EXTENSION OF RAW MILK QUALITY DURING STORAGE THROUGH SUPPLEMENTATION OF HYDROCYANIC ACID FROM FRESH CASSAVA PULP AND FRESH CASSAVA PEEL IN DAIRY CATTLE DIET

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การยืดอายุการเก็บรักษาคุณภาพน้ำนมดิบโดยการเสริมกรดไฮโดรไซยานิคจากกากมันสำปะหลังสดและเปลือกมันสำปะหลังสดในอาหารโคนม

นางสาวสุปรีณา ศรีใสคำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2558

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

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สุปรีณา ศรีใสคำ : การยืดอายุการเก็บรักษาคุณภาพน้ำนมดิบโดยการเสริมกรดไฮโดรไซยานิคจากกากมันสำปะหลังสดและเปลือกมันสำปะหลังสดในอาหารโคนม (EXTENSION OF RAW MILK QUALITY DURING STORAGE THROUGH SUPPLEMENTATION OF HYDROCYANIC ACID FROM FRESH CASSAVA PULP AND FRESH CASSAVA PEEL IN DAIRY CATTLE DIET) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.วิศิษฐิพร สุขสมบัติ, 205 หน้า.

วัตถุประสงค์งานวิจัยนี้คือ เพื่อศึกษาผลการใช้กรดไฮโดรไซยานิคจากกากมันสำปะหลัง-สดและเปลือกมันสำปะหลังสดในอาหารโคนมต่อประสิทธิภาพการผลิตและการเจริญเติบโตของจุลินทรีย์ในน้ำนมดิบ

การทดลองที่ 1 และที่ 2 ดำเนินการใช้แผนการทดลองแบบ 2x4 factorial arrangement in CRD ปัจจัยแรกคือ อุณหภูมิ (25 และ 30 องศาเซลเซียส) และปัจจัยที่สองคือ ระดับของโซเดียมไธโอไซยาเนต (NaSCN) (0 7 14 และ 21 มิลลิกรัม/ลิตร) ในการทดลองที่ 1 และระดับของ NaSCN : $2Na2CO_3 3H_2O_2$ (0 : 0 7 : 15 14 : 30 และ 21 : 45 มิลลิกรัม/ลิตร) ในการทดลองที่ 2 และวัดซ้ำค่าสังเกตจำนวน 8 ซ้ำ ณ เวลาการบ่มที่ 0 3 6 9 และ 12 ชั่วโมงในหลอดทดลอง ผลการทดลองแรกพบว่า ความเข้มข้นของไธโอไซยาเนตและกิจกรรมแลคโตเพอร์ออกซิเดสในน้ำนมดิบเพิ่มขึ้นตามระดับที่เพิ่มขึ้นของ NaSCN ที่เติมลงไป องค์ประกอบของน้ำนมไม่ได้รับผลกระทบใดจากการเติม NaSCN การเจริญเติบโตของจุลินทรีย์ทั้งหมดและโคลิฟอร์มถูกยับยั้งที่ระดับการเติม 14 มิลลิกรัม/ลิตร NaSCN ที่ 25 และ 30 องศาเซลเซียส ส่วนผลการทดลองที่ 2 พบว่าระดับความเข้มข้นไธโอไซยาเนตและกิจกรรมแลคโตเพอร์ออกซิเดสในน้ำนมดิบเพิ่มขึ้นตามระดับของ NaSCN : $2Na2CO_3 3H_2O_2$ โดยไม่ส่งผลกระทบต่อองค์ประกอบของน้ำนม ระดับการเติม NaSCN : $2Na2CO_3 3H_2O_2$ ที่มีฤทธิ์เหมาะสมต่อการต้านจุลชีพทั้งอุณหภูมิที่ 25 และ 30 องศาเซลเซียส คือ 14 : 30 มิลลิกรัม/ลิตร

การทดลองที่ 3 โคนมที่ใช้ในการทดลองเป็นโคนมลูกผสมพันธุ์โฮลสไตน์ฟรีเซียน จำนวน 24 ตัว วางแผนการทดลองแบบสุ่มบล็อกสมบูรณ์ (RCBD) ด้วยระยะการให้น้ำนม ปรับสมดุลจำนวนวันของการให้นม ปริมาณน้ำนมและน้ำหนักตัว จากนั้นทำการวิเคราะห์ความแปรปรวนโดยใช้ Analysis of Variance (ANOVA) แบ่งโคออกเป็น 3 กลุ่มการทดลอง กลุ่มละ 8 ตัว โดยโคทุกตัวได้รับอาหารข้นชนิดเม็ดประมาณ 6 กิโลกรัม ที่มีโปรตีนไม่น้อยกว่า 21% และหญ้าสดเป็นแหล่งอาหารหยาบแบบไม่จำกัดปริมาณ ดังนี้ 1) ควบคุมได้รับอาหารข้น 2) เสริมกากมันสำปะหลังสด 3.5 กิโลกรัม/วัน (35 พีพีเอ็ม HCN) 3) เสริมกากมันสำปะหลังสด 7.0 กิโลกรัม/วัน (70 พีพีเอ็ม HCN) ผลการทดลอง พบว่าการเสริมกากมันสำปะหลังสด 3.5 และ 7.0 กิโลกรัม/วันและมีหญ้าสด

เป็นแหล่งอาหารหยาบแบบไม่จำกัดปริมาณในอาหารโคนม ไม่ส่งผลต่อกระทบต่อน้ำหนักตัวของโค ปริมาณน้ำนมและองค์ประกอบน้ำนม จำนวนจุลินทรีย์ทั้งหมดและโคลิฟอร์มลดลงที่ระดับการเสริมกากมันสำปะหลังสด 3.5 และ 7.0 กิโลกรัม/วัน เปรียบเทียบกับกลุ่มควบคุม การเสริมกากมันสำปะหลังสดที่ 3.5 และ 7.0 กิโลกรัม/วัน เพิ่มประสิทธิภาพการต่อต้านจุลินทรีย์ด้วยระบบแลคโตเพอร์ออกซิเดสในน้ำนม ทั้งนี้การเสริมกากมันสำปะหลังสดที่ 3.5 กิโลกรัม/วัน สามารถใช้ในอาหารโคนมได้ ถึงแม้ว่าความเข้มข้นของไธโอไซยาเนตและกิจกรรมของแลคโต-เพอร์ออกซิเดสในน้ำนมดิบที่การเสริมกากมันสำปะหลังสด 7.0 กิโลกรัม/วัน จะสูงกว่ากลุ่มการทดลองอื่น

การทดลองที่ 4 โคนมที่ใช้ในการทดลองเป็นโคนมลูกผสมพันธุ์โฮลสไตน์ฟรีเชียน จำนวน 24 ตัว วางแผนการทดลองแบบสุ่มบล็อกสมบูรณ์ ด้วยระยะการให้น้ำนม ปรับสมดุลจำนวนวันของการให้นม ปริมาณน้ำนมและน้ำหนักตัว จากนั้นทำการวิเคราะห์ความแปรปรวนโดยใช้ ANOVA แบ่งโคออกเป็น 3 กลุ่มการทดลอง กลุ่มละ 8 ตัว โดยโคทุกตัวได้รับอาหารข้นชนิดเม็ดประมาณ 6.5 กิโลกรัม ที่มีโปรตีนไม่น้อยกว่า 21% และหญ้าหมักเป็นแหล่งอาหารหยาบแบบไม่จำกัดปริมาณ ดังนี้ 1) ควบคุมได้รับอาหารข้น 2) เสริมเปลือกมันสำปะหลังสด 400 กรัม/วัน (65 พีพีเอ็ม HCN) 3) เสริมเปลือกมันสำปะหลังสด 800 กรัม/วัน (130 พีพีเอ็ม HCN) ผลการทดลอง พบว่าการเสริมเปลือกมันสำปะหลังสดไม่ส่งผลต่อกระทบต่อน้ำหนักตัวโค ปริมาณน้ำนมและองค์ประกอบน้ำนม ผลการศึกษานี้แสดงให้เห็นว่าการเสริมเปลือกมันสำปะหลังสด 400 และ 800 กรัม/วัน สามารถเพิ่มประสิทธิภาพกิจกรรมของแลคโตเพอร์ออกซิเดสในน้ำนมดิบ โดยลดจำนวนจุลินทรีย์ทั้งหมดและโคลิฟอร์มลงได้ ดังนั้นการเสริมเปลือกมันสำปะหลังสดที่ระดับ 400 กรัม/วัน สามารถใช้ในอาหารโคนมได้

สาขาวิชาเทคโนโลยีการผลิตสัตว์ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SUPREENA SRISAIKHAM : EXTENSION OF RAW MILK QUALITY DURING STORAGE THROUGH SUPPLEMENTATION OF HYDROCYANIC ACID FROM FRESH CASSAVA PULP AND FRESH CASSAVA PEEL IN DAIRY CATTLE DIET. THESIS ADVISOR : ASSOC. PROF. WISITIPORN SUKSOMBAT, Ph.D., 205 PP.

HYDROCYANIC/FRESH CASSAVA PULP/FRESH CASSAVA PEEL/RAW MILK

The objectives of this research were to extend the raw milk quality during storage by supplementing fresh cassava pulp (FCPu) and fresh cassava peel (FCPe) in dairy cow's diet and to determine the effect on productive performance.

Experiments I and II were conducted as a 2x4 factorial in completely randomized designs (CRD), which factor A was temperature (25ºC vs 30ºC) and factor B with different levels of sodium thiocyanate (NaSCN) (0, 7, 14 and 21 mg/L) in Experiment I, and NaSCN : $2Na2CO_33H_2O_2$ (0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L) in Experiment II with 8 replicates per run using 0, 3, 6, 9 and 12 h incubation time *in vitro* assay. The results from Experiment I showed that the thiocyanate (SCN^-) concentration and lactoperoxidase (LP) activity in raw milk increased with increasing NaSCN. The milk composition was not significantly affected after the addition of NaSCN. The total bacterial count (TBC) and coliform count (CC) growth were inhibited at 14 mg/L NaSCN both at 25°C and 30°C while the results from Experiment II showed that the SCN^- concentrations and LP activities in raw milk increased with increasing NaSCN : $2Na_2CO_33H_2O_2$ levels. The milk composition was not affected after the NaSCN : $2Na_2CO_33H_2O_2$ addition. LP activated milk was an antimicrobial activity at 14 : 30 mg/L NaSCN : $2Na_2CO_33H_2O_2$ both at 25°C and 30°C.

In Experiment III, 24 Holstein Friesian (HF) crossbred lactating dairy cows were assigned into a randomized complete block design (RCBD) with 8 cows in each group. All cows were fed approximately 7 kg/d of 21% crude protein (CP) concentrate and *ad libitum* fresh grass. Treatments were the control concentrate for the 1st group, the 2nd group received the control concentrate supplemented with 3.5 kg/d of FCPu (35 ppm HCN) and the 3rd group received the control concentrate supplemented with 7.0 kg/d of FCPu (70 ppm HCN). The 3.5 and 7.0 kg/d FCPu had no effect on live weight change (LWC), milk yield and milk composition. SCC, leukocytes and neutrophil were lowest at 7.0 kg/d FCPu. TBCs and CCs were lower in 3.5 and 7.0 kg/d FCPu compared to the control. The results showed that at 3.5 and 7.0 kg/d FCPu increased the efficiency of antibacterial activity of the LPs in raw milk. Therefore, 3.5 kg/d FCPu can be used in the concentrate for lactating dairy cows although SCN^- and LP activity of 7.0 kg/d FCPu cows were higher than other treatments.

In Experiment IV, 24 HF crossbred lactating dairy cows were assigned into RCBD. All cows were fed approximately 6.5 kg/d of 21% CP concentrate and *ad libitum* grass silage. Treatments were the control concentrate for the 1st group, the 2nd group received the control concentrate supplemented with 400 g/d FCPe (75 ppm HCN) and the 3rd group received the control concentrate supplemented with 800 g/d FCPe (150 ppm HCN). The FCPe supplementation had no effect on total DMI, LWC, milk yield and milk composition. The results showed that 400 and 800 g/d FCPe enhanced the efficiency of LP activity in raw milk to reduce TBC and CC, therefore 400 g/d FCPe can be used in the concentrate for lactating dairy cows.

School of Animal Production Technology Student's Signature_____

Academic Year 2015 Advisor's Signature_____

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Supreena Srisaikham

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LIST OF ABBREVIATIONS

ADF	=	Acid Detergent Fiber
ADICP	=	Acid Detergent Insoluble Crude Protein
ADIN	=	Acid Detergent Insoluble N
ADL	=	Acid Detergent Lignin
CAC	=	Codex Alimentarius Commission
CC	=	Coliform Count
FAO	=	Food and Agriculture Organization of the United Nations
FCM	=	Fat Corrected Milk
HTST	=	High Temperature Short Time
H_2O_2	=	Hydrogen Peroxide
JECFA	=	Joint FAO/WHO Expert Committee on Food Additives
LPs	=	Lactoperoxidase System
NDICP	=	Neutral Detergent Insoluble Crude Protein
NDIN	=	Neutral Detergent Insoluble N
NE	=	Net Energy
NPN	=	Non Protein Nitrogen
NRC	=	National Research Council
ppm	=	Parts per million
RDP	=	Rumen Degradable Protein
RDP_{req}	=	Rumen Degradable Protein Requirement

LIST OF ABBREVIATIONS (Continued)

RDP_{sup}	=	Rumen Degradable Protein Supply
RUP	=	Rumen Undegradable Protein
RUP_{req}	=	Rumen Undegradable Protein Requirement
RUP_{sup}	=	Rumen Undegradable Protein Supply
TBC	=	Total Bacterial Count
tdCP	=	Truly Digestible Crude Protein
SCN^-	=	Thiocyanate
UHT	=	Ultra-High Temperature (sterilization)/Ultra Heat Treated
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

The raw milk quality preservation during transportation was an exceedingly important factor for milk products because some dairy cattle farms were in areas remote from the Milk Collection Centers, especially for those farming small holdings whereby there was a lengthy duration for transportation and mechanical refrigeration was either unavailable or economically prohibitive. Without refrigeration, milk products can only be transported a distance of up to 20 km, after which they begin to deteriorate, beginning the process of acidification (FAO, 2002). The dispersed nature of the production across diverse farm operations, difficulties with collection, poor handling systems and inadequate transportation and refrigeration systems all create considerable challenge to extended raw dairy cow's milk quality during storage in several developing countries (Devandra, 1999; Seifu et al., 2003). This is especially relevant in Thailand where cooling facilities and poor handling systems, are often unavailable in rural areas which are poorly resourced in terms of technology including the cooling equipment required to maintain milk quality.

It has been reported that lactoperoxidase system (LPs), an innate immune factor which is naturally present in milk, has been recommended for the preservation of raw milk at room temperature as an alternative of cooling in some developing countries (Saha et al., 2003). Milk LP catalyses, lead to oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2) to generate hypothiocyanite (OSCN) and

hypothiocyanous acid (HOSCN : Shin et al., 2001) and the oxidation of I to generate hypoiodite (OI) and hypoiodous acid (HOI; Bosch et al., 2000). These compound which are effective against various living microbes (Kussendrager and Van Hooijdonk, 2000), thus enhancing the microbiological quality of milk (Seifu et al., 2005). As a result, the bacteriological quality of raw milk is safeguarded by LPs during storage and transportation through refrigeration in developing countries and LP is also an alternative option for dairy cattle farmers to preserved raw milk quality instead of the use of cooling systems as some indigenous SCN^- is usually present and certain bacteria produce H_2O_2. FAO/WHO have reported that the LPs of raw milk preservation is currently the only approved method other than relatively expensive artificial cooling systems. However, LP technology can generalize to appropriate for farmers, by using the dietary sources of SCN^-; glucosinolates and cyanogenic glycoside. Cyanogenic glycosides is found in cassava tissues (Wolfson and Sumner, 1993), which that is ruminant feed. Cyanogenic glycoside are disintegrated to hydrocyanic acid after tissues were destroyed. Cyanide is transformed to the non-toxic thiocyanate by rhodanese action in the majority of liver and kidneys of animals (Punthanara et al., 2009). SCN^- will be eliminated via milk (Soto-Blanco and Gorniak, 2003) that useful for maintaining the quality of raw milk. This results in a blocking of bacterial metabolism thereby preventing the multiplication of bacteria present in the raw milk (Joint FAO/WHO, 2005) and inhibit various spoilage and pathogenic organisms thus enhancing the microbiological quality of milk (Seifu et al., 2005).

Thus for farmers operating small farm holdings the presentation of the conclusions of the research will help to develop the understanding that cooling of the product during transportation between farm and dairy plant is a superior method for

the extension of the raw milk quality during both transportation and storage and should assist to reduce the incidence of resorting to chemical additives to achieve the same result. The objective of several parts of this thesis proposal is to study the aspects of safety and effectiveness of LPs treatment to extend raw milk quality during storage (shelf-life), in ambient temperatures, by focusing on different concentrations of SCN^- and/or the activation of SCN^- and sodium percarbonate for retaining the quality of raw cow's milk. These several parts are also to aspects of the effectiveness of extending shelf-life of raw milk during storage by increasing milk SCN^- via fresh cassava pulp and fresh cassava peel supplementation in dairy cows feed.

1.1 Research objectives

1.1.1 To study the inhibitory effect of thiocyanate levels on microorganism growth in raw milk samples.

1.1.2 To study the inhibitory effect of thiocyanate and sodium percarbonate levels on microorganism growth in raw milk samples.

1.1.3 To study the utilisation of fresh cassava pulp in dairy cow's diet on productive performances and microorganism growth in raw milk.

1.1.4 To study the utilisation of fresh cassava peel in dairy cow's diet on productive performances and microorganism growth in raw milk.

1.2 Research hypothesis

1.2.1 Thiocyanate can decrease total bacterial count and coliform count and can extend raw milk quality during storage (shelf-life).

1.2.2 Thiocyanate and sodium percarbonate can decrease total bacterial count and coliform count and can extend raw milk quality during storage (shelf-life).

1.2.3 Fresh cassava pulp can be used in dairy cow's diet, provide thiocyanate, decrease total bacterial count and coliform count, has no negative effect in productive performance and can be used as an effective treatment to extend raw milk storage.

1.2.4 Fresh cassava peel can be used in dairy cow's diet, provide thiocyanate, decrease total bacterial count and coliform count, has no negative effect in productive performance and can be used as an effective treatment to extend raw milk storage.

1.3 Scope of the study

These researches intended to study, 1) the raw milk samples from Suranaree University's dairy farm will be taken and thiocyanate and/or thiocyanate and sodium percarbonate will be added to prevent bacterial spoilage of raw milk quality during storage and 2) Lactating, crossbred, Holstein Friesian dairy cows from Suranaree University's dairy farm will be used in the studies of increasing the efficiency of antibacterial activity of the LPs in raw milk.

1.4 Expected results

1.4.1 Thiocyanate does decrease total bacterial count and coliform count and can extend raw milk quality during storage.

1.4.2 Thiocyanate and sodium percarbonate does decrease total bacterial count and coliform count and can extend raw milk quality during storage.

1.4.3 Extended raw milk quality during storage is realized through supplementation of thiocyanate from fresh cassava pulp in dairy cow's diet, total

bacterial count and coliform count are decreased, has no negative effect in the milk composition, milk yield, or productive performance.

1.4.4 Extended raw milk quality during storage is realized through supplementation of thiocyanate from fresh cassava peel in dairy cow's diet, total bacterial count and coliform count are decreased, has no negative effect in the milk composition, milk yield, or productive performance.

1.5 References

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CHAPTER II

LITERATURE REVIEW

2.1 Background

In 1924, Hanssen, a visionary scientist, was the first to introduce the agricultural and animal husbandry industries to the concept of the involvement of LPs in inhibition of bacterial growth and this was ultimately demonstrated by Wright and Tramer (1958). Known as the “LPs” it was capable of achieving an extended raw milk quality during storage by utilizing a naturally occurring defense mechanism in the body to inhibit bacterial proliferation (Klebanoff and Luebke, 1965; Morrison and Allen, 1966; Slowey, Eidelman and Klebanoff, 1968), a phenomenon which was at first recognized as a hazard in the manufacture of cultured dairy products from normal cow's milk, which contained relatively large amounts of LP and SCN (Hogg and Jago, 1970). The raw milk quality preservation during transportation was an exceedingly important factor for milk products because some dairy cattle farms were in areas remote from the Milk Collection Centers, especially for those small holdings dairy farm whereby there was a lengthy duration for transportation and mechanical refrigeration was either unavailable or economically prohibitive. Without refrigeration, milk products can only be transported a distance of up to 20 km, after which they begin to deteriorate, beginning the process of acidification (FAO, 1990). The dispersed nature of the production across diverse farm operations, difficulties with collection, poor handling systems and inadequate transportation and refrigeration

systems all create considerable challenge to extend raw dairy cow's milk quality during storage in several developing countries (Seifu et al., 2003). This is especially relevant in Thailand where cooling facilities are often unavailable in rural areas which are poorly resourced in terms of technology including the cooling equipment required to maintain milk quality. However, today the LPs has been recommended for the preservation of raw milk at room temperature as an alternative of cooling (The International Dairy Federation, IDF, 1988). During a test in 1957 from the analysis and approval by the Food and Agriculture Organization (FAO), the possibility of using H_2O_2 for conservation of raw milk for human consumption in conditions where refrigeration is not possible in many developing countries, officially or without express approval of national legislation, showed that it could be used for that purpose. A report in 2003 indicated that some developing countries are still using LP as an alternative system of milk preservation.

