioxidant-related enzyme activity (Sakano et al., 2005). Furthermore, anthocyanins are able to modulate antioxidant and detoxifying enzymes by activation of Nrf2 (Speciale et al., 2014). Thus, significant positive correlations were found between enzymes in plasma and NFE2L2. In the current study, stronger correlations were noted between some antioxidant enzymes and the mRNA expression of inflammatory and antioxidant genes, suggesting that the body triggered a natural protective response by modulating these antioxidants and their genes expression. Indeed, antioxidant enzymes form the first line of defense against FRs in organisms (Rodriguez et al., 2004). The levels of antioxidant-related enzyme activity can response for the abundances of mRNA expression of the inflammatory and antioxidant in tissues (Polavarapu et al., 1998). For example, one

study did reveal that the significant correlation coefficients between mRNA and protein levels as well as between protein levels and enzyme activities of the antioxidant enzymes in rat tissues (Tiedge et al., 1997). These findings were in agreement with Aitken et al. (2009) who showed significant positive correlations were observed between some antioxidant enzymes (cytosolic glutathione peroxidase, thioredoxin reductase 1, and heme oxygenase-1) and pro-inflammatory molecules (intercellular vascular adhesion molecule-1, vascular cell adhesion molecule-1) in bovine mammary tissue during the periparturient period. However, several parameters showed the negative correlations or weak correlation coefficients, perhaps because differences in the content of mitochondria in the mammary gland tissues. This point needs to be detected in further additional studies. Stated thus, our data provided evidence that antioxidant enzymes and related genes expression may be cooperatively or differentially involved in the defense mechanisms in plasma and mammary gland of dairy goats exposed to OS and recovery.

4.7 Conclusions

The current study demonstrated that anthocyanin-rich PSS supplementation had no negative effect on DMI, nutrients apparent digestibility, nitrogen utilization and rumen fermentation parameters, whereas it had the potential to alleviate the OS condition by improving DPPH scavenging activity and SOD activity in plasma, and up-regulation of inflammatory mRNA expression of NFE2L2, and antioxidant mRNA expression of SOD2, GPX1, and GPX2 in the mammary gland of dairy goats. However, further study is necessary to clarify the mechanism of individual anthocyanin composition in ruminants.

4.8 References

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CHAPTER V

EFFECTS OF ANTHOCYANIN-RICH PURPLE CORN (*Zea mays* L.) STOVER SILAGE ON ANTIOXIDANT ACTIVITIES IN PLASMA AND MILK, MILK PRODUCTION, AND ANTHOCYANIN COMPOSITION IN MILK OF LACTATING DAIRY GOATS

5.1 Abstract

The study utilized 16 multiparous lactating Saanen dairy goats (body weight (BW), 41.80 ± 2.92 kg; mean ± standard deviation (SD)) with healthy and symmetrical udders. Goats were divided into two blocks of eight goats based on milk yield that averaged 75 day in milk (DIM) in a randomized completed block design (RCBD). There were two treatments: (1) control, fed sticky corn stover silage (SSS; CSSS); and (2) goats fed anthocyanin-rich purple corn (*Zea mays* L.) stover silage (PSS; TPSS). The results showed that goats receiving TPSS showed higher ($P<0.05$) levels of superoxide dismutase (SOD) in plasma and milk compared to the control treatment. TPSS did not differ ($P>0.05$) for the milk yield and milk efficiency, whereas it led to a significant ($P<0.05$) elevation in milk lactose composition relative to the CSSS. The TPSS treatment had no effect ($P>0.05$) on the milk cyanidin-3-glucoside (C3G), delphinidin (Del), cyanidin (Cya), pelargonidin (Pel) and total anthocyanins.

Pelargonidin-3-glucoside (P3G) and malvidin (Mal) were no longer detected between two treatments, whereas TPSS treatment resulted in higher ($P<0.05$) levels of peonidin (Peo) and malvidin-3-O-glucoside (M3G) relative to the control. The present study showed that: (1) anthocyanin composition in anthocyanin-rich PSS can be transferred to the milk; and (2) anthocyanin-rich PSS had the potential to alleviate oxidative stress in lactating Saanen dairy goats. Collectively, anthocyanin-rich PSS can be used as an ideal roughage for ruminants.

Key words: anthocyanin-rich purple corn stover silage, milk production, antioxidant activity, dairy goats

5.2 Introduction

For the sake of looking for the natural antioxidant to ruminants in order to promote their health, anthocyanin-rich plants for ruminants' roughages have been utilized in Thailand. Anthocyanin-rich plants as specific flavouring or functional ingredients, which had the ability to effect on the ruminant health and milk production because they might be effect of the catabolism of proteins by bacteria, contamination with sanitising agents (O'connell and Fox, 2001). Indeed, anthocyanin-rich plants have been used as ruminants' roughage, such as, purple guinea grass (Jaturasitha et al., 2009), purple sweet potato (Chanjula et al., 2003).

As a source of natural antioxidant, anthocyanins exhibit strong antioxidant property. However, anthocyanins might be effect on the palatability due to their bitter

taste, thereby resulting in lower levels of dry matter intake (DMI) and milk yield. Previous studies accomplished by Hosoda et al. (2012a) have indicated that although anthocyanin-rich purple corn silage had no effect on milk composition, it tended to decline milk yield.

Our previous work has shown that non-lactation dairy goats receiving anthocyanin-rich purple corn stover silage (PSS) had the ability to improve the antioxidant activities in blood and modulate some inflammatory and antioxidant gene expression in mammary gland tissue. However, this will definitely affect the sensitivity and response of tissues to hormones when animal in different lactation periods (Ohtani et al., 2012). Anthocyanins showed a low level of bioavailability in animal because the structure of the anthocyanin composition was fairly affected by pH value (Kırca et al., 2007). However, despite the low absorption, the finding of these anthocyanins in milk may be important due to their unique antioxidant properties for human health. To date, there is no information if anthocyanins are absorbed into the milk, improving consumers' health. Accordingly, the current study was conducted to assess the effect of anthocyanin-rich PSS on ruminants' antioxidant capacity and milk production, and transfer the anthocyanin composition to the milk in lactating Saanen dairy goats.

5.3 Materials and Methods

The experiment was conducted at Sukjai Farm, Nakhon Ratchasima, Thailand (14°53'36.7"N, 102°03'21.2"E). All goats were handled in accordance with the Guide for the care and use of laboratory animals (Institute of Laboratory Animal Resources, Commission on Life Sciences).

5.3.1 Animal, diets and experimental design

A total of 16 multiparous lactating Saanen dairy goats (body weight (BW), 41.80 ± 2.92 kg; mean ± standard deviation) that averaged 75 day in milk (DIM) with healthy and symmetrical udders were used. Goats were divided into two blocks based on milk yield during the previous lactation. In each block there were eight animals to which treatments were randomly assigned according to a randomized completed block design (RCBD). The experiment lasted for 8 weeks with the first 2 weeks as the adjustment period, followed by 6 weeks of measurement period. The goats received total mixed ration (TMR; Table 5.1) in two treatments: (1) control of goats fed sticky corn stover silage (SSS; CSSS); and (2) treatment of goats fed anthocyanin-rich PSS (TPSS). Animals were housed in clean pens with free access to water. Ration was offered twice daily at 07:30 and 16:30 for *ad libitum* intake and allowed for approximately 10% refusal. The diets were formulated to meet requirements for dairy goat having 40 kg of BW according to the National Research Council (NRC, 1981). Moreover, nutrient composition and anthocyanin composition in two types of corn stover silage was listed in Table 5.2.

Table 5.1 Ingredient and nutrient composition of experimental diets for dairy goats.

Item[1]	**Treatment**[2]	
	CSSS	**TPSS**
Ingredient (% DM)		
Sticky corn silage	50.0	
Purple corn silage		50.0
Napier Pak Chong 1	10.0	10.0
Soybean hull	7.5	7.5
Soybean residue	17.5	17.5
Concentrate [3]	15.0	15.0
Total	100.0	100.0
Chemical composition		
DM (%)	23.95	24.27
OM (% DM)	92.02	92.63
CP (% DM)	15.02	15.10
GE (Cal/g DM)	4317.75	4339.44
NDF (% DM)	45.32	46.48
ADF (% DM)	26.57	25.87
Hemicellulose (% DM)	18.75	20.61
Ash (% DM)	7.98	7.37

[1] DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy; NDF = neutral detergent fiber; ADF = acid detergent fiber.