After 15 years of field experiments in developing countries, a code of practice was adopted for the use of an alternative system of milk preservation based on the activation of a complex natural antibacterial enzyme in milk (LP) by the Expert Committee on Food Additives of FAO/WHO in 1990 and the Codex in 1991 (Codex Alimentarius Commission, Codex Committee on Milk and Milk Products, 1991). Milk SCN^-, a natural antimicrobial agent of the LPs presented in raw milk (Zapico et al., 1991), can inhibit various spoilage (microorganisms that causes food to deteriorate and develop unpleasant odors, tastes, and textures (USFDA, 2011)) and pathogenic organisms, thus enhancing the microbiological quality of milk (Seifu et al., 2005). LP constitutes a naturally occurring defense mechanism in the body to control microbial proliferation (Shimizu et al., 2011) which found in various mammalian in their

secretions and is a component of saliva, milk, tears, bronchial, nasal cavity, lachrymal glands of mammals, intestinal secretion, milk, harderain glands and the other exocrine secretions (Naidu, 2000). The activity of LP is that the enzyme catalyzes the peroxidation of SCN^- by H_20_2 and some halides to an unstable oxidation product to generate hypothiocyanate or OSCN products which effective against alive microbes (Kussendrager and Van Hooijdonk, 2000). The system damages the inner membrane causing leakage and cessation of uptake of nutrient, leading eventually to death of the organisms and lysis. These products have antibacterial effects, reducing bacterial growth by damaging the cell membranes and inhibiting the activity of many cytoplasmic enzymes (Haddadin, Ibrahim and Robinson, 1996). However, the gram positive and negative bacteria are not inhibited by the LPs only but are also dependent on the medium pH, temperature, incubation time, cell density and the particular electron donor. They may be destroyed provided that H_2O_2 is supplied exogenously, either chemically, enzymatically or by H_2O_2-producing bacteria (Björck et al., 1975). Therefore, the normal mode of action is that the inner membrane of gram-negative bacteria appears to be more damaged by LP treatment than that of gram-positive species (Reiter and Härnulv, 1984). As a result, the bacteriological quality of raw milk is safeguarded by LPs during storage and transportation through refrigeration in developing countries and LPs is also an alternative option for dairy cattle farmers to preserve raw milk quality instead of the use of cooling systems as some indigenous SCN^- is usually present and certain bacteria produce H_2O_2. FAO/WHO have reported that the LPs of raw milk preservation is currently the only approved method other than relatively expensive artificial cooling systems. Many developing country trials

have shown that, in the absence of refrigeration, the shelf life the raw milk can be significantly prolonged by the application of the LPs (El-Agamy et al., 1992).

Thus, for small holding dairy farmers, the presentation of the conclusions of the research will help to develop the understanding that activating of LPs the product during transportation between farm and dairy plant is a superior method for the extension of the raw milk quality during both transportation and storage and should assist to reduce the incidence of resorting to chemical additives to achieve the same result.

2.2 The condition of milk testing and raw milk quality control system of Thai dairy factories before purchasing

With respect to the conditions for milk testing and quality control systems in Thailand, a level of industrial organisation is required for dairy factories before purchasing raw milk, to enable grading of raw milk for processing into various dairy products and to set a purchase price. In the past, the milk is tested and graded (A, B, C) in a number of ways such as; particle containing test, TBC, specific gravity, milk fat content and observations on dairy farms for hygienic quality test, (Ministry of Science and Technology, 1987). In 1996, Dairy Farming Promotion Organisation of Thailand sets the techniques that most dairy factories use this technique in milk quality control testing before purchasing including hygiene quality test (e.g., colour, dreg and particle containing), Methylene blue reduction test (MBRT), clot on boiling test and alcohol test (Food and Agriculture Organisation; FAO, 1999). These tests are widely used as they are quick, simple, low cost and the results can be trusted. As per Dairy Farming Promotion Organisation of Thailand in 2000 regulations for grading

raw milk for processing into dairy products, dairy factories purchased raw milk that was approved by MBRT for decolourisation time to identify the microbiological quality of milk. According to the standard plate count technique (SPC) which is a test that requires longer time, is more complicated to analyse and also requires a specialist to perform the testing. Dairy cooperatives and dairy factories implement sampling and testing of milk delivered from their members twice per month or double check to assure the quality of raw milk in cases where an MBRT result is lower than the specified standard.

However, the situation regarding the purchase of raw milk in Thailand has changed over time. In 2008, Dairy cooperatives are the performed primarily for milk collection center that buy milk from farmers and then milk is sold to milk processing companies. (Rabobank, 2004). Rhone (2008) reviewed that the system of milk pricing in Thailand comprised a base price plus additions or deductions, usually based on the quality of raw milk. Until now the standard price for one kilogram of raw milk sold ranges from approximately 18 to 19 Baht. Due to administration problem of operation and transportation costs of milk collection centers, the base price that farmers actually receive possibly lower than this (MOAC, 2006). Therefore, a large part of the pricing system is depending on what companies and products dairy cooperatives are marketing their milk. To the base price in 2015, as per the regulation of Ministry of Agriculture and Cooperatives, MOAC) sets the techniques that most dairy factories use in milk quality control testing before purchasing based on total solids (TS). According to raw milk pricing system, the dairy farmer can maintain a milk fat content between 3.40 and 3.59 while, TS between 12.00 and 12.59, thus they will be able to achieve the standard price. The rules for milk price based on additions and

deductions for TS that was analyzed by Lactometric or Ultrasonic method or Infrared spectroscopy of MOAC. The TBC in raw milk is found to be more than $5x10^5$ CFU/mL which indicates raw milk quality as unacceptable in milk production processing. TBC are also used for grading raw milk and for setting a purchase price. Where TBC are determined to be higher the value of the milk is reduced commensurately. Milk with TBC less than $3.0x10^5$ CFU/mL, farms will be able to get extra milk price (0.10 Baht/kg) from the standard pricing system, while a price is paid lower for milk with TBC above $7.0x10^5$ CFU/mL or the milk collecting centres may reject milk until the quality is better. If farms can maintain a SCC of less than 200,000 cells/cm^3, farms will be able to achieve or exceed the maximum addition the milk price at 0.20 Baht/kg from the standard pricing system. However, the current situation of most small, medium and large dairy farms is that they are struggling to maintain low bacterial scores and SCC in bulk tank and, as a result, they are receiving lower farm milk revenue and profits.

2.3 Keeping quality (KQ) of raw milk

Bacteria were found in milk mostly from containers (e.g., bulk tank), milking equipment, milkers and barn sanitation. Bacteria constantly found in raw milk are lactobacillaceae and micrococcaceae family, local bacteria are *L. casei*, *L. acidophilus*, *L. brevis*, *S. lactis* and *S. cremoris*. These types of bacteria are non-pathogenic microorganisms which will not cause diseases excluding to create lactic acid and others, which reduce pH in milk. Besides, the other bacteria found in milk, such as *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Bacillus*, *E. coli*, and coliform bacteria (enterobacteriaceae family) can also be found anywhere and also in human

digestive system, grow fast at temperature over 20^{o}C (Walstra et al., 1999). When these bacteria grow rapidly, they will change the milk quality. As milk has a high nutritive value that composes of an ideal for bacterial growth, which will affect the milk quality. Storage of milk at low temperature will prevent or reduce these problems. The greatest temperature to store milk varies as each type of bacteria requires different temperature for their growth showing in Table 2.1. Van Heddeghem and Vlaemynck (1992) had discovered *Bacillus cereus* bacteria, which harm the quality of raw milk. It resists heat and can grow at low temperature, specified standard milking process, and milk manufacturing process can prevent this bacteria in milk. Common bacteria in milk mainly psychrotrophs family can grow at temperature below 15^{o}C and produce protease and lipase enzymes that digest proteins and fat in milk and leave rancid off-flavors and putrid. Although this bacteria family is easily killed off by pasteurization, but protease and lipase enzymes are still left in milk which will change smell and taste of milk. *Pseudomonas* (Cousin, 1982) and mesophiles group, such bacteria will degrade the raw milk quality when store milk at room temperature for long period or during transportation to dairy factory without keeping it cool (Praksangounsri, 1989). Besides, bacteria belonging to thermodurics group, thermophiles, coliform, including pathogenic bacteria are also found. Cleanliness temperature of the milk and directly transportation after milking are essential for keeping the raw milk quality. Besides, cooling is necessary to prevent spoilage rotten of milk. The low temperature will decrease the bacterial growth because bacterial cell will be less active at low temperature. The induction of LP enzyme reaction will affect the cell activity and that will change the structure of proteins, which affect the protoplasm colloidal system and will decrease or destroy

cell metabolism. Bacteria are destroyed slowly when their cells subject to toxic in large doses. Reduction of temperature until the water in cell forming ice crystals can also kill bacteria as its cell will be penetrated.

Table 2.1 The greatest temperature to store milk varies as each type of bacteria requires different temperature for their growth.

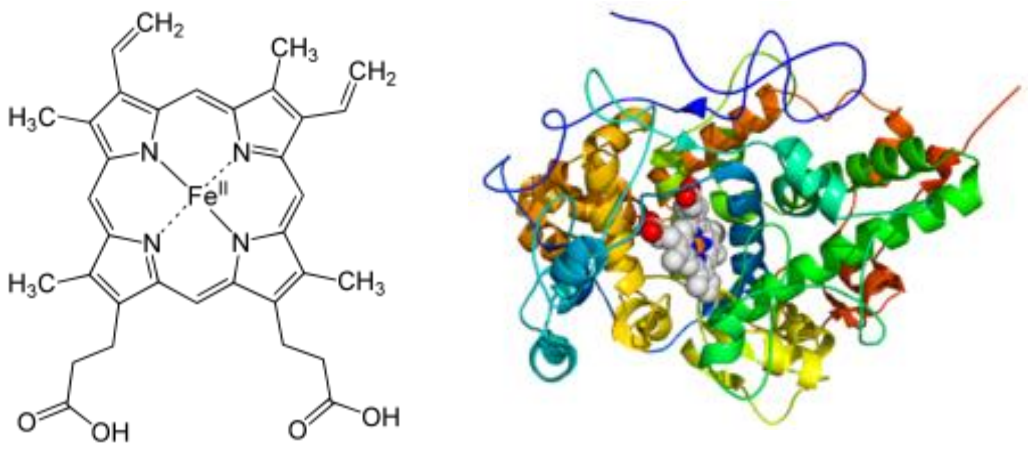
Temperature to store milk (°C)	**Types of bacteria**
0-5	*Pseudomonas spp.*
5-10	*Pseudomonas spp.*, *Proteus vulgaris* and *Micrococcus sp.*, *Alcaligenes viscolctis* and *A. marshallii*
10-15	Various of *Streptococcus*, namely *S. lactic, S. acidominimus, S. faecalis, S. agalactiae, S. durans, S. dysagalactiae* and *S. uberis*
15-30	*S. lacis*
30-40	*Aerobacter aerogenes*, *E.coli* and many types of *Lactobacillus*, namely *L. bulgaricus, L. casei, L. brevis, L. caucasicus, L. fermenti, L. lactis, L. helveticus, L. plantarum, L. thermophilus* and *L. leichmannii*
40-50	Many types of *Lactobacillus*, namely *L. lactis, L. fermenti, L. helveticus, L. bulgaricus, L. caucasicus, L. thermophilus*; *S. faecalis, S. thermophilus* and *yeast*

Source : Suksringam (1982).

2.4 Lactoperoxidase system (LPs)

2.4.1 LPs

LP (alternate names: salivary peroxidase (SPO) and/or EC 1.11.1.7), plays an important role in the innate immune system in protecting the lactating mammary gland and mucosal secretions (Naidu, 2000). The LP enzymes derive from an animal's blood plasma, leukocytes and the apical membrane or cytoplasm of the secretory cells (Fox and Kelly, 2006). The peroxidase includes eosinophilic peroxidase in intestinal tissues (Rytomaa and Teir, 1961), myeloperoxidase in leukocytes (Klebanoff, 1970), and thyroid peroxidase in cell membranes (Ohtaki et al. 1982). It is synthesized and released from alveolar epithelial cells (Harada et al., 1973) and neutrophils (Moldoveanu et al., 1982). The LP has a molecular weight of approximately 77.5 kDa (Sisecioglu et al., 2010) combining with heme. Iodide reacts directly with the heme group, the complex then iodinates the substrate on adding H_2O_2 (Morrison and Bayse, 1973). LP contains 15 half-cysteine residues and carbohydrate moieties that comprise approximately 10% of the molecular weight (Sisecioglu et al., 2009, 2010) as shown in Figure 2.1. LP activity is denatured at pH lower than 4 is denatured at 80^{o}C/2.5 seconds and tolerant on pasteurization at 63^{o}C with 30 minutes or at (high temperature short time (HTST) 161°F (72^{o}C) for about 15 to 30 seconds (Walstra et al., 1999) as shown in table 2.2. Joint FAO/WHO (2006), pasteurization has confirmed the little effect on LP activity, leading to complete destruction of LP enzyme at 80^{o}C or more (UHT sterilization). Correspondingly, Sheehan (2007) showed that HTST pasteurization of cow's milk destroys 30% of the LPs.



(a) (b)

Figure 2.1 An iron-containing molecule that binds with proteins as a cofactor or prosthetic group to form the hemoprotein in the structure of lactoperoxidase enzyme. (a) Formula structure (b) Crystallographic structure of lactoperoxidase.

Source : http://www.answers.com/topic/heme#ixzz2MkG9yc7F

http://metallo.scripps.edu/PROMISE/2CYP.htm

The system of LP consists of three components: LP, SCN^- and H_2O_2 (Reiter and Härnulv, 1984). LP enzyme alone is unable to inhibit the growth of an array of bacteria whereas the combination of LP with H_2O_2 and SCN^- creates the system of LP. The LPs appeared probable that antibacterial activity is catalyzed by the LP enzyme which is naturally present in milk leading to the oxidation of SCN^- by H_2O_2 to form a compound (e.g., hypothiocyanite ($OSCN^-$)) which inhibits microbial growth (Barrett, Grandison, and Lewis, 1999). These substances vary in concentrations in animal's milk, and LP enzyme quantity depends on several factors mainly the breed,

age, season, lactation stage, day of lactation, feed and animal health (Fonteh et al., 2001; 2002; 2006; Mariba, 2006). They also demonstrated that the efficiency of the system (which is generally assessed by determining enzyme activity) varies with a number of substrates present. Usually, LP enzyme presents very low concentration approximately 30 mg/L in cow's milk but rapidly increases 4-5 days after giving birth and at 20 times in human's milk. The LPs of Verata goats is significantly lower than Murciano-Grandina goats (0.95 and 2.15 U/mL respectively). The LP activities of these two breeds are at the highest level of 135-150 and 60-75 days of lactation respectively (Zapico et al., 1991). However, the concentration of LP activity is at 4.45$\pm$1.94 U/mL, SCN^- at 10.29$\pm$4.76 µg/mL and $OSCN^-$ at 1.11$\pm$0.84 µg/mL in goat milk (Schoos et al., 1999). Stage of lactation, age and milk compositions of goat were not affected by the LP enzyme activity although SCN^- milk concentration depends on animal feeding and stage of lactation. In addition, LP activity was correlated with SCC in goat (Seifu et al., 2007). SCC and LP activity were observed to be a positive correlation that suggests the LPs may be used to detect subclinical mastitis in dairy goats. Accordingly, Isobe et al. (2010) indicated that the correlation of LP activity to the SCC in bovine milk may point to the potential use of formers as an indicator of SCC.

Table 2.2 Sources of enzymes, pH and optimum temperature and enzyme inactivation in raw milk.

Name	EC Name	Optimum		Activity[a]		Where in milk	Inactivation[b]
		pH	Temperature (°C)	Potential	Actual		
Xanthine oxidase	1.1.3.22	~8	37	>>40	40	Fat globule membrane	7 min 73°C
Sulfhydryl oxidase	1.8.3.2	~7	~45	-	-	Plasma	3 min 73°C
Catalase	1.11.1.6	7	37	-	300	Leukocytes	2 min 73°C
Lactoperoxidase	1.11.1.7	6.5	20	-	22,000	Serum	10 min 73°C
Superoxidase dismutase	1.15.1.1	-	37	~2,000	-	Plasma	65 min 75°C
Lipoprotein lipase	3.1.1.34	~9	33	3,000	0.3	Casein micelles	30 s 73°C
Alkaline phosphatase	3.1.3.1	~9	37	500	<<500	Fat globule membrane	20 s 73°C
Ribonuclease	3.1.27.5	7.5	37	(C)	-	Serum	-
Plasmin	3.4.21.7	8	37	3	0.05	Casein micelles	40 min 73°C

Source : Walstra et al. (1999).

[a] $\mu mol \cdot min^{-1} \cdot 1^{-1}$.

[b] Heat treatment needed to reduce activity to approximately 1%.

[c] 11-25 mg enzyme per kg of milk., - = not report.

Kussendrager and Hooijdonk (2000) shown that the antibacterial activity of LP occurs from the combination of H_2O_2 and SCN^-. The LPs function is predominant that of defense against microbial infections or activity and bacteriostatic and bactericidal effects against a broad spectrum of micro-organisms were showed *in vitro* studies (Wolfson and Sumner, 1993). FAO/WHO (2005) reported that LPs were bactericidal effect and bacteriostatic on some common milk-borne pathogens (Pitt, Harden and Hull, 2000). Others (Pitt et al., 1999; Gay and Amar, 2005) reported that similar LP effects on the other microorganisms causing human infections and domestic animals. Several applications now exist where the LPs is commercially used as a natural preservative (de Wit and Van Hooydonk, 1996).

2.4.2 Thiocyanate (SCN^-)

SCN^- is recognized as rhodanide which is the anion and conjugate base of thiocyanic acid. Biological chemistry of SCN^- is known to be an important part in the medicine and biosynthesis of hypothiocyanite by LPs (Conner et al., 2007). SCN^- is present in the salivary glands, mammary gland, thyroid gland, adrenal gland and secretions (Reiter and Harnulv, 1984). It is excreted in the urine and kidney (Wolfson and Sumner, 1993) but naturally present in raw milk approximately 4-5 mg/L, thus less than 20 times of plasma (Korhonen et al., 1977). Although H_2O_2 systemic supply is generated by the polymorphonuclear neutrophils during phagocytosis, the flora in an intestine and in the mouth may excrete H_2O_2 to trigger the LPs activity (Reiter and Perraudin, 1991). It presents in saliva approximately 50-300 mg/L and 40-50 mg/L in the digestive enzyme of human. LP activity equivalent to enzyme protein concentrations between 20-40 mg/L is found frequently (Mullan, 2003). These are marked in excess of the levels (0.5 µg/mL in the absence of catalase) required for

efficient LPs activation (Björck, 1978). While the amount of H_2O_2 does not appear in raw milk or find at very low levels, because the natural LPs in raw milk loses its effect within 2 h after milking which acquires from phagocytosis and the microorganism growth of Lactobacilli, Lactococci and Streptococci in aerobic condition (Kussendrager and Hooijdonk, 2000). The amount of milk SCN^- concentration varies according to season of the year, cows healthy and types animal feed (Thomas, 1981), breed and lactation cycle (Zapico et al., 1991) and animal species (de Wit and Van Hooydonk, 1996).

The researches have been reviewed about the utilisation of plant containing cyanide with animal diets in numerous articles (WHO, 1993). Normally, cyanide is presented in various plants such as raw lima beans (100-3100 mg/kg), raw cassava tubers (10-462 mg/kg), raw cassava leaves (68-468 mg/kg), dried cassava root cortex (2450 mg/kg), almonds (6.2 mg HCN/bitter almond), bamboo shoots tips (around 8000 mg/kg), stone fruits and sorghum (around 2500 mg/kg) (FAO, 1990). The action of rhodanese reaction in liver and kidneys of animals transforms cyanide by detoxification to the non-toxic SCN^- (Drakhshan Vaziri and Aminlari, 2004), an enzyme widely distributed in nature. SCN^- is eliminated mainly via the urine, milk, tears and saliva (Soto-Blanco and Górniak, 2003).

SCN^- comes from common dietary sources of 1). glucosinolates which hydrolysed to SCN^- and 2). cyanogenic glucosides, when cyanogenic plant tissues are damaged, ruptured or otherwise injured, the action of endogenous plant enzymes release free cyanide. Genus *Brassica* vegetables (family Crucciferae, an average 100 mg/kg of SCN^-) such as cabbage, cauliflower, clover, brussel sprout, turnips, broccoli and radish are rich in glucosinolates (Kussendrager, 2000). The cyanogenic

glucosides are present in cassava, potatoes, corn, sorghum, sugar cane and beans or the kernel of various fruits, etc. (Oerlemans et al., 2006). Rumen microbial enzymes could digest cyanogenic glycosides and are rapidly absorbed from the rumen (Majak and Cheng, 1984), which, in a reaction with thiocysteine or thiosulfate (metabolic product), is detoxified into SCN^-, these latter were catalyzed by the enzyme rhodanese (Punthanara et al., 2009). The foliages contain high sulfur (S), as feedstuffs for ruminant which these are hydrolyzed to SCN^- and then it appears in raw milk for preserving the milk quality. However, cyanides readily decompose upon heating as sun-drying. Wanapat et al. (2001) reported that increased SCN^- in raw milk was observed in roughage a week before milking when fed cassava hay (CH) as basal diet in lactating cows. HCN and SCN^- concentrations in milk were increased with increasing levels of cassava pulp up to 30% in roughage mixtures (Khungaew, 2009). The SCN^- concentrations and the efficiency of antibacterial activity of the LPs in raw milk increase when CH was supplemented in early-to-mid lactation dairy cow's diets (Punthanara et al., 2009). The concentration of milk SCN^- was increased by increasing level of ensiled cassava foliage supplementation (0, 2 and 4 kg/h/d) in lactating dairy cows, and number of milk SCC was decreased which may improve milk quality and decrease mastitis in lactating cows (Petlum et al., 2012).

2.4.3 Hydrogen peroxide (H_2O_2)

H_2O_2 is the one component in the LPs. It is not normally detected in raw milk (Seifu, Buys and Donkin, 2004b). Seifu et al. (2005) reported that H_2O_2 may be generate endogenously by polymorphonuclear leucocytes in the phagocytosis processing. *Lactobacilli*, *Lactococci*, and *Streptococci* produce sufficient H_2O_2 under aerobic conditions to the LPs-activated (Wolfson and Sumner, 1993). H_2O_2 may

generated by adding H_2O_2, $2Na_2CO_33H_2O_2$ and glucose oxidase to stimulate LPs (Kussendrager and Van Hooijdonk, 2000). High H_2O_2 is toxic for mammalian cells, however, at low concentrations (100 µM or less) of LP and SCN^- mammalian cells are protected from this toxicity (Pruitt and Kamau, 1991).

2.4.4 Mechanism of lactoperoxidase system

LP is an effective antimicrobial agent and is used as an antibacterial agent in reducing bacterial microflora in milk (Thomas, 1981) and milk products (Reiter and Härnulv, 1984). LP reactions are shown below.

$$H_2O_2 + 2SCN^- + 2H^+ \longrightarrow 2H_2O + (SCN)_2 \quad (1)$$

$$(SCN)_2 + H_2O \longrightarrow HOSCN + H^+ + SCN^- \quad (2)$$

$$HOSCN \rightleftharpoons OSCN^- + H^+ \quad (3)$$

Sum of these equations are

$$H_2O_2 + SCN^- \longrightarrow OSCN^- + H_2O \quad (4)$$

From the enzyme's reaction in equation (1), there is thiocyanogen (SCN_2) excluding it non-stable and it is hydrolyzed to hypothiocyanous and SCN^- as in equation (2). The oxidation reaction of SCN^- in condition of neutral pH will have the product as hypothiocyanite ion ($OSCN^-$) as in equation (4).

$$OSCN^- + \text{protein-SH} \longrightarrow \text{protein-S-SCN}+OH^- \quad (5)$$

$$HOSCN + \text{protein-SH} \longrightarrow \text{protein-S-SCN}+H_20 \quad (6)$$

LP is an electron acceptor to catalyzes, in the presence of H_2O_2, the oxidation of SCN^- to attain either $OSCN^-$ and hypothiocyanous acid (HOSCN) as the major products (Shin et al., 2001) and the oxidation of I to yield hypoiodite (OI) and

hypoiodous acid (HOI; Bosch et al., 2000). Both the amount of $OSCN^-$ and HOSCN have reduced agent property against bacterial metabolism and growth. These compounds specifically react with the microbial protein sulfhydryl (SH-group) to sulfenyl SCN^- derivatives (the equation (5) and (6)) to inhibit various cellular functions. It leads to disruption of the cuticle damaging the cell membranes and inhibiting the enzymatic activity of bacterial cytoplasmic membrane (Shin et al., 2001), also directs in leaking of potassium ions, amino acids, peptide, purines and pyrimidines and transport glucose across the plasma membrane, thus subsequently bacteria were killed or denaturalized (Kussendrager and Hooijdonk, 2000).

2.5 Antimicrobial spectrum effect of lactoperoxidase system

Seifu et al. (2005) reviewed and reported that the significance of the LPs can exert both bacteriostatic and/or bactericidal activity on a variety of susceptible microorganisms including bacteria, fungi and viruses. The molecular mechanism(s) of such inhibitory microbial cellular function effects depend on the kind of electron donor, medium, temperature, pH, incubation time, cell density and could range from oxidative killing to blockage of glycolytic pathways or interference in cytopathic effects (Naidu, 2000). The difference of bacteria groups show a varying degree of sensitivity to the LPs (Seifu et al., 2005), it can probably be elucidated by the differences in cell wall structure and their different barrier properties (de Wit and van Hooydonk, 1996). LP present in various secretions oxidizes SCNK by H_2O_2 to produce OSCNK, which is bactericidal for enteric pathogens including multiple antibiotic resistant strains of *E. coli* (Naidu, 2000). The LPs activity seems to be related with microbial sulphydryl groups of *E. coli* (Thomas and Aune, 1978). The

oxidation of sulfhydryls to sulphenyl derivatives inhibits bacterial respiration. Linked to Shin et al. (2001) indicated these OSCN and HOSCN compound against *E. coli* is related to the inhibition of dehydrogenases in the respiratory chain of *E. coli*. LP-activated in goat milk, *E. coli* were low during storage at 8^{o}C for 5 days (Zapico et al., 1995). Although the LPs can elicit bacteriostatic and bactericidal activities against strains of *S. typhimurium* (Purdy et al., 1983), the permeability of the bacterial cell wall was an important factor to be destroy by the bactericidal activity. The LPs was both bactericidal and bacteriostatic against *S. aureus* (Seifu et al., 2004b). Although pasteurisation processing destroyed *S. aureus*, enterotoxins, these strains of this pathogen can withstand pasteurisation and cause food poisoning (Smith, Buchanan and Palumbo, 1983). Denis and Ramet (1989) reported that the effect of high LPs levels, either 20 or 40 ppm are more inhibit inoculated growth of *L. monocytogenes* after 9 and 16 h. Similar results were also reported (Boussouel et al., 2000; Seifu et al., 2004b). The LPs inhibited growth of *E. coli* and the corresponding wild-type, *S. typhimurium*, *P. fluorescens*, *S. aureus*, *L. innocua* and *L. plantarum*, by high hydrostatic pressure in skim milk (Garcia-Graells et al., 2000; 2003). Mclay et al. (2002) reported that the use of LPs and monolaurin in the range of 5-200 mg kg^{-1} and 50-1000 ppm respectively, inhibited *E. coli* O157 : H7 and *S. aureus* growth. Accordingly, Dajanta et al. (2008) reported that the LPs-activated was greatly increased KQ of milk to decline at the rate of 78% (*E. coli* treated milk sample). These all above results clearly demonstrate that the LPs can serve as an alternative method to control the microbial growth in cow milk.

2.6 The applications and preservation of raw milk and milk products using the lactoperoxidase system

FAO/WHO (2005) reported that the LPs of raw milk preservation is the one alternative approved method other than relatively expensive artificial cooling systems. The LPs has been reported to have a wide range of beneficial outcomes for smaller dairy producers in supplying milk and dairy products to milk collection centers, particularly in many developing countries, such as Asia and West Africa (Food and Agriculture Organization of the United Nations, FAO, 2005). LPs is a cooperative term with food safety used to describe one of that maintains the initial milk quality without refrigeration until milk can be processed and to significantly extend its shelf life (IDF, 1988).

LPs efficiency has reliably yielded antibacterial effects to retard bacterial growth and gram-negative bacteria appear to be more damaged than that of gram-positive species. The traits of control characteristics and inhibition of microorganism growth were bactericidal effects which almost are gram-negative bacteria/catalase positive bacteria, (Wolfson and Sumner, 1993) that can inhibit some parts of microorganism growth only. This results in a blocking of bacteria metabolism thereby preventing bacteria multiplication present in the milk (JECFA, 2005). It can be regenerated themselves resembling bacteriostatic effect and does not present a toxicological hazard which evaluated by JECFA (2005) "The bacteriostatic effect of the system means that it neither improves nor disguises poor quality milk (Nichol et al., 1995). However, FAO (2005) demonstrated that the LPs-activated in milk at 10 ppm of SCN^- (powder form) increase the overall level to 15 ppm (at 5 ppm is naturally present). However, the effectiveness in restraining the growth of bacteria

during transportation to milk products factory depended on temperature keeping. The solution is thoroughly mixed for 30 sec and then an equimolar amount 8.5 ppm of H_2O_2 is added (generally in the form of a granulated sodium carbonate peroxyhydrate). The LP activated a bacteriostatic effect and effectively extended raw milk quality to prolong the shelf life for 7-8 h under ambient temperatures at around 30^{o}C or longer at lower temperatures.

Many research documents suggest to add SCN^- and/or H_2O_2 in raw milk or milk products for controlling spoilage bacteria and preservation during storage and/or transportation to processing plants. The addition of SCN^- and H_2O_2 together with 3% glucose and 0.1 U/mL glucoseoxidase in milk, can have antibacterial activity of the LPs against psychrotrophic bacteria, *Pseudomonas* species and other gram-negative bacteria (Kamau et al., 1990a; b). The coliform, *S. aureus*, psychrotrophs bacteria and fungi in raw milk was reduced by LP-activated (Garcia-Garibay et al., 1995). This finding was similar to those reported by other researchers (Pruitt, 2003; Fonteh et al., 2005; Kanloung et al., 2007). The milk pasteurized with LP treatment, can be reactivated to inhibit microbial growth to extend the shelf life of raw milk (Barrett et al., 1999). Heat resistant was used as an indicator of temperature over pasteurized milk (Marks et al., 2008). Although Heuvelink et al. (1998) confirmed that the LPs by adding SCN alone was unable to retain raw milk quality or inhibit the *E. coli* (O157 VTEC strain), it may be used alone or in conjunction with processing of pasteurization to reduce or eliminate the bacterial load in milk products (Musser, 2011).

Nowadays, the use of LPs has been recognized for many years and has been used in countries where unavailable refrigeration or cooling system. It was used extensively as an effective anti-bacteriostatic agent to reduce microflora in milk and milk products (Tan and Ockerman, 2005). Both cow and buffalo maintained milk quality during storage by an addition of SCN as 14 mg NaSCN and H_2O_2 in the form of 30 mg $2Na_2CO_3 3H_2O$. A few studies have been attempted to apply the LPs in meat and poultry products (Kennedy et al., 2000). Currently, it is also applied as antibacterial agents in cosmetics, ophthalmic solutions, dental, wound action as antitumor and antiviral agents as shown in Table 2.3. Finally, FAO also provided knowledge and manual guidance for using LPs under Global Lactoperoxidase Programme. However, the LPs required at least, 40-50 L of milk and the skill persons to handle the process because it required SCN^- quantity testing before adding substance which is needed 10 mg/L higher than normal.

The addition of H_2O_2 and SCN^- extended the shelf life of raw milk at 10^oC for at least three days, with a decrease in bacterial numbers during storage. The LP application is mainly used in milk and dairy products (Tan and Ockerman, 2005).

Table 2.3 Applications of the LPs to utilize as antibacterial agents.

Products	**LP-system**	**Functions**	**Results**
Raw milk	By natural	Preserving	4 Days 4^oC
Raw milk	SCN/Hydrogen peroxide	Shelf life	2 Days 10^oC
Pasteurized milk	SCN/Hydrogen peroxide	Shelf life	21 Days 10^oC
Cheese milk	SCN/Hydrogen peroxide	Shelf life	8 Days 4-10^oC
Yogurt	LPO	Acidity control	14 Days 20^oC

Table 2.3 Applications of the LPs to utilize as antibacterial agents (Continued).

Products	**LP-system**	**Functions**	**Results**
Cosmetics	LPO/KI/SCN/SCN/GO	Preserving	2-4 months
Dental	LPO/SCN/LYS/GO	Healing	Every of day
Ophthalmic solutions	LPO/KI/SCN/GO	Preventing	1 week
Anti-tumor	LPO/GO/antibodies	Healing	According to dead line

Source : Kussendrager and Hooijdonk (2000).

2.7 The safety level application of the components and the intermediary products of the lactoperoxidase system

The safety and toxicological assessment of the safety levels of using LPs are in terms of public health outcomes, pose a significant risk to human health. Providing consumers allergic to milk protein are aware of its presence setting by The Australia New Zealand Food Authority (ANZFA). ANZFA has ruled to organize usage of LPs function and the scope of the application to meat and meat products (including poultry) fish and fish products; and milk and milk products.

2.7.1 LP enzyme is allowed to use with meat at 1-20 mg/kg meat. This level found an average of 30 ppm in milk which is the non toxic risk but may be sensitive to people who are cow's milk allergy.

2.7.2 SCN^- is allowed to used in term of NaSCN or KSCN at the proposed use levels of 5-40 mg/kg meat, this level is low range when compared with

human's liquid (10-20 ppm) or vegetable (Brassia; 100 ppm) (ANZFA, 2002). However, the maximum amount of potentially allergenic milk protein that could be present would be about 1 mg/kg meat.

2.7.3 H_2O_2 is allowed to use at 5-50 mg/kg meat, the substance decomposes to O_2 and water when contacting the tissues. The H_2O_2 is disintegrated by itself or enzyme catalyzes in the epidermis or mucous membrane; thereafter, H_2O_2 is reversed quickly during enzymatic oxidation reaction of SCN to generated SCN^- and water, thus no risk of toxicity.

2.8 References

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CHAPTER III

THE INHIBITORY EFFECT OF THIOCYANATE LEVELS ON MICROORGANISM GROWTH IN RAW MILK SAMPLES

3.1 Abstract

Two experiments were conducted to evaluate the effect of 2 different temperatures and 4 sodium thiocyanate (NaSCN) levels on raw milk samples *in vitro* assay. Experiment 1 (Exp. 1) was to determine the effect of NaSCN addition on thiocyanate (SCN^-) concentration, lactoperoxidase (LP) activity and milk compositions. Experiment 2 (Exp. 2) was to examine whether NaSCN addition decrease total bacterial count (TBC) and coliform count (CC) as an effective treatment to extend raw milk quality during storages. The experiments were conducted as a 2x4 factorial in completely randomized designs (CRD), which factor A was temperature (25ºC vs 30ºC) and factor B with different levels of NaSCN (0, 7, 14 and 21 mg/L) with eight replicates per run using 0, 3, 6, 9 and 12 h incubation time. Raw milk was collected from the bulk tank of collection centres. The milk composition, SCN^- concentration, hydrogen peroxide (H_2O_2), and LP activity in raw milk were determined. Plates containing media nutrient agar (NA) powder and eosin methylene blue (EMB) agar were incubated to encourage bacterial growth, the resulting numbers of colonies were counted and recorded as colony forming units per

ml (CFU/mL) of milk. The milk composition was not significantly affected after the LP activation. The SCN^- concentration and LP activity in raw milk increased when NaSCN solution was added at 0, 7, 14 and 21 mg/L, respectively. In contrast, LP-activated milk had TBC and CC comparatively lower than the control for both 25ºC and 30ºC, consequently 7, 14 and 21 mg/L of NaSCN addition led to improvements in the preservation of the milk quality. An obvious effect for the LP activated milk was the inhibition of TBC and CC at 14 mg/L of NaSCN both at 25ºC and 30ºC. It can be concluded that improved preservation of milk can be achieved through the addition of NaSCN (14 mg/L) both at 25ºC and 30ºC to extend raw milk quality and to decrease TBC and CC in raw milk which are the main causes of the deterioration of milk over time.

Keywords : sodium thiocyanate, microorganism growth, raw milk quality

3.2 Introduction

Increased delivery times due to inadequate transportation, lack of available refrigeration systems and funding all provide considerable challenges to extend the quality of raw dairy cow's milk during storage (Seifu et al., 2003). This is the fundamental problem contributing to the decline in the quality of raw milk produced by a large number of smallholding dairy farmers and the subsequent economic losses associated with it are significant. Therefore, the use of non-immunoglobulin glycoproteins, a naturally occurring enzyme presented in the antimicrobial system in raw milk to preserve the milk quality is known as LP system (LPs) (Siragusa and Johnson 1989). It is a cooperative term with food safety used to describe one of that

maintains the initial quality of milk without refrigeration until milk can be processed and its beneficial outcomes for smallholder dairy producers in supplying milk to milk collecting center (FAO, 2005). LP is synthesized and released from alveolar epithelial cells (Harada et al., 1973) and neutrophils (Moldoveannu et al., 1982). LP and its substrate SCN^- released are secreted in tears and saliva (Thomas et al., 1994) and also appeared to present in milk (Isobe et al., 2009b). While, it is true that LP activity is ubiquitous in cow's milk, the concentration varies widely from 1.2-19.4 U/mL (Pruitt, 2003) and the milk SCN^- concentration ranged from 0.1 to 15 mg/kg (Björck et al., 1979; Perraudin, 1991) or approximately 1-10 ppm (Davidson,1997), it also varies according to animal species (de Wit and Van Hooydonk, 1996), breed, lactation cycle, season of the year, cows' healthy and types animal feed (Zapico et al., 1991).

The addition of small quantities of naturally occurring substances such as SCN^- to raw milk has been demonstrated to stimulate the innate antibacterial system presented in the milk and to significantly extend its shelf life (Joint FDA/WHO, 1991; FSANZ, 2002). These techniques are required to be simple, relatively inexpensive and not represent any risk to consumers. SCN^- is naturally presented in the salivary glands, mammary gland, thyroid gland, adrenal gland and their secretions and in the liquids (Seifu, 2003). Rate of SCN^- excretion relates to the level of SCN^- in the blood, it was increased with increasing outflow rate of SCN^- excretion. Zapico et al. (1991) reported that milk SCN^- is a natural antimicrobial agent of the LPs presented in raw milk which it can cause antimicrobial activity, thus enhancing the microbiological quality of milk (Seifu et al., 2005). The use of SCN^- (in the form of sodium or potassium salt) approximately 14 mg/L added into milk has been reported sufficient to activate the LPs (FAO, 2005). SCN^- is oxidized by H_2O_2 to generate the

hypothiocyanite ($OSCN^-$) and hypothiocyanous acid (HOSCN) products and other intermediates (Shin et al., 2001). These products have antibacterial effects, reducing bacterial growth by damaging the cell membranes and inhibiting the activity of many cytoplasmic enzymes (Haddadin et al., 1996). It has been shown that the LPs can increase storage times of raw milk by delaying bacterial growth (Wolfson and Summer, 1993); perhaps this SCN^- could be the majority precursor for LPs-activated to extend the shelf life of raw milk.

Therefore, the effects of the LPs by adding NaSCN and optimum rates need to screen using *in vitro* methods before NaSCN can be used in raw cow's milk, because *in vitro* method can screen large number of samples and treatments. Thus, this study was to evaluate the inhibitory effect of the LPs by adding various NaSCN levels on microorganism growth in raw cow's milk as an effective treatment to extend raw milk quality during storage.

3.3 Objective

The objective of this experiment was to study the inhibitory effect of thiocyanate levels on microorganism growth in raw milk samples as an effective treatment to extend raw milk quality during storage.

3.4 Materials and methods

Experiment 1 (Exp.1) and Experiment 2 (Exp.2) were conducted as a 2x4 Factorial in completely randomized design (CRD) with 8 treatments combination arranged as a factorial (2 different temperatures x 4 NaSCN levels) with eight replicates per run. In both experiments, the runs were conducted on the same 4 NaSCN levels and separate temperature and time of incubation were used.