[2] CSSS = control of goats fed sticky corn stover silage; TPSS = treatment of goats fed anthocyanin-rich purple corn stover silage.

[3] The concentrate consisted of (per 100 kg of DM): 32 kg of soybean meal, 20 kg of rice bean, 20 kg of cassava chip, 24.6 kg of corn, 1 kg of salt, 0.4 kg of limestone, 2 kg of a minerals and vitamins premix [4].

[4] The premix consisted of (per 1 kg of premix) : vitamin A (500 IU), vitamin D_3 (150 IU), vitamin B_2 (0.25 mg), vitamin E (0.40 mg), folic acid (0.165 mg), choline (3 mg), Ca (378160 mg), P (8400 mg), Fe (3410 mg), Co (88.4 mg), Mn (1280 mg), I (76.1 mg), K (23.6 mg), Zn (4344 mg), Mg (244 mg), Cu (550 mg).

Table 5.2 Nutrient composition and anthocyanin composition in two types of corn stover silage.

Item[1]	Type of corn stover silage[2]	
	SSS	**PSS**
Nutrient composition		
DM (%)	20.48	22.85
OM (% DM)	93.65	94.44
CP (% DM)	7.33	7.37
GE (kJ/g DM)	4174.11	4235.36
NDF (% DM)	61.46	62.37
ADF (% DM)	32.85	31.09
Hemicellulose (% DM)	28.62	31.28
Ash (% DM)	6.35	5.56
Anthocyanin composition		28.88
C3G (mg/kg DM)	37.71	100.78
Del (mg/kg DM)	96.18	101.43
P3G (mg/kg DM)	25.20	132.51
Peo (mg/kg DM)	46.89	73.88
M3G (mg/kg DM)	47.28	68.51
Cya (mg/kg DM)	129.37	125.42
Pel (mg/kg DM)	104.11	118.80
Mal (mg/kg DM)	21.60	140.61
Total (mg/kg DM)	485.66	861.95

[1] DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy; NDF = neutral detergent fiber; ADF = acid detergent fiber; C3G = cyanidin-3-glucoside; Del = delphinidin; P3G = pelargonidin-3-glucoside; Peo = peonidin; M3G = malvidin-3-O-glucoside; Cya = cyanidin; Pel = pelargonidin; Mal = malvidin; - = not detected.

[2] SSS = sticky corn stover silage; PSS = anthocyanin-rich purple corn stover silage.

5.3.2 Sample collection

Around 100 g diets were collected once weekly and kept -20°C at the beginning of the feeding trial. After that, the samples were mixture and dried at 65°C in a vacuum oven for 72 h, then ground and passed through a 1-mm sieve. Feed offered and refused were weighed daily prior to the morning feeding, and then calculated DMI throughout the experiment.

Goats were milked once daily at 19:00 h via a portable milking machine (Condor Company, made in Italy) and the milk yield was recorded. Milk collected during this initial milking was discarded. Milking routine included milking equipment preparation, udder preparation, teat cleaning and disinfecting (1.0% sodium hypochlorite solution), and milking. An operating vacuum of 36 kPa was used and a pulsator was operated at 120 cycles/min with a 50:50 milk : rest ratio. Milk was sampled at the last 2 days of 3^{th} to 8^{th} weeks of the experiment (2 d for each collection period). The samples (100 g per kg of recorded milk yield) were mixed thoroughly and collected at each milking, and the milk of pH value determination was done immediately by a portable pH meter. After that, milk samples were divided into 2 portions. One aliquot was stored at 4°C with a preservative (bronopol tablet; D&F Control System Inc., San Ramon, CA, USA) until analyzed for fat, protein, lactose, total solid (TS), and solid not fat (SNF) using a MilkoScan analyzer (MilkoScan™ FT2, FOSS, Hillerod, Denmark) after incubation for 10 min in a 40°C water bath. The other aliquot was stored at -20°C until analyzed for antioxidant activity and

anthocyanins. Milk and fat outputs were utilized to calculate 3.5% fat-corrected milk (3.5% FCM) using the formula 3.5% FCM = Kg of milk yield × [0.432 + 0.162 × (fat %)] (Hamzaoui et al., 2013). The milk energy content (MEC), milk energy output (MEO), and energy-corrected milk (ECM) and were calculated using the following formulas by Tyrell and Reid (1965), and Kholif et al. (2016).

MEC (MJ/kg) = 4.184 × [(41.63 × fat (g/kg) + 24.13 × protein (g/kg) + 21.60 × lactose (g/kg) – 117.2) ÷ 10000] × 2.204;

MEO (MJ/d) = MEC (MJ/kg) × milk yield (kg/d);

ECM (kg/d) = milk (kg/d) × [38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 16.54 × lactose (g/kg) + 20.7] ÷ 3240.

Blood (around 3 mL) was sampled at 0 and 4 h from jugular vein by vacuette® tubes (Greiner Bio-One, Greiner Bio-One GmbH Bad Haller Str. 324550 Kremsmünster, Austria) with K_3-EDTA at the end of the experiment. After centrifugation at 4000 r/min for 15 minutes at 4°C (Sorvall™ Legend™ XT/XF Centrifuge Series, Thermo Fisher Scientific Pte Ltd., Waltham, USA), the plasma was transferred to a 1.5 mL tube and stored at -80°C until further analysis.

5.3.3 Chemical analysis

The determinations of dry matter (DM), crude protein (CP) and ash of the samples were performed by the method of AOAC (2005). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and hemicellulose of the samples were assayed

according to Van Soest et al. (1991). Organic matter (OM) was calculated from the difference between DM and ash. The gross energy (GE) of the sample was analyzed using an auto-calculating bomb calorimeter (Parr 6200, Moline, Illinois, USA).

5.3.4 Antioxidant activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was analyzed by the spectrophotometrically methods of Wei and Chiang (2009) and Zarban et al (2009) using a stable free radical DPPH (Sigma-Aldrich, Pcode: 101845869) with a minor modification. Briefly, 50 µL of each sample was added to 1 mL of methanolic solution of DPPH reagent (25 µmol/L) in a 1.5 mL tube. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min, and then centrifuged at 4000 r/min for 10 min at 4°C. 200 µL of the supernatant was transferred to a 96-well plate immediately and the absorbance was detected at 517 nm via a microplate reader (Epoch, BioTek, Luzern, Switzerland). The DPPH scavenging activity was calculated by the following formula: DPPH scavenging activity = (Ac − As) × 100 / Ac, where Ac is the absorbance of the control, and As is the absorbance of the sample.

The enzymatic activity of total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) were measured using the kits from Sigma-Aldrich (MAK187-1KT, 19160-1KT-F, CGP1-1KT, and CAT100-1KT, respectively). Moreover, all samples were assayed via a microplate reader.

5.3.5 Anthocyanin composition

The anthocyanin content in silage was performed as described by Hosoda et al. (2009). Briefly, the sample was extracted using 1% hydrochloric acid (HCl) dissolved in 95% methanol solution (15:85, v/v), the supernatant was collected after incubation at 50°C for 24 h. After that, the supernatant was filtered through 13 mm 0.45 μm Nylon Syringe Filter (Xiboshi, TNL1345PP, Tianjin Fuji Science & Technology Co., Ltd., China) for the determination of anthocyanin composition by a high performance liquid chromatography (HPLC; 1260 Infinity II LC, Agilent Technologies, Santa Clara, CA, USA). The anthocyanin composition in milk was analyzed per the methods of Seeram et al. (2006) and Tadapaneni et al. (2012) with a slight modification. Briefly, the milk samples adjusted to pH 4 with 1% hydrochloric acid and were pretreated using liquid-liquid extraction using acetone/water (70:30, v/v), and then centrifuged 10000 r/min for 15 min at 4°C after incubation at room temperature for 4 h, the supernatant was collected for the determination of anthocyanin composition as reported previously.