3.4.1 Milk sampling

Approximately 1 L each of raw cow's milk samples was collected from milk tank of collection centres, at Suranaree University of Technology (SUT) farm. Milk samples were divided into two main portions with one stored at 25^{o}C and another at 30^{o}C to examine before (as control) and after LP activation of the milk by using NaSCN at 0, 7, 14 and 21 mg/L. Exp.1 (first portion) milk samples were analysed for milk composition, SCN^- concentration, H_2O_2 and LP activity immediately. Exp.2 (second portion); milk samples were examined for microbiological properties including TBC and CC before and after LP-activation. Milk samples were diluted and then spread on a medium plate and then incubated at 0, 3, 6, 9 and 12 h to count colony forming units per ml (CFU/mL) of milk.

3.4.2 Analysis of thiocyanate in milk

SCN^- of raw milk samples were determined according to Codex Alimentarius Commission (CAC/GL 13-1991). Four mL of milk was mixed with trichloroacetic acid (TCA) 20% (w/v) solution and then filtered through a suitable filter paper (Whatman No. 40). 1.5 mL of the clear filtrate was then mixed with ferric nitrate reagent [$(Fe(NO_3)_3.9H_2O$] and dissolved in 50 mL 2M HNO_3 obtained by diluting 138.5 mL 65% HNO_3 to 1000 mL with distilled water and then stored in darkness at 4^{o}C. SCN^- concentration was determined in the milk after deproteinisation with TCA, as the ferric complex by measuring the absorbance at 460 nanometres (nm). The measurement was required to be carried out within 10 min from the addition of the ferric nitrate solution as the coloured complex was not stable for any length of time. SCN^- at concentrations of 0, 3, 5, 10, 15, 20 and 25 ppm were used as standards.

3.4.3 Analysis of hydrogen peroxide concentration in milk

H_2O_2 concentration in milk was performed according to a modification method of Bjorck (1978) and Allen and Wrieden (1982). Absorbance was measured at 436 nm by the spectrophotometer using H_2O_2 as solution standard graph.

3.4.4 Activation of lactoperoxidase system

The LPs was activated by the addition of SCN^- (in the form of sodium thiocyanate (NaSCN). NaSCN was prepared from 99.7% purity NaSCN powder (UNIVA, APS, NSW, Australia), then SCN^- solution was added into raw milk at 0, 7, 14 and 21 mg/L respectively, and then stirred thoroughly for 1 min thereby increasing the SCN^- content.

3.4.5 Measurement of lactoperoxidase activity

LP activity in milk was determined by the method described by Wijkstrom-Frei et al. (2003) and Isobe et al. (2009b). Milk fat was removed immediately by centrifugation at 12,000 rpm for 5 min at 4°C. 10 µL of skim milk powder was mixed with 200 µL of tetramethyl benzidine (TMB) solution and incubated at 37°C for 30 min. After brief centrifugation at 6,000 x g for 1 min, the optical density of supernatant was measured at 655 nm wavelength by spectrophotometer. Lactoperoxidase from bovine milk (lyophilized powder) (E.C. 1.11.1.7) (Sigma, St. Louis, MO) at concentrations of 0, 2.4, 3.4, 4.9, 7.0, 10.0, 14.4, 18.9 and 24.0 U were used as standards. The quantitative results were expressed in U/mL of enzyme. (1 unit formed 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C).

3.4.6 Analysis of milk composition

Raw milk samples were collected from milk tank of collection centres both in the evening and morning milking on 2 consecutive days of each 5-d period and stored

at 4°C until analysed for milk composition at 25°C and 30°C using Lactostar (Art. no : 3510; Funke Gerber Labortechnik GmbH, Berlin). The milk compositions were analysed immediately before and after LP-activation including fat, protein, lactose, solid not fat (SNF) and total solid (TS) contents.

3.4.7 Total bacterial count

Total bacterial count was determined by the method described by AOAC (2000). 1.) The medium was prepared by Nutrient Agar powder (NA) (M001; HIMEDIA) by suspending 28 g of NA in 1000 mL of purified water. The medium was boiled to completely dissolve and then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. The medium was repeated shake for suspending the precipitate of the medium. 2.) Milk samples were diluted with deionized water up to 1 : 10, 1 : 100 and 1 : 1000. One mL of milk sample was poured in a semi-solid medium plate and then incubated for 24±3 h at 36°C to encourage bacterial growth. The resulting numbers of a colony were counted and recorded as CFU/mL of milk.

3.4.8 Coliform count

Coliform bacteria were determined by the method described by AOAC (2000). The medium was prepared by using EMB Agar (Levine) (M002; HIMEDIA) by suspending 37.46 g of EMB Agar in 1000 mL of purified water. The medium was sterilized by autoclaving. The experimental procedures were the same as described for TBC, except that medium plate was incubated for 24±2 h.

3.4.9 Statistical analysis

Data analyses were conducted using the General Linear Models procedure of the statistical analysis system (SAS, 1996). Data from Exp.1 and Exp.2 were analyzed separately as a 2x4 Factorial in CRD with temperature, NaSCN levels and their

interaction included in the model as fixed factor effects. When the interaction between temperature and NaSCN levels was significant, orthogonal polynomial contrasts were performed to determine linear, quadratic and cubic responses to the temperature within NaSCN levels. When the main effect of NaSCN levels was significant, an orthogonal polynomial contrast was performed to determine overall linear, quadratic and cubic responses to temperature. Significance was declared at $P<0.05$.

3.4.10 Experimental location

The experiment was conducted at Suranaree University of Technology's Dairy Cattle Farm, the Center for Scientific and Technological Equipment, Building 10, Suranaree University of Technology, Nakhon Ratchasima.

3.4.11 Experimental period

This experiment was carried out during April 2014 to July 2014 at an average ambient temperature of 27.5°C (http://www.e-report.energy.go.th).

3.5 Result and discussion

The present study was undertaken to elucidate the effect of milk SCN^- on counts of microorganism in raw cow's milk. The important findings were : 1) that activation of raw milk LPs by the addition of NaSCN at 0, 7, 14 and 21 mg/L increased concentration of SCN^-, H_2O_2 and LP activity in raw cow's milk samples; 2) that activation of raw milk LPs by the addition of NaSCN had no adverse effects on raw milk compositions; and 3) the inhibitory effect of NaSCN levels on microorganism growth was to decrease TBC and CC and was an effective treatment for extending raw milk quality during storage (shelf-life).

3.5.1 Effect of temperatures and NaSCN levels on SCN^- and H_2O_2 concentrations and LP activity (Exp. 1)

The initial concentration of SCN^-, H_2O_2 and LP activity in raw cow's milk before LPs activation by the addition of NaSCN at 25°C shown in Table 3.1, were 3.43 ppm, non-detectable and 4.08 U/mL of enzyme respectively, whereas at 30°C they were 3.33 ppm, non-detectable and 3.84 U/mL of enzyme respectively. The resulting an initial concentration of SCN^- in natural raw milk was low both at 25°C and 30°C, being similar to that observed in dairy milk (Crossbred Holstein Friesian) at ambient temperature examined from the same collection centres at SUT farm by Maneerate (2006) but lower than those reported by Lambert (2001) and Panthanara et al. (2005) who showed the average SCN^- of 5.00 and 5.16 ppm respectively. Similar results were obtained for cow milk (6-12 mg/L; mean 8.5 mg/L) (Fonteh et al., 2002). The normal levels of SCN^- have been reported to vary between 2.3 and 35 mg/L from individual cows and to be around 8 mg/L in bulked milk (Ponce et al., 2005). However, the result of SCN^- concentration was within the 1-15 ppm normal range of SCN^- concentrations in bulk bovine milk (Reiter and Härnulv, 1984). The SCN^- has been reported to vary considerably depending on udder health, breeds and its precursors in the animals' diet types, including thioglycosides (glucosinolates) and cyanogenic glycosides (Kussendrager and van Hooijdonk, 2000), which could explain the differences observed. Feedstock is the major dietary source of SCN^- substrates; glucosinolates and cyanogenic glucosides (Wolfson and Sumner, 1993) and these sources generate SCN^- in raw milk (Seifu et al., 2005). However, the cows used in the present study were fed on the same ration during the experiment, it would not cause the differences in SCN^-.

SCN^- concentration was increased after LPs activation. The concentration of SCN^- at 25ºC was higher than 30ºC after activation of the LPs. The effect of LPs activation by the addition of NaSCN level on SCN^- concentration depended upon the level (temperature x NaSCN level interactions, $P \leq 0.01$). For 25ºC and 30ºC, the response to NaSCN level were differences of exponential functions to linear, quadratic and cubic functions with highest SCN^- concentration, such that at both temperature all levels increased SCN^- concentration compared to the control (0 mg/L NaSCN). FAO/WHO (The Codex guidelines) have established a guideline recommendation for the use of the LPs activation by the addition of 10-15 mg/L of NaSCN into raw milk, so that overall levels in activated bulk milk would be in the order of 20 mg/L. The present study required 14 and 21 mg/L NaSCN to increase SCN^- to 16.7 and 22.0 ppm which approximately be in the elevated range of Codex guidelines. This is in accordance with the findings of Panthanara et al. (2005) where elevated milk SCN^- concentrations were reported with NaSCN solutions added. Normally, the level of SCN^- in milk of individual cows varies widely, ranging from 2.3 to 35 ppm, that is why the total content of SCN^-, once the LPs is activated in a milk mixture, does not surpass the natural maximal concentration in any particular cow milk (Ponce et al., 2005). Evidence for more than 10 years of undesirable effects were not observed in the populations consuming milk activated with the LPs (Fernandez, Marrero and Capdevila, 2005).

H_2O_2 was not detectable at all levels of NaSCN addition (0, 7, 14 and 21 mg/L). This result shows that raw milk is not contaminated with the bacillus, catalase-negative bacteria, including lactic acid bacteria, namely Lactobacilli, Lactococci and Streptococci even though the mastitis infection (*S. aureus* and *Streptococcus sp*.).

These bacteria possess the ability to produce sufficient amounts of H_2O_2 under anaerobic conditions. The raw milk sampled from the collection centre was confirmed as the source from healthy dairy cattle at SUT's standard farm as certified by Department of Livestock Development (DLD), Thailand. Maneerate (2006) also reported that H_2O_2 was not detectable in dairy raw milk at SUT farm. Other authors reported lower H_2O_2 concentrations in the milk of other species. For instance, Althaus et al. (2001) obtained the average value of 0.39 mg/L for dairy ewe milk while Schiffman et al. (1992) observed 2 to 4 mg/L for sterile cow milk. In fact, H_2O_2 is generally non-detectable in raw milk (Siefu et al., 2005) and also thermodynamically unstable and decomposes to form water and oxygen and is also decomposed biologically by enzyme catalase.

LP activity in raw milk was also moderately low. The LP activity activated by NaSCN showed that the enzyme was lower than Panthanara et al. (2005) who reported the mean value of LP activity was 7.55 U/mL in Chiangmai, Thailand, whereas the result was higher than that of Fonteh et al. (2002) who reported an overall mean of 2.3$\pm$1.0 U/mL. However, the result from the present study was covered within the normal range of LP activity in raw cow's milk of 1.2-19.4 U/mL (Priutt, 2003). Normally, LP activity in milk measured only at 1.44 U/mL which is sufficient to act as a catalyst for an activity of LPs (Marshall et al., 1986). Previous research has demonstrated that variations in LP activity were probably dependent on individual animals, breeds and seasons (Fonteh, 2002; 2006), stages of lactation (primiparous or postpartum) as well as the health of the dairy cow. The LP is produced in large amounts in milk in an early stage of lactation, especially in primiparous cows (Isobe et al., 2011). Fonteh et al. (2002) also reported that some cows showed high LP

activity postpartum, followed by a decrease during lactation. Moreover, lactation of cows and the duration of storage affect the concentration of the LPs because the concentrations of the components are reduced. Furthermore, LP activity and SCN^- concentrations changes over the duration of the cow's lactation, while H_2O_2 concentration is reported as rather more stable. Because H_2O_2 was produced as a result of the presence of microorganisms in raw milk, the LPs in raw milk has not performed to optimum levels in general (Althaus et al., 2001).

The effect of LPs activation by the addition of NaSCN level on LP activity depended upon the NaSCN level (temperature x NaSCN level interactions, $P \leq 0.001$). The LP activity at 25ºC was higher than 30ºC after activation of the LPs. For 25ºC, the responses to NaSCN level was differences of exponential functions to linear and cubic for LP activity. Effects of temperature and NaSCN level were more prominent for the level of SCN^- concentration than for LP activity. SCN^- concentration increased up to 2.99, 4.93 and 6.52 times at 25ºC, while LP activity increased 2.45, 3.93 and 5.41 times at 7, 14 and 21 mg/L NaSCN respectively.

For 30ºC, the response to NaSCN level were linear, quadratic and cubic, such that at both temperatures all NaSCN levels increased LP activity compared to the control (0 mg/L NaSCN). Similarly, SCN^- concentration increased respectively by up to 2.85, 4.92 and 6.51 times at 30ºC and LP activity increased by up to 2.44, 3.92 and 5.40 times. Thus, the increases in SCN^- concentration and LP activity were fairly similar for 25ºC and 30ºC. Other researchers have never observed the result of SCN^- concentration and LP activity by addition of NaSCN (0, 7, 14 and 21 mg/L) in raw milk combined with temperatures between 25ºC and 30ºC. On the other hand, literature data on the effectiveness under different ambient conditions have been

variable. Given that the efficacy of the LPs persists for a limited period of time, it decreases as the ambient temperature increases (CAC, 1991b). This temperature dependence of the effectiveness of the LPs was defined only in a range between 15°C and 30°C. LP remains active during optimum temperature of 20°C (Walstra et al., 1999).

Experimental data and experience from practice indicate that the LPs can be applied beyond the temperature limits (15-30°C) referred to the 1991 Codex guidelines (CAC, 1991b). The kinetics of thermal inactivation of the LP enzyme are well established (e.g. Barrett et al., 1999). Although LP activity tolerated on pasteurization at 63°C with 30 min or at (high-temperature short time (HTST) 72°C/15 seconds and denatured at 80°C with 2.5 seconds (Ramet, 2004; Walstra et al., 1999). HTST pasteurization results in retention of approximately 70% LP while treatment at 80°C or more (including conventional or UHT sterilisation) leads to complete destruction of the enzyme. Similarly, Sheehan (2007) showed that HTST pasteurization of cow's milk destroys 30% of the LPs. Pasteurization at 65°C/30 min or HTST batch pasteurization has confirmed the little effect on enzyme activity and complete destruction of LP enzyme at 80°C or more (including conventional or UHT sterilisation) is noted (Joint FAO/WHO, 2005). However, despite the higher appearance activity of the LPs at low temperature than at high temperature, we find no prior reports on its guarantee or use as indicative for determining an initial LP activity in raw milk and after activation the LPs with 5°C different interval temperatures comparison. Therefore, LP activity at the different temperatures should be considered indicative, because they may probably be affected to a great extent by the initial bacterial load. Temperature is one of the most important factors influencing

microbial growth. Otherwise, LP activity in the present study was decreased at high temperature possibly dependent on the storage temperature of LPs treated milk and also an initial bacterial load.

3.5.2 Effect of temperatures and NaSCN levels on milk composition

Average initial milk fat, protein, lactose, solid-not-fat (SNF) and total solid (TS) contents were 4.01, 4.43, 3.03, 8.34 and 12.35% at 25^{o}C respectively and respective 4.01, 4.43, 3.03, 8.33 and 12.34% at 30^{o}C as shown in Table 3.1. Luanglawan (2005) reported that mean of milk fat, protein, lactose, SNF and TS contents (12 months average) in SUT's dairy cattle farm were 3.59, 2.91, 4.45, 8.26 and 11.84% respectively at an average 27^{o}C. Milk fat being highest in August and lowest in March, August is in the mid of rainy season in Thailand where fresh grasses or fresh cut corn are adequate to supply as SUT Farm recorded. This experiment was carried out overlap during April 2014 to June 2014, an average initially milk composition may probably differ from mean milk composition for 12 months.

Milk fat in this study is higher than the standard range as it is usually considered in the standard regulation set by NACFS.6003, FDA.265, DLD and Dairy Farming Promotion Organization of Thailand (D.P.O., 2000). The high milk fat content verified conversely result in the proportional reduction of other remaining milk content. Milk fat content has not been consistent since the factors affecting change in milk fat include breeds, species, age, cows' milking duration, lactation seasons and feed nutrients (forage-based or concentrate-based diets of dairy cows) (de Wit, 2003).

Responses to increasing dose of NaSCN levels were generally not significant for a content of milk fat, and the all dose levels differed amongst the temperatures

evaluated. The concentration of milk composition at 25ºC was similar to 30ºC after activation of the LPs. The LPs activation by the addition of 16.78 mg/kg KSCN : 30 mg/kg $2Na_2CO_3.3H_2O_2$ was used in a study of Maneerate (2006) in which raw cow's milk samples were collected from milk tank of collection centres at SUT farm. Maneerate (2006) reported that the milk samples were preserved by LPs, the qualities of LP-treated milk were similar to those of raw milk. Milk composition including milk fat, protein, lactose and TS from LP-treated milk was also within the standard level of regular raw milk. Milk composition after LP activation observed in our study using NaSCN compared to the very minor LP activation seen *in vitro* by Maneerate (2006) for the similar ratio. It is possible that NaSCN would result in the same effect direction as *in vitro* study with a regular raw milk.

Protein, lactose, SNF and TS contents were similar the standard of milk composition set by the Ministry of Public Health (Thailand) (No.265) (2002) Food and Drug Administration (FDA) that identified milk fat, milk protein, SNF and TS of not less than 3.20, 2.80, 8.25 and 11.45% respectively and NACFS (2005) identified milk fat, SNF and TS of not less than 3.20, 8.25 and 12.30% respectively. If the content of protein increase the %SNF will also be increased. TS respectively little decreased down to 12.35 and 12.34 at 25ºC and 30ºC, whereas, at 0, 7, 14 and 21 mg/L NaSCN addition, TS did not differ from the control. TS was the range of standard price set by the Ministry of Agriculture and Cooperatives (MOAC, 2015) of not less than 12.00%. Moisture and TS are an inverse relationship, (moisture was low when TS was high).

After activation of the LPs, there was no difference between 25ºC and 30ºC incubation in terms of their effect on fat, protein, lactose, SNF and TS contents in

milk but differed in their effect on concentration of SCN^- and LP activity. Milk protein was similar for all NaSCN levels at 25°C and 30°C. Similarly, Omer Ibrahim and Zawahir (2013) reported that LP activated milk showed no significant difference in the density and milk protein of all the milk samples stored at 37°C. These results agree with the results of other researchers (Fernandez et al., 2005; Ndambi et al., 2008; Ponce, 2010). Evidence of undesirable effects was not observed in the impact of the application of the LP activation using NaSCN for raw milk composition. However, Maneerate (2006) who examined the raw milk preserved by LPs from SUT's farm, also found that measurements for milk protein failed to meet the standard criteria set by either authority and LP activation did not affect milk composition either. It is not unexpected that the results derived for the samples prior to LP activation conformed quite closely to the standard range by Walstra et al. (1999).

Lactose in response to all NaSCN levels was not in pattern of linear, quadratic and cubic both at 25 and 30°C. Lactose in the range of 4.42 to 4.43 is usually considered normal, which it generally is in the range of 3.80 to 5.30 (Walstra et al., 1999), however, these lactose contents are in the middle of such range.

All NaSCN levels at 25°C and 30°C was no response for TS. TS content with the 7, 14, and 21 mg/L NaSCN addition was similar to the control. The present results clearly found that LPs activation via the addition of safety 14 mg/L NaSCN level had no negative effect on change in raw milk compositions. To the best of our knowledge, research has been limited to undertaken. Literature published did not identify changes in milk composition after activation of LPs. As much as the LPs background, LP can preserve the volume of milk available as an important nutritional component of the diet due to reduced losses of milk through microbial spoilage. With respect to the

conditions for raw milk pricing systems in Thailand, the milk price comprised a base price plus additions and deductions that dairy cooperatives give, usually based on the quality of raw milk. To the base price, MOAC (2015) sets milk price based on milk fat content, TS, SCC, TBC and freezing point. If the dairy farmers can maintain milk fat content between 3.40 and 3.59%, TS content between 12.00 and 12.59%, SCC and TBC between 400,001 and 500,000 cells/mL of milk and freezing point less than -0.510, farmers will be able to achieve the standard price. If TS is lower than 12.00% and TBC is higher than 500,000 cells/mL, farmers will be punished by reducing milk price (-0.10 Baht/kg) from the standard pricing system. A price is paid greater for milk with TS above 12.59% and TBC below 400,001 cells/mL. However, the initial TS content in cow's milk samples collected from the collection centre of SUT's Farm before LPs activation was not high. In fact, in many regions of Thailand, handling systems are quite poor and there are a number of technical and management problems together with inadequate good quality feeds, all of which may contribute to affect milk fat content and TS composition.

Table 3.1 Effect of temperature (25ºC vs 30ºC) and NaSCN levels (0, 7, 14 and 21 mg/L) on thiocyanate (SCN^-), lactoperoxidase activity (LP) and milk composition in raw cow's milk. (Experiment 1) (*N* = 8).

Temperature	Level (mg/L)	After activation of the LPs by addition of NaSCN						
		Concentration		Milk composition (%)				
		SCN^- (ppm)	LP (U/mL)	Fat	Protein	Lactose	SNF	TS
25ºC	**Mean**	**13.23^{A}**	**13.07^{A}**	**4.01**	**3.03**	**4.43**	**8.34**	**12.35**
	0	3.43^{d}	4.08^{d}	4.01	3.03	4.43	8.34	12.35
	7	10.24^{c}	10.0^{c}	4.01	3.03	4.42	8.34	12.35
	14	16.91^{b}	16.03^{b}	4.01	3.03	4.43	8.34	12.34
	21	22.35^{a}	22.06^{a}	4.01	3.03	4.43	8.34	12.34
	Contrast	*l, q, c*	*l, c*	*ns*	*ns*	*ns*	*ns*	*ns*
30ºC	**Mean**	**12.73^{B}**	**12.26^{B}**	**4.01**	**3.03**	**4.43**	**8.33**	**12.34**
	0	3.33^{d}	3.84^{d}	4.01	3.03	4.42	8.34	12.34
	7	9.50^{c}	9.37^{c}	4.01	3.03	4.42	8.34	12.34
	14	16.39^{b}	15.06^{b}	4.01	3.03	4.43	8.33	12.34
	21	21.69^{a}	20.75^{a}	4.01	3.03	4.43	8.33	12.34
	Contrast	*l, q, c*	*l, q, c*	*ns*	*ns*	*ns*	*ns*	*ns*
Level addition	0	3.38^{D}	3.96^{D}	4.01	3.03	4.42	8.34	12.35
	7	9.87^{C}	9.69^{C}	4.01	3.03	4.42	8.34	12.35
	14	16.65^{B}	15.55^{B}	4.01	3.03	4.43	8.33	12.34
	21	22.02^{A}	21.41^{A}	4.01	3.03	4.43	8.33	12.34
	Contrast	*l, q, c*	*l, q, c*	*ns*	*ns*	*ns*	*ns*	*ns*
SEM								
Temperature		0.027	0.016	0.001	0.002	0.001	0.002	0.004
Level addition		0.038	0.023	0.001	0.003	0.002	0.003	0.005
Interaction		0.054	0.033	0.002	0.004	0.002	0.004	0.007
P value								
Temperature		<0.001	<0.001	0.111	0.643	0.564	0.643	0.160
Level addition		<0.001	<0.001	0.899	0.140	0.799	0.140	0.702
Temperature x Level		<0.001	<0.001	0.899	0.445	0.87	0.445	1.000

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05.

[A, B, C] Mean within the same column for the main effects of temperature or level having different superscript letters are different at P<0.05.

l, q, c : Within a column, the effect of level for individual temperature or the main effect of level is linear, quadratic, and cubic, respectively, at P<0.05.

3.5.3 Effect of temperatures and NaSCN levels on total bacterial count and coliform count (Exp. 2)

There were no temperature and NaSCN level interactions (P>0.05) for the number of TBC after 0, 3 and 12 h incubation (Table 3.2). At 0 h, TBC was not affected by temperature, NaSCN level or their interactions but at 3, 6 and 9 h, TBC at 30ºC were higher (P<0.01) than at 25ºC. Although there were some small differences in TBC between 25ºC and 30ºC for Exp.2, generally Exp.2 confirmed the results observed in Exp.1. The results in the present study revealed that the difference in elevated temperature was influential in the quality of activated raw milk tested (milk samples at 30ºC were extended less than the samples at 25ºC). Furthermore, TBCs were reduced with increasing NaSCN at 3, 6, 9 and 12 h incubation. TBC were decreased linearly (3, 6, 9 and 12 h), quadratically (6, 9 and 12 h) and cubically (9 and 12 h) with increasing NaSCN level such that the higher NaSCN level (14 and 21 mg/L NaSCN) differed from the intermediate levels (7 mg/L NaSCN), which all differed from the control both at 25ºC and 30ºC. However, there was no effect of temperature x NaSCN level interactions on TBC at 12 h.

The addition of NaSCN in the raw cow's milk caused the milk SCN^- concentrations to increase. The increasing of SCN^- concentration in raw milk could enhance the efficiency of antibacterial activity of the LPs. In greatly, the ability to reduce bacteria by activation of the LPs was high with significantly decreased at 6 and 9 h and at 25ºC and 3 and 6 h at 30ºC compared with the control. TBC for the controls has already exceeded those range and the level that unacceptable for use in milk production processing. Slightly higher findings were recorded by Barabas (1994); Vivek-Sharma et al., (1999); Lin and Chow (2000) and Bennett (2000). Garcia-

Garibay et al. (1995) determined the antimicrobial effect of the activated LPs in milk by using immobilized lactase and glucose oxidase to produce H_2O_2 to activate the LPs, the results showed that LPs reduced CC, *S. aureus*, psychrotrophs bacteria and fungi by 79%, 68%, 91% and 100% respectively. This finding was similar to those reported by other researchers (Pruitt, 2003; Fonteh et al., 2005; Kanloung et al., 2007). Eyassu et al. (2004) and Nigussie and Seifu (2008) reported similar results of the inhibition of the growth of microbiological organisms through the use of LPs activation in goat's milk in ambient temperature at 7 h of storage.

Normally, TBC in raw milk is found to be more than $5.0x10^5$ CFU/mL described as for a bad grade set by MOAC (2015), indicating that raw milk quality is unacceptable for milk production processing. Where TBC are high, the milk price is reduced commensurately. Milk with TBC less than $3.0x10^5$ CFU/mL, farmers set get greater milk price (+0.10 Baht/kg) from the standard pricing system, while a price is paid lower for milk with TBC above $5x10^5$ CFU/mL. The result of TBC revealed that the significant difference on shelf life extension for raw milk after activation of LPs at 7, 14 and 21 mg/L NaSCN both at 25ºC and 30ºC for 6 h, and 14 and 21 mg/L NaSCN both at 25ºC and 30ºC between 6 to 9 h. From this study, the raw milk quality was prolonged shelf life after an addition of NaSCN between 6 to 9 h during storage, hence the farmers will receive the standard milk price. FAO (2005) reported that the LP activated a bacteriostatic effect and effectively extended raw milk quality to prolong the shelf life for 7 to 8 h under ambient temperatures at around 30ºC or longer at lower temperatures. However, that effectiveness in restraining the growth of bacteria during transportation to milk products factory also depended on temperature keeping.

The result of CC revealed that the difference in elevated temperature were influential in the quality of activated raw milk tested (milk samples at 30ºC were extended less than the samples at 25ºC). For CC, there was temperature x level interactions ($P<0.001$) after LPs activation at 3, 9 and 12 h (Table 3.3). At 0, 3 and 6 h, CC was decreased linearly both at 25ºC and 30ºC, but only at 3 h and at 30ºC, CC was reduced cubically as well. At 9 h, CC was decreased linearly and quadratically at 25ºC whereas it was reduced linearly and cubically at 30ºC. By 12 h, CC was reduced linearly, quadratically and cubically at both 25ºC and 30ºC. CC was significantly decreased with increasing NaSCN at both 25ºC and 30ºC and at all incubation times.

According to Reiter et al. (1976), the bactericidal effect of the LPs against *E. coli* increased when the SCN^- concentration rose from 0.015 to 0.15 mM. The increasing of SCN^- concentration, together with H_2O_2 resulted in a substantial reduction in the bacteria flora and prevented the psychrotrophic bacteria growth for up to five days (Björck et al., 1975), decreasing the number of Salmonella in the acidified (pH 5.3) raw milk (Wray and McLaren, 1987). The activation also reduced standard plate, CC and psychrotrophic counts below those of untreated milk (Zajac et al., 1983). LPs showed an antimicrobial activity against *E. coli* O157 : H7 (Heuvelink et al., 1998). However, Heuvelink et al. (1998) confirmed that the LPs by adding SCN^- alone was unable to retain raw milk quality or inhibit the *E. coli* (O157 VTEC strain), it may be used alone or in conjunction with processing of pasteurization to reduce or eliminate the bacterial load in milk products (Musser, 2011). The milk pasteurized with LP treatment, can be reactivated to inhibit microbial growth to extend the shelf life of raw milk (Barrett et al., 1999). A bacteriostatic effect of the

LPs against *E. coli* in Saanen and South African Indigenous goat milk kept at 30ºC (Seifu et al., 2004b).

Eventually, to evaluate the inhibitory action of previously activated LPs in cow milk in relation to bacteria growth, Gaya et al. (1991) observed a significant decrease in the SCN^- concentration from 8 to 48 h after sampling, confirming the considerable drop a few hours after sampling. These authors also demonstrated that the decrease of an antimicrobial capacity of LPs due to the continuous decrease in the concentration of its components, because that a lower LP activity rate is linked to an increase in the number of microorganisms. Indeed, decreases were observed in LP activity when the analyses were performed at 48 h and in the SCN^- and H_2O_2 concentrations at 12 h compared with those carried out earlier (Althaus et al., 2001). However, the antimicrobial effect of LPs on any particular organism depends on the reaction conditions (Pruitt and Reiter, 1985). Similarly, Wolfson and Sumner (1993) reported that inhibitor reaction of the LPs depends on the type, a number of microorganisms, media conditions of microorganisms and incubation temperature. Another influence on the effectiveness of the stimulated LPs is the microbiological condition of the milk prior to treatment (Härnulv and Kandasamy, 1982). The optimum effectiveness for LPs activation is reported to occur if the system is not activated immediately but delayed slightly until after the multiplication of existing bacteria has begun and when the indigenous antibacterial system remains significantly effective (Reiter, 1978). Further, the concept which involves the activation of LPs in milk should be considered as the fundamental method for inhibiting the growth of acid-producing micro-organisms in un-chilled raw milk (Swedish, 1975).

Table 3.2 Effect of temperature (25ºC vs 30ºC) and NaSCN levels (0, 7, 14 and 21 mg/L) on total bacterial count (TBC) in raw cow's milk at incubation time (0, 3, 6, 9 and 12 h). (Exp. 2) (*N* = 8).

Temperature	Level (mg/L)	Number of TBC after activation of the LPs by addition of NaSCN				
		Time (h)				
		0	**3**	**6**	**9**	**12**
25ºC	**Mean**	**$1.85x10^{5A}$**	**$3.25x10^{5B}$**	**$2.20x10^{5B}$**	**$1.05x10^{6B}$**	**$3.48x10^{7A}$**
	0	$1.63x10^{5}$	$3.56x10^{5a}$	$4.99x10^{5a}$	$3.39x10^{6a}$	$1.20x10^{8a}$
	7	$1.77x10^{5}$	$3.29x10^{5b}$	$2.47x10^{5b}$	$6.71x10^{5b}$	$1.82x10^{6b}$
	14	$2.33x10^{5}$	$3.12x10^{5bc}$	$4.31x10^{4d}$	$5.91x10^{4c}$	$4.14x10^{5c}$
	21	$1.68x10^{5}$	$3.02x10^{5c}$	$8.87x10^{4c}$	$7.96x10^{4c}$	$6.38x10^{5c}$
	Contrast	*ns*	*l*	*l, q*	*l, q, c*	*l, q, c*
30ºC	**Mean**	**$1.77x10^{5A}$**	**$3.53x10^{5A}$**	**$2.69x10^{5A}$**	**$1.11x10^{6A}$**	**$3.92x10^{7A}$**
	0	$1.81x10^{5}$	$3.84x10^{5a}$	$7.28x10^{5a}$	$3.54x10^{6a}$	$1.41x10^{8a}$
	7	$1.59x10^{5}$	$3.64x10^{5b}$	$2.69x10^{5b}$	$7.95x10^{5b}$	$1.86x10^{7b}$
	14	$1.57x10^{5}$	$3.48x10^{5c}$	$1.64x10^{4d}$	$3.35x10^{4d}$	$4.67x10^{4b}$
	21	$2.11x10^{5}$	$3.18x10^{5d}$	$6.13x10^{4c}$	$7.32x10^{4c}$	$2.18x10^{5b}$
	Contrast	*ns*	*l*	*l, q, c*	*l, q, c*	*l, q, c*
Level addition	**Mean***	**$1.81x10^{7B}$**	**$3.39x10^{6B}$**	**$2.44x10^{5B}$**	**$1.08x10^{5B}$**	**$3.70x10^{5A}$**
	0	$1.72x10^{5}$	$3.70x10^{5A}$	$6.14x10^{5A}$	$3.47x10^{6A}$	$1.29x10^{8A}$
	7	$1.68x10^{5}$	$3.47x10^{5B}$	$2.58x10^{5B}$	$7.33x10^{5B}$	$1.84x10^{7B}$
	14	$1.95x10^{5}$	$3.30x10^{5C}$	$2.98x10^{4D}$	$4.63x10^{4D}$	$2.30x10^{5C}$
	21	$1.90x10^{5}$	$3.10x10^{5D}$	$7.50x10^{4C}$	$7.64x10^{4C}$	$4.28x10^{5C}$
	Contrast	*ns*	*l*	*l, q, c*	*l, q, c*	*l, q, c*
SEM						
Temperature		0.501	0.257	0.425	0.210	0.266
Level addition		0.709	0.363	0.601	0.296	0.377
Interaction		1.002	0.514	0.850	0.419	0.533
P value						
Temperature		0.715	<0.001	<0.001	<0.001	0.253
Level addition		0.788	<0.001	<0.001	<0.001	<0.001
Temperature x Level		0.273	0.174	<0.001	<0.001	0.240

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C] Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C] Mean within the same column for the main effects of temperature or level having different superscript letters are different at P<0.05.

Table 3.3 Effect of temperature (25ºC vs 30ºC) and NaSCN levels (0, 7, 14 and 21 mg/L) on coliform count (CC) in raw cow's milk at incubation time (0, 3, 6, 9 and 12 h). (Exp. 2) (*N* = 8).

Temperature	Level (mg/L)	Number of CC after activation of the LPs by addition of NaSCN				
		Time (h)				
		0	**3**	**6**	**9**	**12**
25ºC	**Mean**	**$2.13x10^{3B}$**	**$6.10x10^{3B}$**	**$1.02x10^{4B}$**	**$7.19x10^{4B}$**	**$1.02x10^{6B}$**
	0	$2.34x10^{3a}$	$6.90x10^{3a}$	$1.65x10^{4a}$	$1.86x10^{5a}$	$3.23x10^{6a}$
	7	$2.26x10^{3a}$	$6.21x10^{3b}$	$1.30x10^{4b}$	$6.78x10^{4b}$	$4.70x10^{5b}$
	14	$1.98x10^{3b}$	$6.07x10^{3b}$	$4.07x10^{3d}$	$1.20x10^{4d}$	$1.52x10^{5d}$
	21	$1.92x10^{3b}$	$5.20x10^{3c}$	$7.31x10^{3c}$	$2.21x10^{4c}$	$2.31x10^{5c}$
	Contrast	*l*	*l*	*l*	*l, q*	*l, q, c*
30ºC	**Mean**	**$3.84x10^{3A}$**	**$1.20x10^{4A}$**	**$2.39x10^{4A}$**	**$2.13x10^{5A}$**	**$2.23x10^{6A}$**
	0	$4.10x10^{3a}$	$1.38x10^{4a}$	$3.20x10^{4a}$	$4.05x10^{5a}$	$6.32x10^{6a}$
	7	$3.91x10^{3ab}$	$1.21x10^{4b}$	$2.85x10^{4a}$	$3.68x10^{5a}$	$1.42x10^{6b}$
	14	$3.70x10^{3b}$	$1.20x10^{4b}$	$1.51x10^{4c}$	$3.74x10^{4b}$	$4.05x10^{5d}$
	21	$3.67x10^{3b}$	$1.01x10^{4c}$	$2.01x10^{4b}$	$4.02x10^{4b}$	$7.74x10^{5c}$
	Contrast	*l*	*l, c*	*l*	*l, c*	*l, q, c*
Level addition	**Mean***	**$2.99x10^{3C}$**	**$9.04x10^{3C}$**	**$1.71x10^{4C}$**	**$1.42x10^{5B}$**	**$1.63x10^{6A}$**
	0	$3.22x10^{3A}$	$1.04x10^{4A}$	$2.42x10^{4A}$	$2.95x10^{5A}$	$4.78x10^{6A}$
	7	$3.08x10^{3A}$	$9.16x10^{3B}$	$2.08x10^{4B}$	$2.18x10^{5B}$	$9.47x10^{5B}$
	14	$2.85x10^{3B}$	$9.01x10^{3B}$	$9.57x10^{3D}$	$2.47x10^{4C}$	$2.79x10^{5D}$
	21	$2.79x10^{3B}$	$7.63x10^{3C}$	$1.37x10^{4C}$	$3.11x10^{4C}$	$5.03x10^{5C}$
	Contrast	*l*	*l*	*l*	*l*	*l, q, c*
SEM						
Temperature		0.409	0.514	0.543	0.336	0.194
Level addition		0.579	0.727	0.768	0.475	0.274
Interaction		0.818	1.028	1.086	0.672	0.388
P value						
Temperature		<0.001	<0.001	<0.001	<0.001	<0.001
Level addition		<0.001	<0.001	<0.001	<0.001	<0.001
Temperature × Level		0.919	0.024	0.115	<0.001	<0.001

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C] Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C] Mean within the same column for the main effects of temperature or level having different superscript letters are different at P<0.05.

3.6 Conclusion

All 4 NaSCN levels evaluated increased SCN^- concentration and LP activity, with the highest level (21 mg/L) being more supply than lower levels. For 25°C and 30°C, maximum response was observed at the highest level and 7, 14 mg/L NaSCN increased SCN^- concentration, LP activity, with a further increase with increasing level rate.

Therefore, optimum level rate was 21 mg/L NaSCN, because a further increase in NaSCN addition failed to further decrease TBC and CC in raw milk which are the main causes of the deterioration of milk over time. Thus, the addition NaSCN of 21 mg/L can serve as an effective treatment to extend raw milk quality during storage.

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CHAPTER IV

THE INHIBITORY EFFECT OF SODIUM THIOCYANATE AND SODIUM PERCARBONATE LEVELS ON MICROORGANISM GROWTH IN RAW MILK SAMPLES

4.1 Abstract

Preservation of raw milk quality during storage by activation of LPs was studied for the inhibition of microorganism growth through activation by addition of sodium thiocyanate (NaSCN) and sodium percarbonate ($2Na_2CO_33H_2O_2$) levels as an effective treatment at 0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L, respectively. Two experiments were conducted to evaluate the effect of 2 different temperatures and 4 NaSCN : $2Na_2CO_33H_2O_2$ levels on raw milk samples *in vitro* assay. Experiment 1 (Exp. 1) Thiocyanate (SCN^-) concentration, hydrogen peroxide (H_2O_2), lactoperoxidase (LP) activity and the milk compositions were examined at different the NaSCN : $2Na_2CO_33H_2O_2$ levels. Experiment 2 (Exp. 2), the inhibitory effect of LPs activation was studied whether it can decrease total bacterial count (TBC) and coliform count (CC) as an effective treatment to extend raw milk quality during storages. The experiments were conducted as a 2x4 factorial in completely randomized designs (CRD), which factor A was temperature (25^{o}C vs 30^{o}C) and factor B with different levels of NaSCN : $2Na_2CO_33H_2O_2$ (0 : 0, 7 : 15, 14 : 30 and 21

: 45 mg/L) with eight replicates per run using 0, 3, 6, 9 and 12 h incubation time. Raw milk was collected from the bulk tank of collection centres. The milk composition, SCN^- concentration, H_2O_2, and LP activity in raw milk were determined. The SCN^- concentrations and LP activities increased with increasing the NaSCN : $2Na_2CO_33H_2O_2$ levels. The milk composition was not significantly affected after LP activation of the milk by NaSCN : $2Na_2CO_33H_2O_2$ levels. LP activated milk was an antimicrobial activity at 14 : 30 mg/L of NaSCN : $2Na_2CO_33H_2O_2$ levels at 25°C and 30°C. These results indicate the inhibitory effect by the addition of NaSCN : $2Na_2CO_33H_2O_2$ by decreasing TBC and CC growth in raw milk as an effective treatment to preserve raw milk quality during storage.