The sample analysis was carried out with a HPLC and Diode Array (DAD) Detector. Moreover, separation of anthocyanin composition was accomplished on a Symmetry C_{18} column (mobile phase: A, acetonitrile (CH_3CN); B, 10% acetic acid/ 5% CH_3CN/ 1% phosphoric acid in deionized water). The run time was 30 min and followed by a 5-min delay prior to the next injection. The other conditions were as

follows: sample temperature of 4°C, and injection volume of 20 µL, flow rate of 0.8 mL/min, column temperature of 25°C and DAD detector wavelength of 520 nm.

5.4 Statistical analysis

All statistical calculations were analyzed using the General Linear Model (GLM) procedure of Statistical Analysis System 9.1.3 (SAS Inst. Inc., Cary, NC) according to a RCBD and using milk yield as block. Each block as the experimental unit according to the following general model: $y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$, where y_{ij} = an observation in treatment i (i = 1 − 2) and block j (j = 1 − 8), μ = the overall mean, τ_i = the effect of treatment i (i = 1 − 2), β_j = the fixed effect of block j (j = 1 − 8), ε_{ij} = residual. Significant differences were set at $P<0.05$.

5.5 Results

5.5.1 Antioxidant activity

No differences ($P>0.05$) were detected for DPPH scavenging activity, TAC, GPX, and CAT in plasma and milk, whereas the inclusion of TPSS resulted in higher ($P<0.05$) levels of SOD in plasma and milk compared to the CSSS (Table 5.3).

Table 5.3 Effects of diets on antioxidant activity in plasma and milk of dairy goats.

Item[1]	Treatment[2]		SEM	P-value
	CSSS	TPSS		
Plasma				
DPPH scavenging activity (%)	19.10	21.58	1.1445	0.1389
TAC (nmole/µL)	37.20	42.02	3.2322	0.3408
SOD (inhibition rate %)	91.64[b]	96.40[a]	0.8526	0.0016
GPX (mmol/min/mL)	51.64	50.36	0.6904	0.2199
CAT (µmoles/min/mL)	230.82	231.28	4.3908	0.9430
Milk				
DPPH scavenging activity (%)	23.14	23.44	1.4575	0.8851
TAC (nmole/µL)	109.24	111.38	1.8854	0.4314
SOD (inhibition rate %)	82.72[b]	90.22[a]	0.8820	<0.0001
GPX (mmol/min/mL)	50.94	50.24	0.1231	0.6789
CAT (µmoles/min/mL)	225.93	228.26	1.6027	0.3438

Values for plasma and milk represented the means of 8 replicates (n = 8) and 12 replicates (n = 12), respectively.

Means with different letters within a row are significantly different (P<0.05).

[1] DPPH = 2, 2-diphenyl-1-picrylhydrazyl; TAC = total antioxidant capacity; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase.

[2] CSSS = control of goats fed sticky corn stover silage; TPSS = treatment of goats fed anthocyanin-rich purple corn stover silage. The same as below.

5.5.2 Feed intake, milk yield and milk efficiency

No significant difference (P>0.05) was observed for DMI between control and TPSS (Table 5.4). Similarly, Milk yield also did not differ (P>0.05) in both of treatments. However, CSSS treatment displayed a greater (P<0.05) level of SNF production compared with that in the TPSS treatment. In addition, TPSS diet had no effect (P>0.05) on the milk efficiency**.**

5.5.3 Milk composition

There was no difference (P>0.05) in the milk of pH values between two treatments (Table 5.5). All milk composition were unaffected (P>0.05) with the exception of milk lactose, which showed a higher (P<0.05) concentration in TPSS treatment.

Table 5.4 Effects of diets on DMI, milk yield and milk efficiency of dairy goats.

Item[1]	Treatment		SEM	P-value
	CSSS	TPSS		
DMI (g/d)	886	875	22.3109	0.5744
Milk production				
Milk yield (kg/d)	1.57	1.53	0.0203	0.1451
3.5% FCM (kg/d)	1.58	1.52	0.0295	0.1341
Fat (g/d)	55.75	52.90	1.6196	0.2262
Protein (g/d)	52.28	50.34	0.7063	0.0654
Lactose (g/d)	70.58	69.43	0.9942	0.4220
TS (g/d)	189.89	181.22	3.0398	0.0562
SNF (g/d)	134.14[a]	128.32[b]	1.9636	0.0480
MEC (MJ/kg)	2.89	2.87	0.0403	0.6250
ECM (kg/d)	1.47	1.41	0.0242	0.1162
MEO (MJ/d)	4.54	4.37	0.0758	0.1255
Milk efficiency				
Milk-DMI	1.78	1.72	0.0229	0.0738
ECM-DMI	1.66	1.59	0.0274	0.0679

Values represented the means of 12 replicates (n = 12).

[1] DMI = dry matter intake; FCM = fat-corrected milk; TS = total solid; SNF = solid not fat; MEC = milk energy content; ECM = energy-corrected milk; MEO = milk energy output.

Table 5.5 Effects of diets on milk composition of dairy goats.

Item[1]	Treatment		SEM	P-value
	CSSS	TPSS		
pH	6.49	6.45	0.0135	0.0741
Fat (%)	3.55	3.47	0.1038	0.5800
Protein (%)	3.33	3.30	0.0245	0.3593
Lactose (%)	4.50[b]	4.55[a]	0.0151	0.0207
TS (%)	12.10	11.88	0.1342	0.2537
SNF (%)	8.55	8.41	0.0496	0.0583

Values represented the means of 12 replicates (n = 12).

[1] TS = total solid; SNF = solid not fat.

5.5.4 Milk anthocyanin composition

As shown in Table 5.6. No differences (P>0.05) were noted in cyanidin-3-glucoside (C3G), delphinidin (Del), cyanidin (Cya), pelargonidin (Pel) and total anthocyanins in milk between two treatments. Pelargonidin-3-glucoside (P3G) and malvidin (Mal) were unable to be detected throughout the experiment. Of interest, the inclusion of TPSS resulted in higher (P<0.05) levels of peonidin (Peo) and malvidin-3-O-glucoside (M3G) relative to the control.

Table 5.6 Effects of diets on anthocyanin composition in milk of dairy goats.

Item[1]	Treatment		SEM	P-value
	CSSS	TPSS		
C3G (mg/kg)	0.338	0.330	0.0048	0.2489
Del (mg/kg)	0.959	0.959	0.0025	0.9342
P3G (mg/kg)	-	-	-	-
Peo (mg/kg)	0.371[b]	0.388[a]	0.0045	0.0105
M3G (mg/kg)	0.446[b]	0.457[a]	0.0026	0.0066
Cya (mg/kg)	1.283	1.290	0.0036	0.1636
Pel (mg/kg)	1.042	1.041	0.0024	0.8332
Mal (mg/kg)	-	-	-	-
Total (mg/kg)	4.439	4.466	0.0119	0.1147

Values represented the means of 12 replicates (n = 12).

5.6 Discussion

5.6.1 Antioxidant activity in plasma and milk

Anthocyanins can be absorbed into the blood (VISKUPIČOVÁ et al., 2008). He et al. (2006) have been reported to the range of total anthocyanins in plasma was 0.2-2 μmol/L in rats. OS is caused by an imbalance between oxidants and antioxidants, potentially leading to animal damage (Sies, 1997). Various literatures have been reported to the anthocyanins not only have the function of directly scavenging oxygen free radicals (FRs) by providing the hydroxyl group, but also

improve the body's antioxidant-related enzyme activity to achieve the purpose of improving the body's antioxidant capacity (Soobrattee et al., 2005; Aseervatham et al. 2013). Thus, supplementation of anthocyanins in the ruminant diets has potential to alleviate animals' OS status. Moreover, one study did show that the level of total anthocyanins in the blood was directly correlated with an increase in blood antioxidant capacity (Mazza et al., 2002). Accordingly, TPSS treatment resulted in higher SOD activity, suggesting that the feeding of anthocyanins in dairy goats could be improve antioxidant in plasma. Indeed, SOD is considered the first defence against prooxidants, which can indicate antioxidant potential in ruminants. Consistent with our results, Hosoda et al. (2012) showed that lactating dairy cows receiving anthocyanin-rich corn silage had the potential to alleviated OS conditions as suggested by enhanced of the plasma SOD activity. In addition, superoxide radicals ($O_2^{\cdot-}$) as precursors of mitochondrial hydrogen peroxide (H_2O_2), which had the relative high toxicity compared to the H_2O_2 (Loschen et al., 1974; Watts et al., 2003). Of interest, the DPPH scavenging activity, GPX, and CAT in plasma were unaffected, perhaps because the high toxicity of $O_2^{\cdot-}$ is converted in the low toxicity of H_2O_2 by SOD enzyme, thereby alleviating OS status in ruminants (Jin et al., 2017).

Tijerina-Sáenz et al. (2009) indicated that the level of antioxidant capacity value could be reflected oxidative stability in milk. Furthermore, one study did find that the inclusion of anthocyanins with selenium (Se), vitamin E (Vit E) showed a synergistic effect in rats (Li et al., 2016). Hence, anthocyanin-rich PSS had the

potential to improve the absorption of other antioxidants, thereby increasing SOD value. However, we did not assay other antioxidants in milk and need to be further confirmed. Of interest, the levels of GPX and CAT enzymes did not differ between two treatments. This is probably because GPX and CAT enzymes in milk not only can degrade H_2O_2, they also had to reduce lipid peroxides, such as fatty acid hydroperoxides and phospholipid hydroperoxides (Lindmarkmånsson and Akesson, 2000). However, it is unclear that anthocyanins can be improved antioxidant capacity directly in goats, because anthocyanins not only are poorly absorbed by the intestine, but also anthocyanin-rich PSS had several kinds of antioxidant compounds, such as flavonoids and vitamin family.

5.6.2 Feed intake, milk yield and milk composition

It is known that nutrient digestibility and energy intake were the two main factors that affected on the milk yield (Oba and Allen, 1999). In the current study, milk yield did not differ in two treatments, whereas CSSS treatment showed a greater level of SNF production. One study did reveal that anthocyanins had been reported to show a negative effect on the intake of the animals because of their bitter taste (Jöbstl et al., 2004). Indeed, anthocyanin-rich PSS did not affect DMI, but the feeding time of goats increased while intake velocity reduced in the TPSS treatment during whole trial period. Moreover, anthocyanins as a kind of phenolic compounds, which might be bind to the nutrients to be digested, inhibiting the digestive enzyme and exerting anti-microbial effect (Acosta-Estrada et al., 2014; Kamalak et al., 2015).

Accordingly, the inclusion of anthocyanin-rich plant may be displayed a slight decline trend in the milk production.

Propionic acid is precursor the milk lactose content (Rigout et al., 2003). In the present study, higher level of lactose was noted in TPSS treatment, perhaps because anthocyanin-rich PSS might be able to affect rumen fermentation, especially inhibiting acetic acid and increasing the proportion of propionic acid in the ruminal fluid. Of interest, the structure of anthocyanin in purple corn is bonded to sugars (Aoki et al., 2002). Hence, the sugars in anthocyanin might be broken down in the digestive tract and involved in lactose synthesis. These results concur with Jordán et al. (2010) who indicated that polyphenolic-rich distilled rosemary leaves can be proposed as an ingredient in ruminant feed because they both alter neither the yield nor the quality of Murciano-Granadina goats' milk.

5.6.3 Milk anthocyanin composition

One report did show that anthocyanin absorption appears to be at least an order of magnitude lower than for the flavonol quercetin due to its poorly absorbed from the gut and is subject to degradation by intestinal micro-organisms (Formica and Regelson, 1995). Indeed, various studies have been shown that the low bioavailability of anthocyanins in human (Xiao et al., 2017; Mueller et al., 2017). This may be not just because the structure of anthocyanin is a highly conjugated system, but also because the anthocyanins in the plant will not be absorbed by the animals unless they are through in its cell wall (Cavalcanti et al., 2011). Thus, the amounts of C3G, Del,

Cya, Pel, and total anthocyanins in milk were identical. However, despite the low absorption, the finding of these anthocyanins in milk may be important due to their unique antioxidant properties for human health. Jordán et al. (2010) indicated that polyphenolic components transferred to the milk is dependent on the component molecular weight. This study also indicated that the Cya and Pel were the major anthocyanin composition in milk, perhaps due to the molecular weights of Cya and Pel under 300 g/moL; In contrast, other anthocyanin composition with the molecular weights above 300 g/moL.

It is reported that anthocyanin can be stable in acidic aqueous solutions (Passamonti et al., 2003). However, anthocyanins are unstable and quickly decolorize by hydration at the two-position of the anthocyanidin skeleton in high pH values (Brouillard, 1988). Oldfield et al. (2000) showed that the milk of pH values were in the range of weak acidity (6.48-6.83). In agreement with our results, the milk pH values ranged from 6.45 to 6.49. Therefore, P3G and Mal were unable to be detected, perhaps due to these two kinds of anthocyanin composition are not absorbed into the milk after ruminant metabolism as well as they had extremely low stability in milk due to their hydration.

Acylated anthocyanins served as a major source of natural food colours due to the characteristics of high stability (Cevallos-Casals and Cisneros-Zevallos, 2004; Netzel et al., 2007). One report has been shown to anthocyanins can be absorbed in their intact glycosylated and possibly acylated forms in human subjects (Mazza et al.,

2002). He et al. (2006) showed that the type of sugar substitution had significant impact on the absorption and excretion of individual anthocyanins in rats. Accordingly, these previous reports make us assume that Peo and M3G might be took part in the acylation reaction of anthocyanin absorption and metabolic mechanism in dairy goats. Anthocyanin-rich PSS showed high level of anthocyanin composition, resulting in high concentrations of Peo and M3G in milk. However, information about the absorption and metabolism of anthocyanin composition in ruminants is scarce. In addition, the sugar residues may be occur acylated with aromatic acids, whereas anthocyanins containing rather complicated acylation patterns attached on different sugar moieties (Giusti and Wrolstad, 2003). Therefore, the exact reasons remain unclear and further observation is required.

Otsuki et al. (2002) indicated that twelve acylated anthocyanins were isolated from the red radish (*Raphanus sativus* L.). Thus, the authors hope that it might encourage those who are interested in acylated anthocyanins in purple corn to make further studies in this field. Hertog et al. (1993) suggested that adult human the intake of anthocyanins have been estimated at more than 100 mg/day. He and Giusti (2010) suggested that the daily requirement of anthocyanins for human was estimated to be 2.5 mg/kg body weight. Thus, the concentration of anthocyanins in milk has never been enough to protect against OS for human. Therefore, the author on this topic is necessary to think about how to improve anthocyanin was absorbed in milk.

5.7 Conclusions

Under the condition of the current experiment, anthocyanin-rich PSS can be proposed as an ideal feedstuff for ruminants because: (1) anthocyanin composition in anthocyanin-rich PSS can be transferred to the milk; and (2) anthocyanin-rich PSS had the potential to alleviate oxidative stress in lactating Saanen dairy goats.

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CHAPTER VI

OVERALL CONCLUSION AND IMPLICATION

6.1 Conclusion

This study provided an insight into anthocyanins in anthocyanin-rich PSS as a source of antioxidant that can enhance antioxidant activities in Saanen dairy goats.

The first part was focused on the chemical composition, anthocyanin stability, and antioxidant activity during the storage period and *in vitro* rumen fermentation of anthocyanin-rich PS and SS. The results revealed that: (1) anthocyanins could be stable in silage; (2) anthocyanin-rich PSS showed excellence silage fermentative quality and stronger DPPH scavenging activity; and (3) anthocyanin-rich PSS had no negative effect on rumen fermentation parameters.

The second part was to investigate the effect of anthocyanin-rich PSS on digestibility, rumen fermentation, antioxidant activity in plasma, and the mRNA expression of inflammatory and antioxidant genes in the mammary gland of dairy goats. The results indicated that the consumption of anthocyanin-rich PSS by dairy goats had no negative effect on DMI, apparent digestibility, nitrogen utilization and rumen fermentation parameters, whereas it had the potential to alleviate the oxidative stress condition by improving antioxidant capacity in plasma, and up-regulation of inflammatory mRNA expression of NFE2L2 and antioxidant mRNA expression of SOD2, GPX1, and GPX2 in the mammary gland of dairy goats.

The third part was to observe the effect of anthocyanin-rich PSS on milk production, antioxidant activity in plasma and milk, and milk anthocyanin composition in lactating dairy goats. The results showed that the addition of anthocyanin-rich PSS had no negative effect on the milk yield and milk composition, whereas it can improve antioxidant capacity in plasma, and anthocyanin composition can be transferred to the milk in lactating Saanen dairy goats.