Keywords : sodium thiocyanate, sodium percarbonate, lactoperoxidase system, microorganism growth, raw milk quality

4.2 Introduction

The historical practice, the tropical countries especially for Thai smallholding dairy farmers, was to store the raw milk in unrefrigerated conditions in cans or tanks which would be later collected in series from each smallholder and transported (unchilled) in bulk collection to a processing plant or larger milk collection centre. This method of collection would often result in delays between milking and final point of delivery at dairy processing plants of more than four to five hours which would have significant negative consequences for the milk quality. These issues contribute to a higher frequency of raw milk being rejected by the dairy factories as the product would not be acceptable to consumers (Barabas, 1994). Alternative

preservation techniques are clearly required to maintain the quality of raw milk long enough to ensure that it can be transported from the producer to processing plants and preferably through to final consumers without significant deterioration. These techniques are required to be simple, relatively inexpensive and not represent any risk to consumers (Barabas, 1995; Ryoba et al., 2000). In seeking to address this problem, an alternative way to enhance the storage stability of raw milk at high ambient temperatures has been developed (Seifu et al., 2005). The method makes use of a naturally occurring enzyme present in the antimicrobial system in milk known as LPs. LP is active against both Gram-positive and Gram-negative bacteria to varying extents (Siragusa and Johnson 1989) and leads to the formation of antimicrobial compounds.

The LPs alone is unable to exhibit sufficient antibacterial activity to maintain raw milk quality during storage for extended periods. The system consists primarily of three components; LP, SCN^- and H_2O_2 and is active only in the presence of all three components (Seifu et al., 2005). Together increasing concentrations of two components or activator with SCN^- and H_2O_2 reacting with each other are added which is sufficient to activate the LPs according to FAO (2005). Basically, SCN^- was log into LPs, SCN^- is oxidized by H_2O_2 in milk and LP has the ability to catalyze this oxidative reaction with antibacterial hypothiocyanite product and the other intermediates (Modi et al., 1991). These products have effectiveness in reducing bacterial growth activity (Naidu, 2000) by damaging the cell membranes and inhibiting the activity of metabolic enzymes (Haddadin et al., 1996). The degradation of the membrane damages the bacteria's ability to transport glucose and promotes the loss of potassium ions, amino acids and peptides within the cell's structure (Aune and

Thomas, 1978). The activity of the system as described above has shown that the LPs can increase storage times and extend the shelf life of raw milk by delaying bacterial growth (Wolfson and Summer, 1993). Thus, this study was to evaluate the inhibitory effect of activation of the LPs by using different NaSCN : $2Na_2Co_33H_2O_2$ levels on microorganism growth in raw cow's milk as an effective treatment to extend raw milk quality during storage.

4.3 Objective

To study the inhibitory effect of sodium thiocyanate and sodium percarbonate levels on microorganism growth in raw milk samples as an effective treatment to extend raw milk quality during storage.

4.4 Materials and methods

Experiment 1 (Exp. 1) and Experiment 2 (Exp. 2) were conducted as a 2x4 factorial in completely randomized design (CRD) with 8 treatments combination arranged as a factorial (2 different temperatures x 4 NaSCN : $2Na_2CO_33H_2O_2$ levels) with eight replicates per run. In both experiments, the runs were conducted on the same 4 NaSCN : $2Na_2CO_33H_2O_2$ levels and separate temperature and time of incubation were used.

4.4.1 Milk sampling

Approximately 1 L each of raw cow's milk samples was collected from milk tank of collection centres, at Suranaree University of Technology (SUT) dairy farm. Milk samples were divided into 2 main portions with one stored at 25^{o}C and the other at 30^{o}C to examine before (as control) and after LP activation of the milk by using the

NaSCN : $2Na_2CO_33H_2O_2$ at 7 : 15, 14 : 30 and 21 : 45 mg/L. Exp. 1 (first portion) milk samples were analysed for milk composition using Lactostar (Art. no : 3510; Funke Gerber Labortechnik GmbH, Berlin), SCN^- concentration (Codex Alimentarius Commission (CAC/GL 13-1991b), H_2O_2 (Bjorck, 1978; Allen and Wrieden, 1982) and LP activity (Isobe et al., 2009b) immediately. Exp. 2 (second portion); milk samples were examined for microbiological properties including TBC (NA agar) and CC (EMB agar) (AOAC, 2000) and then incubated at 0, 3, 6, 9 and 12 h before and after LP activation (See chapter III).

4.4.2 Activation of lactoperoxidase system

The LPs was activated by the addition of SCN^- (in the form of sodium thiocyanate (NaSCN) : sodium percarbonate ($2Na_2CO_33H_2O_2$). 1.) NaSCN was prepared from 99.7% purity NaSCN powder (UNIVA, APS, NSW, Australia), then SCN^- solution was added into raw milk at 0, 7, 14 and 21 mg/L respectively, and then stirred thoroughly for 1 min thereby increasing the SCN^- content. 2.) $2Na_2CO_33H_2O_2$ (Eisen-Golden Laboratories) was added at 0, 15, 30 and 45 mg/L of milk, then the raw milk was stirred thoroughly for 2-3 min. In contact with distilled water, percarbonate decomposes into carbonate and H_2O_2 that corresponds to 0, 15, 30 and 45 mg/L of H_2O_2 (Zajac et al., 1983a, b) recommended by IDF, 1988 code of practice. LP-activated milk was plunged for about one minute and then stirred for 2 min. The enzymatic reactions were completed in the milk within 5 min after addition of H_2O_2 donor (Na_2CO_3), and stored at 4-7^oC. The activation of LPs was carried within 2-3 h after milking by reactions of SCN^- and H_2O_2.

4.4.3 Statistical analysis

Data analyses were conducted using the General Linear Models procedure of the statistical analysis system (SAS, 1996). Data from Exp. 1 and Exp. 2 were analyzed separately as a 2x4 factorial in CRD with temperature, the NaSCN : $2Na_2CO_33H_2O_2$ levels and their interaction included in the model as fixed factor effects. When the interaction between temperature and the NaSCN : $2Na_2CO_33H_2O_2$ was significant, orthogonal polynomial contrasts were performed to determine linear, quadratic and cubic responses to the temperature within the NaSCN : $2Na_2CO_33H_2O_2$ levels. When the main effect of NaSCN : $2Na_2CO_33H_2O_2$ levels were significant, orthogonal polynomial contrast were performed to determine overall linear, quadratic and cubic responses to temperature. Significance was declared at $P<0.05$.

4.4.4 Experimental location

The experiment was conducted at Suranaree University of Technology's Dairy Cattle Farm, the Center for Scientific and Technological Equipment Building (F10), Suranaree University of Technology, Nakhon Ratchasima.

4.4.5 Experimental period

This experiment was carried out during August 2014 to November 2014 at an average ambient temperature of 27.5ºC (http://www.e-report.energy.go.th).

4.5 Results and discussion

4.5.1 Effect of temperatures and NaSCN : $2Na_2CO_33H_2O_2$ levels on SCN^- concentration, H_2O_2 and LP activity (Exp. 1)

The difference initial concentration of SCN^- in raw cow's milk samples were observed (3.63 and 3.60 ppm at 25ºC and 30ºC respectively) shown in Table 4.1. The

SCN^- concentrations are lower than the average value obtained by Fonteh et al. (2002) for the milk of cows (6-12 mg/L; mean 8.5 mg/L). Similar results were obtained for bulked milk tank from the same collection centres at SUT's dairy farm (approximately 2.4 ppm) (Maneerate, 2006). In contrast, Lambert (2001) and Panthanara et al. (2005) concluded that the average SCN^- concentrations were 5.00 and 5.16 ppm respectively. Literature data on the values of SCN^- concentration in milk have been variable. The concentration of SCN^- in milk is reported to be affected by a vast number of factors, including feed (thioglycosides (glucosinolates) and cyanogenic glycosides) (Wolfson and Sumner, 1993), animal species (de Wit and Van Hooydonk, 1996), breeds of animal (Pruitt and Reiter, 1985), lactation cycle (Zapico et al., 1991), seasons of the year (Dabur et al., 1996) and individual animals and differences in flocks (Medina et al., 1989). A similar report was obtained for SCN^- concentration varying markedly depending on the feed if cows are on grass during the spring and summer (Ekstrand, 1989). SCN^- concentrations have been reported to vary between 2.3 and 35 mg/L in milk from individual cows and to be around 8 mg/L in bulked milk (Ponce et al., 2005). However, the experimental cows were fed on the same levels during the experiment, it would not cause of differences in milk SCN^- concentration.

Due to the previous research studies, the LPs alone is the incomparable performance to SCN^- and H_2O_2 combination in sufficient antibacterial activity to maintain raw milk quality during storage for extended periods. Similarly, Kussendrager and Hooijdonk (2000) revealed the antibacterial activity of LP activation by using H_2O_2 and SCN^-. The system consists primarily of LP, SCN^- and H_2O_2 which that more effectiveness in the presence of all three components (Seifu et

al., 2005). The LPs function is predominant that of defense against microbial infections or activity and the bacteriostatic and bactericidal effect against a broad spectrum of micro-organisms were showed on *in vitro* studies (Pruitt and Reiter, 1985; Wolfson and Sumner, 1993). Indeed, Heuvelink et al. (1999) confirmed the application of LPs by adding SCN^- alone was unable to retain raw milk quality or inhibit the *E. coli* of serogroup O157 (O157 VTEC strain). However, Ewais et al. (1985) reported that a greater concentration of H_2O_2 was necessary for preservation of milk at temperatures as high as 35°C, level of H_2O_2 (>80 mg/kg) extended the keeping quality for buffalo and cow milk at that temperature. The current study was in agreement with most literature reports that showed effects of the other substrate of SCN^- and peroxide (either in the form of H_2O_2 or $2Na_2CO_33H_2O_2$) for activating LPs. SCN^- (in the form of Na or K salt) are the substrate for LP and are normally added at a level of approximately 14 mg/L (SCN^-) and 30 mg/L ($2Na_2CO_33H_2O_2$) to milk as recommended by Codex guidelines. Others (Gregory et al., 1989; Haddadin et al., 1996; Fonteh et al., 2005; Seifu et al., 2005) have reported similar effects when raw milk samples were added with NaSCN and H_2O_2 with different ratios.

The effect of temperature on the concentration of SCN^- depended upon the interaction of temperature x NaSCN : $2Na_2CO_33H_2O_2$ level ($P<0.001$) after LP activation. The SCN^- concentration was increased both at 25°C and 30°C when NaSCN : $2Na_2CO_33H_2O_2$ was added at 0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L respectively. For 25°C and 30°C, SCN^- concentration at 0 : 0, 7 : 15 and 14 : 30 mg/L NaSCN : $2Na_2CO_33H_2O_2$ was decreased when compared with 21 : 45 mg/L NaSCN : $2Na_2CO_33H_2O_2$. Thus, the highest concentration of SCN^- was observed at the highest level after LPs activation at 25°C. Different activation strategies and substrate levels

have been used in earlier studies to activated LPs in the milk for controlling spoilage bacteria and preservation during storage. IDF (1994) recommends that 10 : 10 mg/L SCN^- and peroxide should be used to render the LPs effective, however, LP activity in milk measured only at 1.44 U/mL, sufficient to act as a catalyst for LP activity (Marshall et al., 1986). Adamson and Clarsson (1981) reported that LPs inhibited *E.coli*, *S. mutans*, *S. salivai* and *S. sanguis* from H_2O_2 at ambient temperature. Garcia-Garibay et al. (1995) suggested that immobilized lactase and glucose oxidase were used to produce H_2O_2 to evaluate the inhibitory action of the activated LPs in milk in relation to coliform, *S. aureus*, psychrotrophs bacteria and fungi. Panthanara et al. (2005) reported that the SCN^- concentration in raw milk increased linearly with increasing SCN^- solution added (from 5.16 to 11.11, 15.48 and 19.89 ppm) at 0, 5, 10 and 15 ppm respectively, however, these authors did not measure H_2O_2 and LP activities after LP activation. In this CAC-1991a regard, FAO/WHO (2005) provided that the method of activating the LPs in milk is to add about 10 ppm SCN^- (preferably in powder form) to the raw milk to increase the overall level to 15 ppm (around 5 ppm is naturally present). In general, these results suggest that the inhibitory effect of microorganism growth through LPs activation in milk varies with the amount of added the substrate type of SCN^- and temperature.

The H_2O_2 values determined in this work was non-detectable in all period. H_2O_2 is known to be unstable and generally non-detectable in raw milk (Seifu et al., 2005). H_2O_2 only exists temporarily, being absorbed by the substrate of the system in a few hours with H_2O as the only product and does not pose any hazards. This result shows bacteria did not contaminate in milk, such that the catalase-negative bacteria, or mastitis infection (*S. aureus* and *Streptococcus sp*.). These bacteria possess the

ability to produce sufficient amounts of H_2O_2 under anaerobic conditions. In contrast, Wilkins and Board (1989) and Schiffman et al. (1992) observed that values of H_2O_2 performed range from 2 to 4 mg/L for sterile cow milk.

LP activities analysed at 0 h both at 25ºC and 30ºC in this work were lower than the 7.55 U/mL reported by Panthanara et al. (2005) in cow milk samples after being taken from the milk tank at Chitralada Gardens Dairy Farm Project in Thailand. The normal range of LP activity in raw cow's milk is between 1.2 and 19.4 U/mL (Priutt, 2003). Reiter (1985) reported that the LP activity is generally low in cow's colostrum followed by a rapidly increase at 4 to 5 days after calving, declining to a constant level during the rest lactation. Similar values cited for goat milk between Verata and Murciano-Grandina that low LP activity in the first 24 h after kidding and continue to increase during 75 d postpartum (Zapico et al., 1991). Given that Verata goats with an average of LPs is significantly lower than Murciano-Grandina goats (0.95 and 2.15 U/mL, respectively). However, LP activity cited for goat milk was approximately 4.45$\pm$1.94 U/mL (Schoos et al., 1999). Therefore, values of LP activity is known to depend on many several factors mainly the breeds, age, seasons, lactation stage, day of lactation, fed and animal health (Althaus et al., 2001; Fonteh et al., 2001; 2002; Fonteh, 2006; Mariba, 2006). Moreover, the period of storage and lactation of cows affect the composition of the LPs because the concentrations of the components are reduced.

The effect of temperature on LP activity depended upon the temperature x NaSCN : $2Na_2CO_3 3H_2O_2$ level interaction ($P<0.001$) after LP activation. Compared with the control, LP activity was increased both at 25ºC and 30ºC when NaSCN : $2Na_2CO_3 3H_2O_2$ was added at 7 : 15, 14 : 30 and 21:45 mg/L respectively. The LP

activities of 0 : 0, 7 : 15 and 14 : 30 mg/L NaSCN : $2Na_2CO_33H_2O_2$ were lower than of 21 : 45 mg/L NaSCN : $2Na_2CO_33H_2O_2$ both at 25ºC and 30ºC. Although, the highest LP activity were observed at the highest NaSCN : $2Na_2CO_33H_2O_2$ level after LPs activation at 25ºC, the changes in LP activity at different temperatures were moderately low after LPs activation. LP activity tended to linearly increase with increasing NaSCN : $2Na_2CO_33H_2O_2$. Siva et al. (1991) reported that the keeping quality (pH, titratable acidity, ethanol stability, clot on boiling) was extended when raw milk was treated with 10 : 10, 20 : 20 and 30 : 30 mg/kg of SCN^- : H_2O_2 at 9.9, 12.2, and 13.6 h respectively, at 30°C. Barraquio et al. (1994) reported that the quality of milk treated with SCN^- : H_2O_2 (14 : 30 mg/L) at 27 to 32ºC to prolong shelf life (7 h). Whereas, 3 h extension in the shelf life of buffalo milk at 37ºC with 15 : 10 mg/kg SCN^- : H_2O_2 was recorded by Chakraborty et al. (1986). Similarly, Eyassu et al. (2004) reported that LPs inhibited the bacterial pathogens (*E. coli*, *L. monocytogenes* and *B. melitensis*) in the milk of Saanen and Indigenous goats at 30ºC. Maneerate (2006) reported that the raw cow's milk preserved by LPs (KSCN : $2Na_2CO_3.3H_2O_2$ ratios) as recommended by Codex (1991) showed average values of 0.03 U/mL of enzyme LP activity. From the previous studies cited, it appears that changes in titratable acidities are closely related to the effects of LP activation. This could be due to an optimum concentration of SCN^- : H_2O_2 (increase the effectiveness of LPs) which decrease acid development and hence increase the keeping quality of the milk. However, some of those activities of milk-borne pathogens, namely *E. coli*, *Salmonella spp*., *Campylobacter spp*., *S. aureus*, *L. monocytogenes*, *Y. enterocolitica* and *B. melitensis* depend on temperature, length of incubation and strain also shown *in vivo* studies (Pitt et al., 1999; Gay and Amar, 2005).

4.5.2 Effect of temperatures and NaSCN : $2Na_2CO_33H_2O_2$ levels on milk composition

Average initial milk fat, protein, lactose, solid-not-fat (SNF) and total solid (TS) contents were 4.12, 3.03, 4.52, 8.40 and 12.52% at 25ºC and 30ºC respectively as shown in Table 4.1. The raw milk of SUT's dairy cattle farm (12 months average) has been reported by Lounglawan (2005) that the mean of milk fat, protein, lactose, solid-not-fat (SNF) and total solid (TS) contents were 3.59, 2.91, 4.45, 8.26 and 11.84% at an average 27ºC respectively. Milk fat being highest in August and lowest in March, August is in the mid of rainy seasons in Thailand where fresh grasses or fresh cut corn are adequate to supply as SUT Farm recorded. This experiment was carried out overlap during August 2014 to November 2014, an average initial milk composition may higher from mean milk composition for 12 months. A research also reported milk composition of Holstein Friesian crossbred (>87.5% Holstein Friesian) lactating dairy cows at SUT farm were variables, due to many the factors including days in milk, milk yield and body weight. Others (Maneerate, 2006 (raw milk tank); Noosen, 2014; Suksombat et al., 2014; Thanh and Suksombat, 2015) have reported similar milk composition when cows were held at the SUT experimental farm. These variations of milk composition is known to be affected by a great number of factors, including species and breeds of animal, (Pruitt and Reiter, 1985), age of cows, health, milking duration, lactation, seasons, feed nutrients (de Wit, 2003). The nutrition affects the quality of the milk and that the milk fat is variable and correlated with the inclusion rate, degree of unsaturation, physical form, concentration of fiber in the basal diet composition of dairy cows (Shingfield et al., 2010; Saad et al., 2013).

For the concentration of milk composition, there were not affected by all temperatures, NaSCN : $2Na_2CO_33H_2O_2$ level or their interactions before and after activation of the LPs both at 25^oC and 30^oC. However, it is well understood about which factors regulating its relation and activity on preservation of raw cow milk using NaSCN : $2Na_2CO_33H_2O_2$ with no negative effect for milk composition. Hamid and Musa (2013) revealed that both levels of NaSCN and $2Na_2CO_3.3H_2O$ (12, 16, 20 and 20, 30 40 mg/L) treated, there were no significant differences on raw cow milk composition (fat, protein, density and TS) compared with the control stored at 37^oC for 8 h. Evidence of undesirable effects were not greatly observed in the milk composition activated with the LPs. Maneerate (2006) reported that the bulk milk composition and qualities of LP-treated milk at 16.781 mg/kg KSCN : 30 mg/kg $2Na_2CO_33H_2O_2$ were similar to the regular of raw milk. Kumar and Mathur (1989a, b) reported that the activation of LPs by pure H_2O_2 did not destroy the nutrition quality of milk proteins. Others (Kumar and Mathur, 1999; Seifu et al., 2004b; Fernandez et al., 2005; Ndambi et al., 2008 and Ponce, 2010) have also been reported the activation of the LPs with different concentration of SCN^- and H_2O_2 in raw milk samples had no effect on milk composition. Indeed, Saad et al. (2013) also revealed that the treated LPs sheep milk did not show any significant different chemical properties in acidity.

Table 4.1 Effect of temperature (25°C vs 30°C) and NaSCN : $2Na_2CO_3 3H_2O_2$ levels (0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L) on thiocyanate (SCN^-) concentration, lactoperoxidase activity (LP) and milk composition in raw cow's milk after LPs activation by the addition of NaSCN : $2Na_2CO_3 3H_2O_2$ (Exp. 1) (*N* = 8).