Collectively, anthocyanin-rich PSS can be used as an excellence feedstuff for dairy goats, because: (1) anthocyanins in anthocyanin-rich PSS could be stable in silage; (2) the consumption of anthocyanin-rich PSS had the potential to alleviate the oxidative stress condition by improving antioxidant activity in plasma and modulating the expression of inflammatory and antioxidant genes in the mammary gland; and (3) anthocyanin composition in anthocyanin-rich PSS can be absorbed into the milk in Saanen dairy goats.

6.2 Implication

The metabolism of anthocyanins in ruminants is also unclear at present. Moreover, anthocyanin-rich PSS not only contains anthocyanin compounds, it also has other natural antioxidants, such as, flavonoids, polyphenols, and vitamin family. Consequently, further studies are encouraged to determine the degradation pathways of absorption, metabolism, and excretion of individual anthocyanin composition in ruminants.

APPENDICES

APPENDIX A

Solution preparation for *in vitro* gas production technique

A1 Macromineral solution

Na_2HPO_4	5.7 g
KH_2PO_4	6.2 g
$MgSO_4$	0.6 g
Make up to 1 L with distilled water	

A2 Buffer solution

$NaHCO_3$	35 g
$(NH_4)\ HCO_3$	4 g
Make up to 1 L with distilled water	

A3 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 L with distilled water	

A4 Resazurin aqueous: (100mg/100ml)

A5 Preparation of artificial saliva

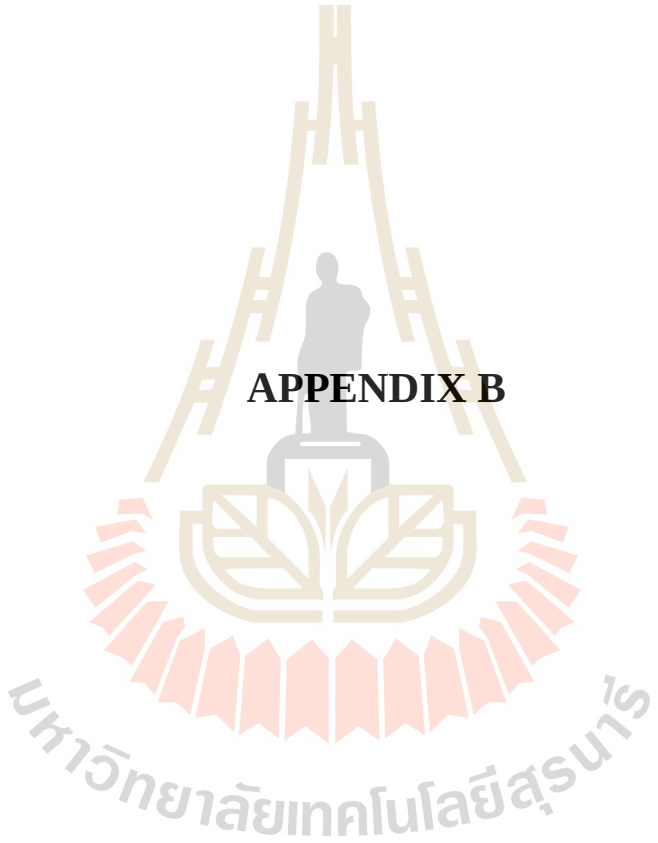
Artificial saliva

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
$Na_2S_9.H_2O$	336 mg

APPENDIX B



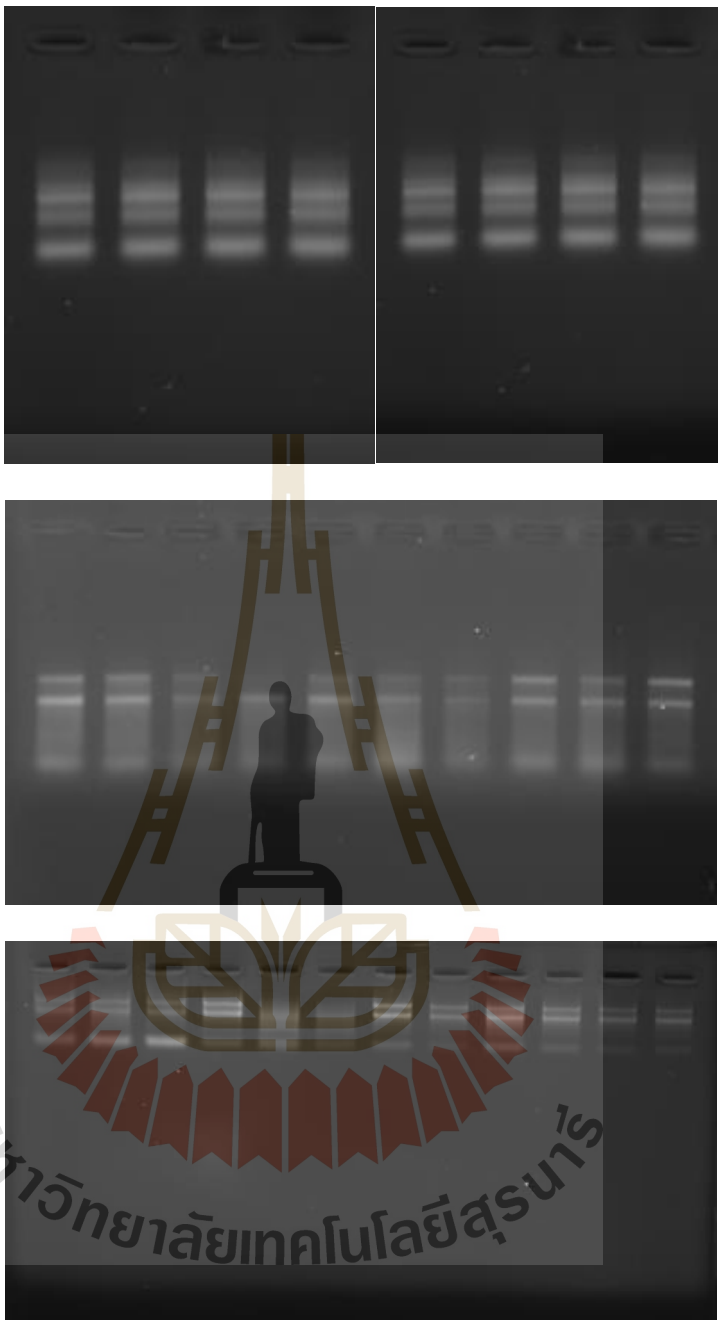


Figure B1 Total RNA agarose gel electrophoresis analysis.