Temperature	Level (mg/L)	After activation of the LPs by addition of NaSCN : $2Na_2CO_3 3H_2O_2$						
		Concentration		Milk composition (%)				
		SCN^- (ppm)	LP (U/mL)	Fat	Protein	Lactose	SNF	TS
25°C	**Mean**	**13.72^{A}**	**13.99^{A}**	**4.12**	**3.03**	**4.52**	**8.40**	**12.52**
	0 : 0	3.63^{d}	4.08^{d}	4.12	3.04	4.52	8.39	12.51
	7 : 15	10.17^{c}	10.66^{c}	4.12	3.03	4.53	8.40	12.52
	14 : 30	17.37^{b}	17.35^{b}	4.12	3.04	4.52	8.40	12.52
	21 : 45	23.72^{a}	23.89^{a}	4.12	3.03	4.52	8.40	12.52
	Contrast	*l, q, c*	*l, c*	*ns*	*ns*	*ns*	*ns*	*ns*
30°C	**Mean**	**12.66^{B}**	**13.36^{B}**	**4.12**	**3.03**	**4.52**	**8.40**	**12.52**
	0 : 0	3.60^{d}	4.00^{d}	4.12	3.04	4.52	8.40	12.51
	7 : 15	8.67^{c}	9.79^{c}	4.12	3.03	4.53	8.39	12.52
	14 : 30	15.50^{b}	16.96^{b}	4.12	3.04	4.52	8.40	12.52
	21 : 45	22.85^{a}	22.70^{a}	4.12	3.04	4.52	8.40	12.52
	Contrast	*l, q*	*l, c*	*ns*	*ns*	*ns*	*ns*	*ns*
Level	0 : 0	3.62^{D}	4.08^{D}	4.12	3.04	4.52	8.40	12.51
addition	7 : 15	9.42^{C}	10.22^{C}	4.12	3.03	4.53	8.39	12.52
	14 : 30	16.43^{B}	17.15^{B}	4.12	3.04	4.52	8.40	12.52
	21 : 45	23.29^{A}	23.29^{A}	4.12	3.04	4.52	8.40	12.52
	Contrast	*l, q*	*l, c*	*ns*	*ns*	*ns*	*ns*	*ns*
SEM								
Temperature		0.027	0.013	0.001	0.002	0.001	0.001	0.001
Level addition		0.038	0.018	0.001	0.002	0.002	0.002	0.002
Temperature × Level		0.054	0.026	0.002	0.003	0.003	0.003	0.002
P value								
Temperature		<0.001	<0.001	0.827	1.000	0.599	0.185	0.249
Level addition		<0.001	<0.001	0.821	0.683	0.353	0.181	0.187
Temperature × Level		<0.001	<0.001	0.912	0.888	0.780	0.647	0.717

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05

[A, B, C] Mean within the same column for the main effects of temperature or treatment having different superscript letters are different at P<0.05.

4.5.3 Effect of temperatures and NaSCN : $2Na_2CO_3 3H_2O_2$ levels on total bacterial count and coliform count (Exp. 2)

According to Exp. 1 above, together increasing concentrations of two components or activator with SCN^- and H_2O_2 reacting with each other are added which is sufficient to activate the LPs following by FAO (2005). Basically, SCN^- was log into LPs, SCN^- is oxidized by H_2O_2 in milk by LPs activation has the ability to catalyze this oxidative reaction with antibacterial hypothiocyanite product and the other intermediates (Modi et al., 1991). These products have more effectiveness in reducing bacterial growth activity (de Wit and van Hooydonk, 1996; Naidu, 2000) by damaging the cell membranes and inhibiting the activity of metabolic enzymes (Haddadin et al., 1996). The present study was undertaken to elucidate the effect of LP activity as a proportion of NaSCN : $2Na_2CO_3 3H_2O_2$ on counts of microorganism in raw cow's milk. The important findings were : 1) that after activation of its LPs, increased the concentration of SCN^- and LP activity in raw cow's milk samples with no negative effects on milk compositions from Exp. 1 and Exp. 2) resulting from Exp. 1, increasing concentration of SCN^- and LP activity in the milk through the addition of NaSCN : $2Na_2CO_3 3H_2O_2$ would be expected to decrease TBC and CC as an effective treatment for extending raw milk during storage.

As shown in Table 4.2, TBCs were influenced by all levels of addition (0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L) NaSCN : $2Na_2CO_3 3H_2O_2$ and at all incubation times (0, 3, 6, 9 and 12 h), such that TBCs were decreased with increasing addition level. There was no temperature effect on TBC at 0 and 9 h incubation whereas at 3, 6 and 12 h incubation. TBCs at 30°C were higher than 25°C. For 25°C, TBCs were reduced quadratically and cubically by levels of addition at 0 h incubation and they were

reduced linearly, quadratically and cubically at the rest incubation times. For 30ºC, TBCs were decreased linearly and quadratically by addition level at 0 h incubation and they were decreased linearly by a level of addition at 3 h incubation. At 6, 9 and 12 h incubation for 30ºC, there were the reduction in TBCs linearly, quadratically and cubically by levels of addition. There was no temperature and addition level interaction on TBC at 9 h incubation, however, at 0, 3, 6 and 12 h incubation the responses of TBC to addition level depended upon temperatures. Regardless of the temperature effect, the response of TBC to levels of addition depended on the time of incubations, TBCs increased with increasing incubation times particularly at 9 and 12 h incubation.

According to the colonies count of TBC growth of the original milk calculated *in vitro* has been shown to be the indicator of the potential of LPs activation to preservation raw milk during storage. From the results obtained in this study of the notable differences in bacterial count between LPs activation in the milk and control raw milk samples, it is concluded that the benefits of LPs activation in the milk are evidential. Addition of $NaSCN^-$ and H_2O_2 can inhibit *L. monocytogenes* (Gram-positive bacteria) and aerobic bacteria at 20ºC for 8.9 h and 30ºC for up to 2.8 h (Gregory et al., 1989). Apart from controlling psychrotrophic spoilage bacteria, CAC (1991) reported that LP inhibited mesophilic spoilage bacteria, *Pseudomonades* and *E. coli*, especially in bovine milk (Kamau and Kroger, 1984; Reiter and Härnulv, 1982; Zajac et al., 1983b). Buffalo milk preservation by the activation of the LPs has also been reported both at ambient and refrigeration temperatures (Chakraborty et al., 1986; Thakar and Dave, 1986). LPs activation milk has provided an extension for the shelf-life by reducing the acidity caused by microbial flora at 30ºC and 35ºC for 6 h

(Stefano et al., 1995). LPs present in various secretions, oxidizes SCNK by H_2O_2 to produce OSCNK, which is bactericidal for enteric pathogens including multiple antibiotic resistant strains of *E. coli* (Naidu, 2000). LPs can inhibit *E. coli*, *L. monocytogenes* and *B. melitensis* at 30°C for 6 h in the milk of both Saanen and indigenous species of goats (Eyassu et al., 2004). (Seifu et al., 2004b) indicated that the LPs was bactericidal and bacteriostatic against *S. aureus* ATCC 25923, *L. monocytogenes* and *E. coli* in Saanen and South African Indigenous goat milk respectively, at 30°C. Dajanta et al. (2008) reported that the active LPs was found to greatly increase the keeping quality of milk to decline at the rate of 87% (untreated milk) and 78% (*E. coli* treated milk), respectively. Nigussie and Seifu (2008) also reported that similar results when goat milk was activated by LP during storage in ambient temperature to 7 h.

Therefore, the inhibitor reaction or phagocytosis of the LPs depends on the type, number of microorganisms, media conditions (pH, temperature, incubation time, cell density) of microorganisms and incubation temperature (Wolfson and Sumner, 1993). Seifu et al. (2005) reviewed that the difference of bacteria groups shows a varying degree of sensitivity to the LPs, it can probably be elucidated by the differences in cell wall structure and their different barrier properties (de Wit and van Hooydonk, 1996). For bacteria that survive the initial bactericidal activity of the LPs, there is an extended lag phase or recovery period. The length of this lag period is highly temperature dependent, being much longer at cold storage than at high temperatures (Björck et al., 1979; Kamau and Kroger, 1984; Zajac et al., 1983b). After recovery, most bacteria resume normal growth. The length of the antibacterial effect achieved by activation of the LPs is inversely related to the storage temperature

of the milk (IDF, 1988). When milk is stored at 30, 25, 20 and 15^{o}C, the antibacterial effect of the LPs lasts for 7-8, 11-12, 16-17 and 24-26 h, respectively (IDF, 1988).

CCs were higher at 30^{o}C than at 25^{o}C in all incubation times. There were no temperature and levels of addition incubation at 0 h incubation, however, at the rest incubation times the responses of CC to addition levels depended on temperature. For 25^{o}C, CCs were not influenced by addition levels at 0 h and 3 h incubation, however, the responses were linearly (6, 9 and 12 h), quadratically (9 and 12 h) and cubically (12 h) decreased with increasing addition levels. For 30^{o}C, the responses of CC to addition levels were linear (all incubation times), quadratic (3, 6, 9 and 12 h) and cubic (3, 9 and 12 h). Regardless of the temperature effect, CCs were increased with increasing time of incubation. CCs were reduced linearly (all incubation times), quadratically (3, 9 and 12 h) and cubically (3 and 12 h) with increasing addition levels.

As each temperature provides a regular condition of antibacterial activity to LPs activation in the milk for NaSCN : $2Na_2CO_3 3H_2O_2$ levels activities, differences in the responses among temperatures and optimum level, was anticipated. Similarly, Wolfson and Sumner (1993) reported that *in vitro* and *in vivo* studies (Pitt, Harden and Hull, 2000) appearance of against microbial infections or activity and bacteriostatic and bactericidal effect against a broad spectrum of micro-organisms were increased by LPs, but the response depended upon the temperature, length of incubation, strain and its level, with some levels effective for both temperatures when added (Gay and Amar, 2005).

Under storage condition (from 0 to 12 h), although the TBC of four treatments reached their peak at the end (12 h) of the experiment, their pattern during the storage

was slightly different due to the narrow range of temperature. These desirable effects confirm previous observations when the active LPs decreases the microbial population in raw milk samples at 37°C for 8 h (Dajanta et al., 2008). In contrast, the higher number of CC of this result was found much different in their pattern when during the storage at higher. The effect of temperature depended on NaSCN : $2Na_2CO_3 3H_2O_2$ level (temperature x level interaction, $P \leq 0.001$) after LP activation with incubation times on CC (3, 6, 9 and 12 h) (Table 4.3). CC (0 and 3 h) response to level was no effects at 25°C and linear at 30°C with the same incubation time. Each CC multiplies and form a colony of cells which become in linear, quadratic and cubic at 12 h at 25°C and 9 and 12 at 30°C. Thus, the effect of NaSCN : $2Na_2CO_3 3H_2O_2$ level on decreasing CC differed between temperatures. CC increased with increasing incubation times after LP activation. The mean CC revealed that the difference in elevated temperature was influential in the quality of LPs activation in the milk tested by level, 30°C were extended less than 25°C. Thus Addition of NaSCN : $2Na_2CO_3 3H_2O_2$ levels in raw milk samples with higher (14 : 30 and 21 : 45 mg/L) level had effect to decrease in both TBC and CC compared with that at 0 h. Similarly, Naidu et al. (2000) reported that although the LPs has been found inhibit a variety of both gram-positive and gram-negative bacteria, its activity to be either bacteriostatic or bactericidal effect depending on bacterial species and environment conditions.

However, TBC is remained within acceptable standards set by MOAC (2015) (10^5 CFU/mL) for up to 7 : 15 mg/L of NaSCN : $2Na_2CO_3 3H_2O$ levels from 0 h to 9 h and raised at 12 h. Therefore, 14 : 30 and 21 : 45 mg/L of NaSCN : $2Na_2CO_3 3H_2O$ levels were gradually decreased to 10^4 at 6 and 9 h. Nevertheless, after LP activation, for all intervals responded to be lower throughout the experiment. In particular at 6 h,

TBC was decreased linearly, quadratically and cubically when compared with that of before activation. Whereas, the LPs activation has been shown to inhibit the increase of CC such that it did not exceed the acceptability standard at $1x10^4$ CFU/mL to 6 h, (MOAC, 2005) for up to 7 : 15 and 14 : 30 mg/L NaSCN : $2Na_2CO_33H_2O$ levels. CC is inhibited to remain within acceptable standards for up to 21 : 45 mg/L NaSCN : $2Na_2CO_33H_2O$ levels from 0 h to 6 h and raised at 9 to 12 h. Thus, after LP activated, CC tended to slightly increase between 6 to 9 h and the growth rate is indicated as up to 12 h at 7 : 15 and 14 : 30 mg/L of NaSCN : $2Na_2CO_33H_2O$ levels. Although after LP activation, significantly different bacteria was maintained for all study intervals after 6 h. Average TBC increased gradually until 12 h finally increasing to above from 10^5-10^8 CFU/mL and 10^3-10^6 CFU/mL for CC. In basically, the ability LPs activation to reduce bacteria was highly significant with decreased by 6 to 9 h at 25ºC, and approximately 6 h at 30ºC compared with control counts similar with Khanlueng et al. (2007). TBC for the controls has already exceeded this level at 6 h which is above allowable standards and, therefore, unacceptable for use in milk production processing. Slightly higher findings were recorded by Patel and Sannabhadti (1993); Barabas (1995); Vivek-Sharma et al., (1999); Lin and Chow (2000) and Bennett (2000).

Table 4.2 Effect of temperature (25ºC vs 30ºC) and NaSCN : $2Na_2CO_3 3H_2O_2$ levels (0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L) on total bacterial count (TBC) in raw cow's milk after LPs activation by the addition of NaSCN : $2Na_2CO_3 3H_2O_2$ levels at incubation time (0, 3, 6, 9 and 12 h) (Exp. 2) (*N* = 8).

Temperature	**Level (mg/L)**	**Number of TBC after activation of the LPs by addition of NaSCN : $2Na_2CO_3 3H_2O_2$ levels**				
		Time (h)				
		0	**3**	**6**	**9**	**12**
25ºC	**Mean**	**$1.56x10^{5A}$**	**$3.37x10^{5B}$**	**$2.10x10^{5B}$**	**$1.05x10^{6A}$**	**$4.53x10^{7B}$**
	0 : 0	$1.52x10^{5b}$	$3.53x10^{5a}$	$4.97x10^{5a}$	$3.40x10^{6a}$	$1.62x10^{8a}$
	7 : 15	$1.75x10^{5a}$	$3.27x10^{5b}$	$2.44x10^{5b}$	$6.67x10^{5b}$	$1.80x10^{7b}$
	14 : 30	$1.53x10^{5b}$	$3.67x10^{5a}$	$3.98x10^{4d}$	$5.40x10^{4c}$	$4.00x10^{5c}$
	21 : 45	$1.45x10^{5b}$	$3.01x10^{5c}$	$5.96x10^{4c}$	$7.12x10^{4c}$	$4.57x10^{6c}$
	Contrast	*q, c*	*l, q, c*	*l, q, c*	*l, q, c*	*l, q, c*
30ºC	**Mean**	**$1.62x10^{5A}$**	**$3.51x10^{5A}$**	**$4.11x10^{5A}$**	**$5.33x10^{6A}$**	**$4.74x10^{7A}$**
	0 : 0	$1.79x10^{5a}$	$3.82x10^{5a}$	$7.26x10^{5a}$	$3.26x10^{6a}$	$1.71x10^{8a}$
	7 : 15	$1.57x10^{5b}$	$3.62x10^{5b}$	$2.67x10^{5b}$	$7.93x10^{5b}$	$1.86x10^{7b}$
	14 : 30	$1.56x10^{5b}$	$3.46x10^{5c}$	$1.46x10^{4d}$	$3.17x10^{4c}$	$4.14x10^{4c}$
	21 : 45	$1.57x10^{5b}$	$3.16x10^{5d}$	$6.29x10^{4c}$	$7.56x10^{4c}$	$2.49x10^{5c}$
	Contrast	*l, q*	*l*	*l, q, c*	*l, q, c*	*l, q, c*
Level	**Mean**	**$1.59x10^{5D}$**	**$3.44x10^{5C}$**	**$2.39x10^{5D}$**	**$1.04x10^{6B}$**	**$4.64x10^{7A}$**
addition	0 : 0	$1.66x10^{5a}$	$3.68x10^{5a}$	$6.12x10^{5a}$	$3.33x10^{6a}$	$1.67x10^{8a}$
	7 : 15	$1.66x10^{5a}$	$3.44x10^{5c}$	$2.56x10^{5b}$	$7.31x10^{5b}$	$1.83x10^{7b}$
	14 : 30	$1.54x10^{5ab}$	$3.56x10^{5b}$	$2.74x10^{4d}$	$4.29x10^{4c}$	$2.21x10^{5c}$
	21 : 45	$1.51x10^{5b}$	$3.09x10^{5d}$	$6.12x10^{4c}$	$7.34x10^{4c}$	$3.53x10^{5c}$
	Contrast	*l, q*	*l, q, c*	*l, q, c*	*l, q, c*	*l, q, c*
SEM						
Temperature		0.282	0.273	0.360	0.332	0.322
Level addition		0.399	0.387	0.509	0.469	0.456
Temperature × Level		0.564	0.547	0.720	0.663	0.644
P value						
Temperature		0.145	<0.001	<0.001	0.891	<0.001
Level addition		0.015	<0.001	<0.001	<0.001	<0.001
Temperature × Level		0.002	<0.001	<0.001	0.287	<0.001

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C] Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C] Mean within the same column for the main effects of temperature or level having different superscript letters are different at P<0.05.

Table 4.3 Effect of temperature (25°C vs 30°C) and NaSCN : $2Na_2CO_33H_2O_2$ levels (0 : 0, 7 : 15, 14 : 30 and 21 : 45 mg/L) on coliform count (CC) in raw cow's milk after LPs activation by the addition of NaSCN : $2Na_2CO_33H_2O_2$ levels at incubation time (0, 3, 6, 9 and 12 h) (Exp. 2) (*N* = 8).

Temperature	Level (mg/L)	Number of CC after activation of the LPs by addition of NaSCN : $2Na_2CO_33H_2O_2$ levels				
		Time (h)				
		0	**3**	**6**	**9**	**12**
25°C	**Mean**	**$1.77x10^{3B}$**	**$5.46x10^{3B}$**	**$9.85x10^{3B}$**	**$6.64x10^{4B}$**	**$9.77x10^{5B}$**
	0 : 0	$1.86x10^{3}$	$5.82x10^{3}$	$1.63x10^{4a}$	$1.83x10^{5a}$	$3.23x10^{6a}$
	7 : 15	$1.72x10^{3}$	$5.18x10^{3}$	$1.30x10^{4b}$	$5.27x10^{4b}$	$3.12x10^{5b}$
	14 : 30	$1.78x10^{3}$	$5.86x10^{3}$	$3.02x10^{3d}$	$1.10x10^{4d}$	$1.52x10^{5d}$
	21 : 45	$1.71x10^{3}$	$4.98x10^{3}$	$7.11x10^{3c}$	$1.90x10^{4c}$	$2.15x10^{5c}$
	Contrast	*ns*	*ns*	*l*	*l, q*	*l, q, c*
30°C	**Mean**	**$3.82x10^{3A}$**	**$1.38x10^{4A}$**	**$2.44x10^{4A}$**	**$2.87x10^{5A}$**	**$2.42x10^{6A}$**
	0 : 0	$4.04x10^{3a}$	$2.16x10^{4a}$	$4.29x10^{4a}$	$7.11x10^{5a}$	$7.13x10^{6a}$
	7 : 15	$3.89x10^{3ab}$	$1.19x10^{4b}$	$2.76x10^{4b}$	$3.60x10^{5b}$	$1.40x10^{6b}$
	14 : 30	$3.80x10^{3ab}$	$1.18x10^{4b}$	$1.30x10^{4c}$	$3.61x10^{4c}$	$4.16x10^{5d}$
	21 : 45	$3.57x10^{3b}$	$9.76x10^{3b}$	$1.40x10^{4c}$	$3.99x10^{4c}$	$7.57x10^{5c}$
	Contrast	*l*	*l, q, c*	*l, q*	*l, q, c*	*l, q, c*
Level addition	**Mean**	**$2.80x10^{3C}$**	**$9.62x10^{3C}$**	**$1.71x10^{4C}$**	**$1.76x10^{5B}$**	**$1.70x10^{6A}$**
	0 : 0	$2.95x10^{3a}$	$1.37x10^{4a}$	$2.96x10^{4a}$	$4.47x10^{5a}$	$5.18x10^{6a}$
	7 : 15	$2.81x10^{3ab}$	$8.55x10^{3b}$	$2.03x10^{4b}$	$2.06x10^{5b}$	$8.56x10^{5b}$
	14 : 30	$2.79x10^{3ab}$	$8.85x10^{3b}$	$8.02x10^{3c}$	$2.35x10^{4c}$	$2.83x10^{5d}$
	21 : 45	$2.64x10^{3b}$	$7.37x10^{3b}$	$1.06x10^{4c}$	$2.94x10^{4c}$	$4.86x10^{5c}$
	Contrast	*l*	*l, q, c*	*l*	*l, q*	*l, q, c*
SEM						
Temperature		0.405	0.372	0.408	0.257	0.416
Level addition		0.573	0.526	0.576	0.364	0.589
Temperature × Level		0.810	0.744	0.815	0.515	0.832
P value						
Temperature		<0.001	<0.001	<0.001	<0.001	<0.001
Level addition		0.004	<0.001	<0.001	<0.001	<0.001
Temperature × Level		0.178	<0.001	<0.001	<0.001	<0.001

[a,b,c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A,B,C] Means* within the same row within time having different superscript letters are different at P<0.05; [A,B,C] Mean within the same column for the main effects of temperature or level having different superscript letters are different at P<0.05.