APPENDIX C

The genes of reference sequence

C1 NFE2L2

Capra hircus nuclear factor, erythroid 2 like 2 (NFE2L2), mRNA

NCBI Reference Sequence: NM_001314327.1

ttggatttag cgtttcggaa ttgcaattcc aaaatgtgta aggcaggata ttctgttctc

tgctgtcaag ggacatggat ttgattgaca tactttggag gcaagatata gatctcgggg

taagtcgaga ggtatttgac ttcagtcaac gacagaagga gcatgagctg gaaaaacaga

aaaaacttga aaaggaaaga caagaacaac tccaaaagga gcaagagaaa gccttttttg

ctcagttaca actagatgaa gagaccggcg agttcctccc cattcagcca gcacagcaca

caccatcaga agccagtggg tctgccaact acgagaccgg cgagttcctc cccattcagc

cagcacagca cacagcatca gaagccagtg ggtctgccaa ctactcccag gcagcccaca

ttcccaaagc agatgatttg tacttcgatg actgcatgca gcttttggca gagacattcc

tatttgtaga tgacaatgag gtttcttcgg ccacgtttca atcacctgtt cccgatattc

gcagccacat cgagagccca gtcttcaatg ctccttctca ggctcagtca cctgaaactc

tgttcgttca ggtagccact gctgatttag acgatatgca ggaggacatt gagcaagttt

gggaggaact actatccatt ccagaattac agtgtcttaa tattcaaaat gacaagctgg

ctgagactag tacagttcca agtccagaaa ccaaactgac agaaattgac agttataatt

tctattcatc aatgccctca ctggataaag aagttggtaa ctgcagcccg cattttctca

atgcttttga ggattccttc aacagcatcc tctccactga agattccagc cggttgacag

tgaattcatt aaattcaact gccacagtaa atacagattt tggtgatgaa ttttattctg

cttttatagc agagcccagt accagcaaca gcatgccctc ctctgctact ttaagccagt

cactctctga acttctaaac gggcccattg acctctctga tctgtcactt tgtaaagcct

tcaatcaaaa ccaccctgaa agcacaacag cagaattcaa tgattctgac tctggcattt

cactgaacac aacaagtcca agcatggcat caccagacca ctcagtggaa tcttctgtct

atggagacac attgcttggc ttcagtgatt ctgaaatgga agagatagat agtgcccctg

gaaatgtcaa acagaagggt cccaaaacac agtcagtgtg gccttctggg gacccagtcc

aacctttgtc atcatcacaa gggaacagca ctgcagcatg tgattcccag tgtgaaaatg

caccaaagaa agaagtgcct gtaagtcccg gtcatcggaa aaccccattc acaaaagaca

aacattcaag ccgcttggag gctcacctca caagagatga gctacgggca aaagctctcc

atatcccatt ccctgtagaa aagatcatta acctcccagt tgaggacttc aatgaaatga

tgtccaagga gcaattcaac gaggctcaac ttgcattaat cagagatata cgtaggaggg

gtaagaataa agtggctgct cagaattgca gaaaaagaaa actggaaaat atagtggaac

tggagcaaga tttagatcac ttaaaagatg aaaaagaaaa attgctcaaa gaaagagggg

aaaatgacaa aagcctccat ctcctgaaaa aacaactcag caccttgtat cttgaagtct
tcagcatgct acgtgatgaa gatggaaagc cttactctcc aagtgaatac tccttgcagc
aaacaggaga tggcaatata ttccttgttc ccaaaagtaa gaggccagat attaagaaaa
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actatgtact tttttataaa taccatgtga acaaaaaaaa ggcatttttt taatattaaa
ttgtttaact ctggcaaaag acaatttagt tgaaaagctg gtactaataa aggaatatca
ggaccgt

C2 HSF1

Capra hircus heat shock transcription factor 1 (HSF1), mRNA

NCBI Reference Sequence: NM_001314344.2

gcctggtatg gatctgcccg tgggcccggg cgcggcgggg cccagcaacg tcccggcctt
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C3 hspA1A

Capra hircus heat shock 70kDa protein 1A (hspA1A) mRNA, complete cds

NCBI Reference Sequence: JN656104.1

atggcgaaaa acatggctat cggcatcgac tggggcacca cctactcctg cgtgggggtg
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ggctttgggg ctcaggcccc taaagggggc tctgggtctg gcccctccat tgaggacggg
gattag

C4 TNF

Capra hircus tumor necrosis factor (TNF), mRNA

NCBI Reference Sequence: NM_001286442.1

gtctcccgtc tggacttgga tccttctgaa aaagacagca tgagcaccaa aagcatgatc
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C5 GPX1

Capra hircus glutathione peroxidase 1 (GPX1), mRNA

NCBI Reference Sequence: XM_005695962

gtcgcccgcc ttttaaaagc aggcgcatcc cgtccccctc cttgttcttc gagtccgcgc
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gtgagcagaa

C6 GPX2

Capra hircus glutathione peroxidase 2 (GPX2), mRNA

NCBI Reference Sequence: XM_005685982.3

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C7 SOD2

Capra hircus superoxide dismutase 2, mitochondrial (SOD2), mRNA

NCBI Reference Sequence: XM_018053428.1

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tattgtaggt gtgaagtttt ttgattgcca ataaaagaat ctgtttgcca

C8 SOD3

Capra hircus superoxide dismutase 3 (SOD3), mRNA

NCBI Reference Sequence: NM_001285675.1

atgccggcgc tgctctgtgc ctctctgctc ctggtggcct gtgcctcggc cgcctcggcc
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tgtaaggacg tctga

C9 CAT

Capra hircus catalase (CAT), mRNA

NCBI Reference Sequence: XM_005690077.3

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tgcaaagttc tgcagcgccg ctcagacacc atggcggaca accgggatcc agccagcgac

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C10 GAPDH

Capra hircus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

NCBI Reference Sequence: XM_005680968.3

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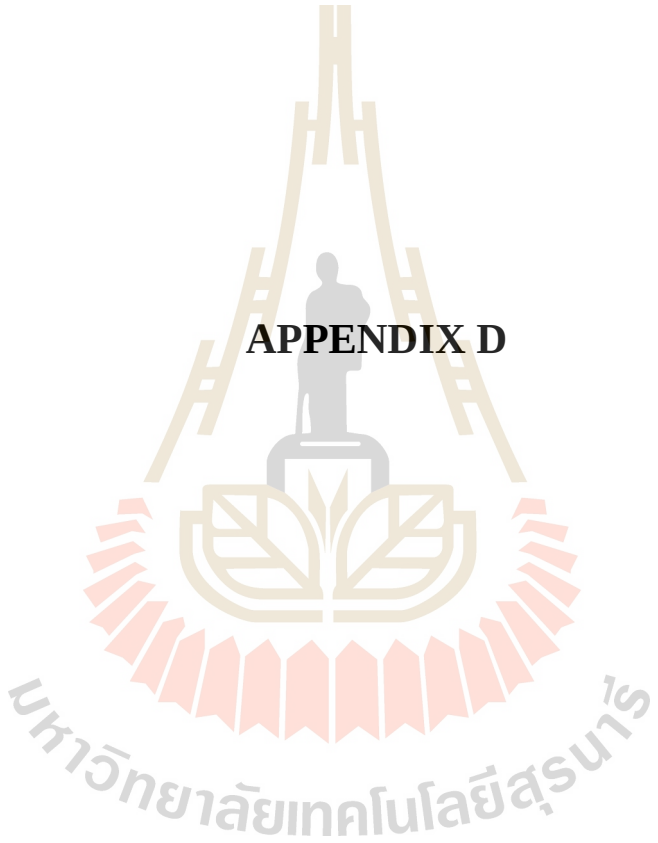
APPENDIX D



The genes for sequencing results

All samples sent to Pacific Science Co., Ltd. (Bangkok, Thailand) for sequencing.

D1 NFE2L2

NFE2L2-R: TGATGCCATGCTTGGACTTG

Company result:

CGGCTGAGGCTTCATCAAACCACCCTGAAGCACAACAGCAGAATTCAATGATTCTGACTCTGGCATTTCACTGAACACAACAAGTCCAAGCATGGCATCAA

Complement result:

TTGATGCCATGCTTGGACTTGTTGTGTTCAGTGAAATGCCAGAGTCAGAATCATTGAATTCTGCTGTTGTGCTTCAGGGTGGTTTGATGAAGCCTCAGCCG

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X2, mRNA	154	154	93%	4e-34	96%	XM_013968675.2
PREDICTED: Capra hircus nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X1, mRNA	154	154	93%	4e-34	96%	XM_018054369.1
PREDICTED: Ovis aries nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X2, mRNA	154	154	93%	4e-34	96%	XM_012132956.2
PREDICTED: Ovis aries nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X3, mRNA	154	154	93%	4e-34	96%	XM_012132935.2
PREDICTED: Ovis aries nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X1, mRNA	154	154	93%	4e-34	96%	XM_015093345.1
PREDICTED: Ovis aries musimon nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X2, mRNA	154	154	93%	4e-34	96%	XM_012143673.2
PREDICTED: Ovis aries musimon nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X5, mRNA	154	154	93%	4e-34	96%	XM_012143671.2
PREDICTED: Ovis aries musimon nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X1, mRNA	154	154	93%	4e-34	96%	XM_012143670.2
Ovis canadensis canadensis isolate 43U chromosome 2 sequence	154	154	93%	4e-34	96%	CP011887.1
PREDICTED: Ovis aries musimon nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X6, mRNA	154	154	93%	4e-34	96%	XM_012143672.1
PREDICTED: Ovis aries nuclear factor, erythroid 2 like 2 (NFE2L2), transcript variant X4, mRNA	154	154	93%	4e-34	96%	XM_012132949.1
Capra hircus nuclear factor, erythroid 2 like 2 (NFE2L2), mRNA	154	154	93%	4e-34	96%	NM_001314327.1

D2 HSF1

HSF1-R: CAGCGAGATGAGGAACTGGA

Company result:

GTGGGTGGCGAAGTGGCAGCCTGCGGCAGAAGCACGCCCAGCAACAG
AAAGTCGTCAACAAGCTCATCCAGTTCCTCATCTCGCTGA

Complement result:

TCAGCGAGATGAGGAACTGGATGAGCTTGTTGACGACTTTCTGTTGCT
GGGCGTGCTTCTGCCGCAGGCTGCCACTTCGCCACCCAC

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Odocoileus virginianus texanus heat shock transcription factor 1 (HSF1), mRNA	137	137	94%	3e-29	96%	XM_020884361.1
PREDICTED: Bos indicus heat shock transcription factor 1 (HSF1), mRNA	137	137	94%	3e-29	96%	XM_019973630.1
Capra hircus heat shock transcription factor 1 (HSF1), mRNA	137	137	94%	3e-29	96%	NM_001314344.2
PREDICTED: Capra hircus heat shock factor protein 1 pseudogene (LOC108635580), misc_RNA	137	137	94%	3e-29	96%	XR_001917842.1
PREDICTED: Capra hircus heat shock transcription factor 1 (HSF1), transcript variant X6, mRNA	137	137	94%	3e-29	96%	XM_018058072.1

D3 hspA1A

hspA1A-R: TGGACGTGTTGAAGTCGATG

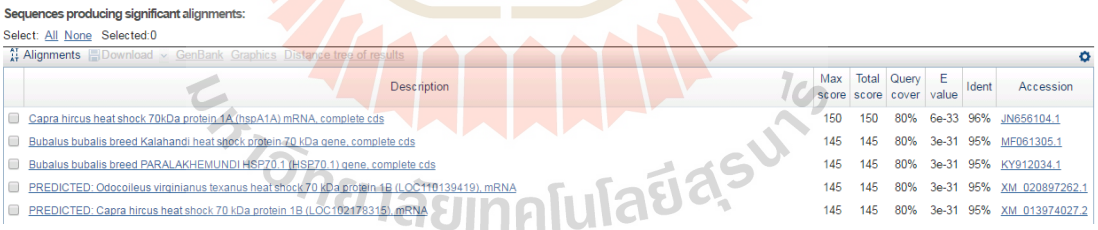
Company result:

GACCCTTAGCGCTGCTTCTGGTCGCGCGAGCCGGACGAAGAGGACCTTGTCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTCGAGGGCATCGACTTCAACACGTCCAA

Complement result:

TTGGACGTGTTGAAGTCGATGCCCTCGAACAGGGAGTCGATCTCCAGGCTGGCCTGGGTGCTGGACGACAAGGTCCTCTTCGTCCGGCTCGCGCGACCAGAAGCAGCGCTAAGGGTC

BLAST result:



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Capra hircus heat shock 70kDa protein 1A (hspA1A) mRNA, complete cds	150	150	80%	6e-33	96%	JN656104.1
☐	Bubalus bubalis breed Kalahandi heat shock protein 70 kDa gene, complete cds	145	145	80%	3e-31	95%	MF061305.1
☐	Bubalus bubalis breed PARALAKHEMUNDI HSP70.1 (HSP70.1) gene, complete cds	145	145	80%	3e-31	95%	KY912034.1
☐	PREDICTED: Odocoileus virginianus texanus heat shock 70 kDa protein 1B (LOC110139419), mRNA	145	145	80%	3e-31	95%	XM_020897262.1
☐	PREDICTED: Capra hircus heat shock 70 kDa protein 1B (LOC102178315), mRNA	145	145	80%	3e-31	95%	XM_013974027.2

D4 TNF

TNF-R: ATGAGGTAAAGCCCGTCAGT

Company result:

GAGCCCGGACTGAAGAACCAGCTGGTGGTGCCCACTGACGGGCTTTACCTCATA

Complement result:

TATGAGGTAAAGCCCGTCAGTGGGCACCACCAGCTGGTTCTTCAGTCCGGGCTC

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus tumor necrosis factor (TNF), transcript variant X1, mRNA	71.3	71.3	70%	1e-09	100%	XM_005696606.3
PREDICTED: Ovis aries tumor necrosis factor (TNF), transcript variant X1, mRNA	71.3	71.3	70%	1e-09	100%	XM_012100437.2
PREDICTED: Pantholops hodgsonii tumor necrosis factor (TNF), transcript variant X2, mRNA	71.3	71.3	70%	1e-09	100%	XM_005965396.1
PREDICTED: Pantholops hodgsonii tumor necrosis factor (TNF), transcript variant X1, mRNA	71.3	71.3	70%	1e-09	100%	XM_005965395.1
Ovis canadensis tumor necrosis factor alpha mRNA, complete cds	71.3	71.3	70%	1e-09	100%	HM017825.1
Ovis canadensis tumor necrosis factor alpha mRNA, complete cds	71.3	71.3	70%	1e-09	100%	EF524262.1
Ovis aries tumor necrosis factor (TNF-alpha) gene, complete cds	71.3	71.3	70%	1e-09	100%	EF446377.1
Capra hircus tumor necrosis factor alpha mRNA, complete cds	71.3	71.3	70%	1e-09	100%	AY304502.1
Capra hircus tumor necrosis factor alpha mRNA, partial cds	71.3	71.3	70%	1e-09	100%	AF276985.1
Ovis aries tumor necrosis factor (TNF), mRNA	71.3	71.3	70%	1e-09	100%	NM_001024860.1
O.aries mRNA for tumor necrosis factor alpha (TNF-alpha)	71.3	71.3	70%	1e-09	100%	X55152.1
Ovine TNF-alpha gene for tumor necrosis factor alpha	71.3	71.3	70%	1e-09	100%	X55966.1
C.hircus mRNA for tumour necrosis factor-alpha	71.3	71.3	70%	1e-09	100%	X14828.1
Capra hircus tumor necrosis factor (TNF), mRNA	71.3	71.3	70%	1e-09	100%	NM_001286442.1

D5 GPX1

GPX1-R: TCGGACGTACTTCAGGCAAT

Company result:

GAACTTGGGCTCAGCTATATGCCATCAACGAGGAGATCCTGAATTGCC
TGAAGTACGTCCGAA

Complement result:

TTCGGACGTACTTCAGGCAATTCAGGATCTCCTCGTTGATGGCATATA
GCTGAGCCCAAGTTC

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	PREDICTED: Capra hircus glutathione peroxidase 1 (GPX1), mRNA	75.0	75.0	92%	2e-10	90%	XM_005695962.3
☐	PREDICTED: Odocoileus virginianus texanus glutathione peroxidase 1 (GPX1), mRNA	69.4	69.4	92%	7e-09	88%	XM_020915885.1
☐	Mammalian expression vector pCAGGSbGPx, complete sequence	69.4	69.4	92%	7e-09	88%	LT727206.1
☐	PREDICTED: Bos indicus glutathione peroxidase 1 (GPX1), mRNA	69.4	69.4	92%	7e-09	88%	XM_019984632.1
☐	PREDICTED: Ovis aries glutathione peroxidase 1 (GPX1), mRNA	69.4	69.4	92%	7e-09	88%	XM_004018462.3
☐	PREDICTED: Ovis aries musimon glutathione peroxidase 1 (GPX1), mRNA	69.4	69.4	92%	7e-09	88%	XM_012122111.2
☐	PREDICTED: Bos mutus glutathione peroxidase 1 (GPX1), mRNA	69.4	69.4	92%	7e-09	88%	XM_005909375.2

D6 GPX2

GPX2-R: AAAGACACTCAGGGCAGGAG

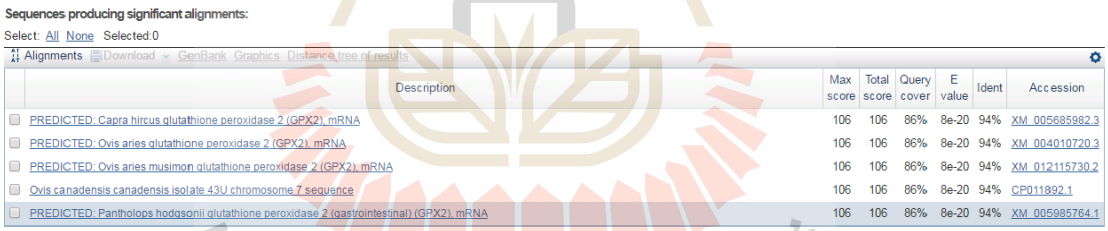
Company result:

GTGGGACCGTCATCAGCGCCTCCTCAAGTTCGCTATATAGACACTAGC AGGTCTATTTCCTCCTGCCCTGAGTGTCTTTA

Complement result:

TAAAGACACTCAGGGCAGGAGGAAATAGACCTGCTAGTGTCTATATA GCGAACTTGAGGAGGCGCTGATGACGGTCCCAC

BLAST result:



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus glutathione peroxidase 2 (GPX2), mRNA	106	106	86%	8e-20	94%	XM_005685982.3
PREDICTED: Ovis aries glutathione peroxidase 2 (GPX2), mRNA	106	106	86%	8e-20	94%	XM_004010720.3
PREDICTED: Ovis aries musimon glutathione peroxidase 2 (GPX2), mRNA	106	106	86%	8e-20	94%	XM_012115730.2
Ovis canadensis canadensis isolate 43U chromosome 7 sequence	106	106	86%	8e-20	94%	CP011892.1
PREDICTED: Pantholops hodgsonii glutathione peroxidase 2 (gastrointestinal) (GPX2), mRNA	106	106	86%	8e-20	94%	XM_005985764.1

D7 SOD2

SOD2-R: AATAAGCATGCTCCCACACG

Company result:

GGGGGGTGTGCTGCTCATAGGAGCAGGGACGCTTGCAGATTGCTGCTTGTTCTAACCAGGATCCCCTGCAAGGAACAACAGGTCTTATCCCCCTGCTGGGGATTGACGTGTGGGAGCATGCTTATT

Complement result:

AATAAGCATGCTCCCACACGTCAATCCCCAGCAGGGGGATAAGACCTGTTGTTCCTTGCAGGGGATCCTGGTTAGAACAAGCAGCAATCTGCAAGCGTCCCTGCTCCTATGAGCAGCACACCCCCC

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus superoxide dismutase 2, mitochondrial (SOD2), mRNA	200	200	85%	7e-48	100%	XM_018053428.1
Capra hircus manganous superoxide dismutase (MnSOD) mRNA, partial cds	200	200	85%	7e-48	100%	GQ204787.1
PREDICTED: Odocoileus virginianus texanus superoxide dismutase 2 (SOD2), transcript variant X2, mRNA	195	195	85%	3e-46	99%	XM_020876404.1
PREDICTED: Odocoileus virginianus texanus superoxide dismutase 2 (SOD2), transcript variant X1, mRNA	195	195	85%	3e-46	99%	XM_020876396.1
PREDICTED: Bos indicus superoxide dismutase 2, mitochondrial (SOD2), mRNA	195	195	85%	3e-46	99%	XM_019967653.1
Bos indicus breed Vechur mitochondrial superoxide dismutase 2 (SOD2) mRNA, complete cds; nuclear gene for mitochondrial product	195	195	85%	3e-46	99%	KT387216.1

D8 SOD3

SDO3-R: ACGGACATCGGGTTGTAGTG

Company result:

GCGGGTAAGGGACTGCGACTCACCGGGCCGCACTACAACCCGATGTC
CGTA

Complement result:

TACGGACATCGGGTTGTAGTGCGGCCCGGTGAGTCGCAGTCCCTTACC
CGC

BLAST result:

PREDICTED: Bubalus bubalis superoxide dismutase 3, extracellular (SOD3), transcript variant X2, mRNA	69.4	69.4	84%	5e-09	95%	XM_006041478.1
PREDICTED: Bubalus bubalis superoxide dismutase 3, extracellular (SOD3), transcript variant X1, mRNA	69.4	69.4	84%	5e-09	95%	XM_006041477.1
PREDICTED: Pantholops hodgsonii superoxide dismutase 3, extracellular (SOD3), transcript variant X2, mRNA	69.4	69.4	84%	5e-09	95%	XM_005980239.1
PREDICTED: Pantholops hodgsonii superoxide dismutase 3, extracellular (SOD3), transcript variant X1, mRNA	69.4	69.4	84%	5e-09	95%	XM_005980238.1
Capra hircus superoxide dismutase 3 (SOD3), mRNA	69.4	69.4	84%	5e-09	95%	NM_001285675.1
Bos taurus superoxide dismutase 3 (SOD3), mRNA	69.4	69.4	84%	5e-09	95%	NM_001082610.1

D9 CAT

CAT-R: 5’AACTGCCTCTCCATTGGCAT3’

Reverse of CAT-R: 3’TACGGTTACCTCTCCGTCAA 5’

Company result:

CGCGATCGGCCATGACGGATATGGATCGCATACTTTCAAGCTGGTTAATGCCAATGGAGAGGCAGT

Complement result:

GCGCTAGCCGGTACTGCCTATACCTAGCGTATGAAAGTTCGACCAATTACGGTTACCTCTCCGTCA

BLAST result:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus catalase (CAT), mRNA	100	100	89%	3e-18	97%	XM_005690077.3
PREDICTED: Ovis aries catalase (CAT), transcript variant X2, mRNA	100	100	89%	3e-18	97%	XM_012096208.2
PREDICTED: Ovis aries catalase (CAT), transcript variant X1, mRNA	100	100	89%	3e-18	97%	XM_004016396.3
PREDICTED: Ovis aries musimon catalase (CAT), transcript variant X2, mRNA	100	100	89%	3e-18	97%	XM_012134220.2
PREDICTED: Ovis aries musimon catalase (CAT), transcript variant X1, mRNA	100	100	89%	3e-18	97%	XM_012134219.2
Ovis canadensis canadensis isolate 43U chromosome 15 sequence	100	100	89%	3e-18	97%	CP011900.1

D10 GAPDH

GAPDH-R: GTACTCAGCACCAGCATCAC

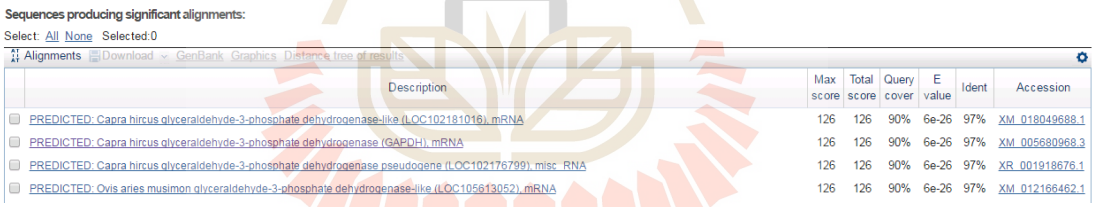
Company result:

GGGGGTCTAATGGAGGCCATCACCATCTTCCAGGAGCGAGATCCTGC CAACATCAAGTGGGGTGATGCTGGTGCTGAGTAC

Complement result:

GTACTCAGCACCAGCATCACCCCACTTGATGTTGGCAGGATCTCGCTC CTGGAAGATGGTGATGGCCTCCATTAGACCCCC

BLAST result:



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Capra hircus glyceraldehyde-3-phosphate dehydrogenase-like (LOC102181016), mRNA	126	126	90%	6e-26	97%	XM_018049688.1
PREDICTED: Capra hircus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA	126	126	90%	6e-26	97%	XM_005680968.3
PREDICTED: Capra hircus glyceraldehyde-3-phosphate dehydrogenase pseudogene (LOC102176799), misc_RNA	126	126	90%	6e-26	97%	XR_001918676.1
PREDICTED: Ovis aries musimon glyceraldehyde-3-phosphate dehydrogenase-like (LOC105613052), mRNA	126	126	90%	6e-26	97%	XM_012166462.1

BIOGRAPHY

Mr. Tian Xingzhou was born in July 15, 1989 in Guizhou province, P. R. China. He received Bachelor Degree (Animal Science) from Southwestern University, Chongqing, China in 2012. He received Master Degree (Ruminant Nutrition) from Guizhou University, Guiyang, China in 2015. In 2015-2018, he received SUT-OROG scholarship for his Ph.D study in School of Animal Production, Suranaree University of Technology.