4.6 Conclusion

All 4 NaSCN : $2Na_2CO_3 3H_2O_2$ levels evaluated increased SCN^- concentration and LP activity but showed no effect on milk composition, with the highest level (21 mg/L) being more supply than lower levels.

This study clearly demonstrates that an optimum level rate was 14 : 30 mg/L NaSCN : $2Na_2CO_3 3H_2O_2$ levels as an effective treatment to extend raw milk quality during storage both at 25ºC and 30ºC. The increase concentration of SCN^- with increasing LP activity was mainly inhibitory effect on microorganism growth, such TBC and CC in raw milk. Although the microorganism growth increased at 30ºC, the variation in a number of bacteria was low.

4.7 References

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CHAPTER V

THE UTILISATION OF FRESH CASSAVA PULP IN DAIRY COW'S DIET ON PRODUCTIVE PERFORMANCE AND MICROORGANISM GROWTH IN RAW MILK

5.1 Abstract

The objective of this study was to investigate the effects of fresh cassava pulp (FCPu) supplement in concentrate as HCN supply source on productive performance, microorganism growth in raw milk of lactating dairy cows. Twenty four Holstein Friesian crossbred lactating dairy cows were blocked by lactation first and then stratified random balanced for day in milk, milk yields and body weight. Cows were assigned into randomized complete block design (RCBD) and divided into 3 treatment groups with 8 cows in each group. The first group (control) received approximately 7 kg of 21% CP concentrate together with *ad libitum* fresh grass (FG), the 2nd group received the control concentrate supplemented with 3.5 kg/d of FCPu (35 ppm HCN) together with *ad libitum* FG; and the 3rd group received the control concentrate supplemented with 7.0 kg/d of FCPu (70 ppm HCN) together with *ad libitum* FG. All cows were individually fed *ad libitum* fresh grass and FCPu according to the treatments. The experiment lasted for 44 days including the first 14 d as the adjustment period, followed by 6 periods of 5 d in each period of measurement period.

The results showed that supplementing 3.5 and 7.0 kg/d FCPu (35 and 70 ppm HCN) had no effects on live weight change, milk yield and milk composition. However, SCN^- concentration and LP activity of 7.0 kg/d FCPu cows were higher than other treatments. TBCs and CCs were lower in 3.5 and 7.0 kg/d FCPu compared to the control. The present study indicated that at 3.5 and 7.0 kg/d FCPu supplementation increased the efficiency of antibacterial activity of the LPs in the raw milk. Therefore, 3.5 kg/d FCPu can be used in the concentrate for lactating dairy cows.

Keywords : Fresh cassava pulp, Cyanide, Raw milk, Lactating dairy cows

5.2 Introduction

Utilisation of cassava (*Manihotesculenta,* Cranzt) in ruminant diets has potential to extend raw milk quality during storage by increasing milk thiocyanate (SCN^-). Cassava is rich in cyanogenic glycosides (Wolfson and Sumner, 1993) which these cyanogens yield cyanide (HCN) following hydrolysis (Keresztessy et al., 2001; Siritunga and Sayre, 2003). HCN is transformed to the non-toxic SCN^- by the action of rhodanese in liver and kidneys of animals. SCN^- is eliminated via raw milk (Soto-Blanco and Gorniak, 2003). Milk SCN^- is used in the lactoperoxidase system (LPs) as an effective antimicrobial system presented in raw milk (Zapico et al., 1991), due to LPs consist of major components i.e. LP, SCN^- and H_2O_2. LPs were generated by H_2O_2 from generating the oxidation of SCN^- giving the product of the antibacterial hypothiocyanite ($OSCN^-$) (Modi et al., 1991). It becomes superior for alternative preservation technology that reliable results and trustworthy quantitative method. Now a day, LPs have been recommended for extending the shelf life of raw milk in

room temperature as an alternative of cooling and inadequate transportation (Björck, 1978; IDF, 1988; 1989), especially small holder farmers. Some studies have shown that SCN^- additives activated the LPs in raw milk under *in vitro* (Gürsel et al., 1999; Fonteh et al., 2005) and *in vivo* (Srinetra, 2001; Buaphan, 2003; Punthanara et al., 2009) to achieve antimicrobial action. It is important to establish optimum inclusion rate of specific HCN because inclusion rate directly affects the cost: benefit ratio of feeding cassava pulp. Therefore, the effects of fresh cassava pulp and optimum inclusion rates need to screen before fresh cassava pulp can be used in dairy cow's diet.

This part of the study focused on the utilisation of fresh cassava pulp in dairy cow's diet on the milk compositions, milk yield, productive performance and microorganism growth in raw milk as an effective treatment to extend raw milk quality during storage.

5.3 Objective

The objective of this experiment was to investigate the utilisation of fresh cassava pulp in dairy cow's diet on productive performance and microorganism growth in raw milk as an effective treatment to extend raw milk quality during storage.

5.4 Materials and methods

Experiment 1 (Exp. 1) Dairy cows were statistically analyzed using a randomized complete block design (RCBD) and treatment means were compared using Duncan’s new multiple range test (DMRT).

Experiment 2 (Exp. 2) TBC and CC were conducted as a 2x3 factorial in RCBD with 6 treatments combination arranged as a factorial (2 different temperatures x 3 HCN levels) with eight replicates per run. In both experiments, the runs were conducted on the same 3 HCN levels from FCPu and separate temperature and times of incubation were used.

5.4.1 Fresh cassava pulp collection

FCPu samples were collected from Korat Flour Industry CO., LTD., Nakhon Ratchasima in the Northeastern region of Thailand. FCPu were divided into 3 parts : the first part was placed in airtight plastic bag with immersed immediately in ice slurry immediately until analyzed HCN content by Pyridine Pyrazorone method (O'Brien, 1991) at Cassava and Starch Technology Research Unit (CSTRU), Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Bangkok, Thailand). The second part; after naturally sundried for 4 or 5 d on a concrete floor, FCPu samples were ground through 1 mm screen and subjected to proximate analysis, detergent analyses and *in sacco* disappearance trial (nylon bag technique).

5.4.2 Animals, experimental design and treatments

All experimental procedures were conducted following the Ethical Principles and Guidelines for the Use of Animals issued by National Research Council of Thailand. Twenty-four crossbred Holstein Friesian lactating dairy cows (early-mid lactation), averaging 50±27 days in milk (DIM), 13.6±3.5 kg of milk and 394±40 kg body weight, were blocked by lactation first and then stratified randomly balanced based on DIM, milk yield and body weight into three groups of 8 cows each. All cows were fed approximately 7 kg/d of 21% CP concentrate. The treatments were: control concentrate together with *ad libitum* fresh grass (FG); control concentrate

supplemented with 3.5 kg/d of FCPu together with *ad libitum* FG; and control concentrate supplemented with 7.0 kg/d of FCPu together with *ad libitum* FG. All cows had free access to clean water, were individually housed in a free-stall unit, and individually fed according to treatments. The experiment lasted for 44 days with the first 2 periods (14 d) was the adjustment period, followed by 30 days (6 periods of 5 d) of a measurement period.

5.4.3 Laboratory analyses

Measurements, sample collection and chemical analysis

Concentrate and roughage were ground through a 2 mm screen for *in sacco* ruminal disappearance determination. Approximately 5 g of 2 mm ground samples were placed into 8x11 cm nylon bags with 47 µm pore size. Samples of feeds were suspended in the rumen of each fistulated non-lactating dairy cow for 0 (pre-feeding), 2, 4, 6, 12, 24, 48 (concentrate) and 72 h (roughage), and then removed and washed in water and then dried at 65ºC for 48 h. After weighing each bag individually, the residues were subjected to dry matter (DM), crude protein (CP) determination. The degradability value was obtained by subjecting nutrient losses at arbitrary of time using NEWAY EXCEL (Chen, 1996).

Feeds offered and residues were weighed for two consecutive days weekly. Feed samples were taken and dried at 60ºC for 48 h. At the end of the experimental period, feed samples were composited and subsamples were taken for further chemical analysis. Samples were ground through a 1 mm screen and subjected to proximate analysis. Crude protein content was determined by Kjeldahl method (procedure 928.08, AOAC, 1998). Ash content was determined by burning in a muffle furnace at 550ºC for 3 h in the muffle furnace (procedure 942.05; AOAC, 1995). The

ether extract was determined by using petroleum ether in a Soxtec System (procedure 948.15, AOAC, 1998). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined using the method described by Van Soest et al. (1991), adapted for Fiber Analyzer. Chemical analysis was expressed on the basis of final DM. Energy values were evaluated using equations recommended by the NRC (2001).

Milk production and compositions

Cows were milked twice daily at 05.00 and 15.00 h, and milk yields were recorded daily for each cow. Milk samples from both the evening and morning milking were collected on two consecutive days of each period and stored at 4°C until analyzed for SCN^- concentration (Codex Alimentarius Commission (CAC/GL 13-1991b), LP activity (Isobe et al., 2009b); milk fat (%), milk protein (%), lactose (%), solid not fat (%) and total solid (%) using Foss MilkoScan^{TM} FT2 infrared automatic analyser (Fourier Transform Infrared Spectroscopy) (Foss Analytical; DK-3400 Hillerøed, Denmark) at The Center for Scientific and Technological Equipment Building (F1), SUT. The second portion of milk samples were examined for microbiological properties including TBC (NA agar) and CC (EMB agar) (AOAC, 2000) and then incubated at 0, 3, 6, 9 and 12 h before and after LP activation. All cows were weighed at the start and end of the experiment (See Chapter III).

Leukocyte culture

Blood was collected and centrifuged at 1500×g for 15min. The erythrocytes were lysed with 0.17mol/L NH_4Cl in Tris solution, and the remaining leukocytes were washed twice with phosphate-buffered saline (PBS). The leukocytes were cultured at concentrations of 10^8 cells/mL with Dulbecco's modified Eagle's medium/F-12 HAM

medium (Sigma Aldrich, St. Louis, MO, USA), at 37ºC for 0-48h with or without 10 μg/mL LPS from *E. coli* O111 : B4 (Wako Pure Chemical Industries, Osaka, Japan). After culture, the cathelicidin-2 concentrations in the conditioned media were measured with an enzyme immunoassay as described previously by Zhang et al. (2014a).

Infusion of E. coli LPS cultivation

LPS in saline solution (0.25mg/5mL) was infused into the jugular veins of the goats. The blood samples were collected and centrifuged immediately at 1500×g for 3 min at 4ºC and the supernatants were collected to measure the cathelicidin-2 concentrations, as described previously (Zhang et al., 2014a). The precipitated red blood cells were ruptured by the addition of H_2O and the remaining leukocytes were washed three times with PBS. The blood leukocytes were spread onto glass slides for immunostaining with an anti-bovine cathelicidin-2 antibody, as described previously (Zhang et al., 2014a; goat cath-2 antibody). The proportions of cathelicidin-2-positive cells in the total leukocytes were calculated.

5.4.4 Statistical analysis

Measured data of intake, milk production, the milk composition and body weight change were analyzed by ANOVA for RCBD using the statistical analysis system (SAS, 1996) (except TBC and CC were analyzed by ANOVA for 2x3 factorial in RCBD). Significant differences among treatment were assessed by DMRT. A significant level of $P<0.05$ was used (Steel and Torrie, 1985).

5.4.5 Experimental location

The experiment was conducted at SUT's Cattle Farm, The Center for Scientific and Technological Equipment Building 10, SUT, Nakhon Ratchasima.

5.4.6 Experimental period

This experiment was carried out during December 2014 to February 2015. (The average of temperature = 25.80±3.33 °C)

5.5 Result and discussion

5.5.1 Feed chemical composition

The analyzed values of chemical composition of the feeds (control concentrate (21%CP), FCPu and FG) are in the range widely reported (Khang et al., 2000, NRC, 2001, Preston, 2004, Suksombat, Luanglawan and Noosen, 2006; Thanh and Suksombat, 2015). The average ash of the FCPu was higher than the concentrate diet but lower than FG. Control had lowest TDN_{1X} and DE_{1X}; in contrast, the FCPu and FG showed lower than control. However, when NE_{LP} is taken into account, the energy values were increased with increasing levels of FCPu supplementation. Variations in chemical composition and energy value of feeds reflect the differences in breeds, harvesting processes, soil types, fertilizer applications, weather, season etc.

The average HCN content of FCPu was 72.20±1.64 ppm (DM basis). Tewe and Lyayi (1989) reported that hydrocyanic acid content of fresh pulp was 34.3 to 301.3 ppm. This level of HCN of FCPu is safe for dairy cows. According to Larson (2006), HCN levels in forage that were potentially toxic were 600 to 1000 ppm (DM basis), or above could cause death in the cattle. Tangkawanit et al. (2014) reported that cassavas in different areas had different levels of cyanogenic glycoside, which may involve the minerals in soils, season and cultivation practices.

Table 5.1 Chemical compositions of feed used in the experimental diets.

Item	Concentrate[1]	Fresh cassava pulp	Fresh Grass
	---------- % of DM ----------		
Dry matter	91.03	21.29	12.22
Ash	9.59	12.38	12.76
Crude protein	21.12	2.18	10.39
Ether extract	3.02	2.16	1.23
Crude fiber	12.64	12.69	37.82
Neutral detergent fiber	43.98	61.62	63.38
Neutral detergent insoluble N	1.44	0.30	0.25
Acid detergent fiber	21.92	15.79	35.25
Acid detergent insoluble N	0.82	0.30	0.46
Acid detergent lignin	6.32	5.61	2.82
TDN_{1x}(%)[2]	61.91	52.32	54.90
DE_{1x} (Mcal/kg)[3]	2.95	2.31	2.52
DE_p (Mcal/kg)[4]	2.87	2.38	2.56
ME_p (Mcal/kg)[5]	2.45	1.96	2.14
NE_{lp} (Mcal/kg)[6]	1.53	1.19	1.31
Cyanide content (mg/kg dry solid)	-	72.20±1.64	-

[1]Contained (as DM basis): 4% cassava 27.5% cassava distillers dried meal, 16% soybean meal (solvent extract), 2% corn gluten feed, 8% rice bran A, 8% cassava ethanol, 6% molasses, 24% palm kernel meal, 2.5% urea and 1.6% mineral (dicalcium phosphate), 0.4% vitamin mineral mix and 0.02% covatak 570. Mineral and vitamin mix : provided per kg of concentrate including 2,000,000 IU Vit. A; 640,000 IU Vit. D3; 64,000 IU Vit. E; 160 g Ca; 99 g S; 80 g P; 16 g Fe; 16 g Mn; 12 g Zn; 3 g Cu; 0.2 g I; 0.05 g Co; 0.05 g Se.

The second and third group (supplementing FCPu at 3.5 and 7.0 kg/h/d respectively).

[2]Total digestible nutrients, TDN_{1X} (%) = tdNFC + tdCP + (tdFA x 2.25) + tdNDF-7 (NRC, 2001)

[3]Digestible energy, DE_{1X} (Mcal/kg) = [(tdNFC/100)x4.2]+[(tdNDF/100) x 4.2]+[(tdCP/100) x 5.6]+[(FA/100) x 9.4]-0.3

[4]DE_P (Mcal/kgDM) = DE_{1X} x Discount (NRC, 2001)

[5]Metabolisable energy at production level, ME_p = [1.01 x (DE_p)-0.45] + [0.0046 x (EE-3)] (NRC, 2001)

[6]Net energy for lactation at production level, NElp = ([0.703 x MEp (Mcal/kg)]-0.19) + ([(0.097 x MEp + 0.19)/97] x [EE-3]) (NRC, 2001)

ADF = Acid-detergent fiber, ADL = Acid-detergent lignin, ADIN = Acid-detergent insoluble nitrogen, DINCP = Acid-detergent insoluble crude protein, NDF = Neutral-detergent fiber, NDIN = Neutral-detergent insoluble nitrogen, NDICP = Neutral-detergent insoluble crude protein TDN_{1X} = total digestible nutrient at maintenance level; DE_P = digestible energy at production level; ME_P = metabolizable energy at production level; NE_{LP} = net energy for lactation at production level; dg = degradability.

5.5.2 Intake and live weight

The average values of nutrient intake and live weight change of lactating dairy cows are presented in Table 5.2. FCPu was used as a source of HCN content because it contains cyanogenic glycosides which yield the HCN 3.5 and 7.0 kg/h/d FCPu were chosen to increase respective HCN of 35 and 70 ppm/d respectively.

Cows supplemented with FCPu had greater total DM, CP and NE_{LP} intake than those fed the control diets. The intakes of DM in the current study were in agreement with literature reports where HCN content was about 2 mg/kg BW of cow which is considered non-toxic to the cattle (Majak and Cheng, 1984). However, Wanapat et al. (1997) reported that cows could consume whole upper part of cassava crop hay up to 11.2 kg/head/d or 3.20% of live weight without any adverse effect to their performance. The effect of HCN content from cassava utilization on feed intake has been variable among previous studies. Hai and Preston et al. (2009) reported that the cattle fed with dried cassava root peelings at 0, 0.25, 0.50 and 0.75 kgDM/100 kg LW decreased intake of the grass but linearly increased overall DMI. In addition, Luanglawan et al. (2012) reported that DM and NE_{LP} intake were decreased in the group supplemented with 40% of cassava peel in lactating dairy cows, while no negative effect were found on milk yield, milk composition, milk fatty acid composition and body weight change (BWC). In contrast, Nitipot et al. (2004) reported that the replacement of cassava chip by cassava pulp at a rate of 0, 50 and 100% in the concentrates and fed to crossbred Holstein Friesian (>87.5% Holstein Friesian) heifers at 2% BW plus *ad libitum* rice straw. The results showed that rice straw and total DM intake, eating behavior, fiber digestibility, rumen fermentation end-products, blood metabolite and body weight gain were similar in all treatments.

Feeding concentrates containing the respective cassava pulp, 35%, 40% and 45% in lactating dairy cows found that DMI (15.3 vs 15.8 kg/d), milk yield (14.2 vs 14.1 kg/d), milk composition and body weight change were unaffected by the treatments (Suksombat et al., 2006).

Table 5.2 Effects of treatment diets on mean values for nutrient intake and performance values of the experimental dairy cows (dry matter intake; DMI, crude protein intake; CPI; NE_{LP} intake), live weight (initial live weight; ILW, final live weight; FLW and live weight change; LWC).

Item	Control	35 ppm HCN[2]	70 ppm HCN[3]	SEM	*P*-value
DM, kg/cow/d					
Concentrate	6.13	6.13	6.13	-	-
Fresh grass	5.27	5.32	5.25	0.02	0.118
Fresh cassava pulp	0	0.48	0.96	-	-
Total	11.40[c]	11.93[b]	12.34[a]	0.02	<0.001
CP, g/cow/d					
Concentrate	1295	1295	1295	-	-
Fresh grass	548	553	545	14.23	0.273
Fresh cassava pulp	0	10.46	20.93	-	-
Total	1842[b]	1858[a]	1861[a]	14.26	0.005
NE_{LP}[1], Mcal/cow/d					
Concentrate	9.38	9.38	9.38	-	-
Fresh grass	6.90	6.97	6.88	0.03	0.102
Fresh cassava pulp	0	0.57	1.14	-	-
Total	16.28[c]	16.92[b]	17.40[a]	0.03	<0.001
Initial live weight, kg	393	396	394	22.42	0.995
Final live weight, kg	385	390	389	22.84	0.987
Live weight change, g/d	-250	-192	-154	58.47	0.517

[1]NE_{LP} = net energy for lactation at the production level.

[2]control concentrate plus 3.5 kg/h/d of fresh cassava pulp (FCPu) together with *ad libitum* FG with approximately 35 ppm HCN (dry matter basis) by calculated.

[3]control concentrate plus 7.0 kg/h/d of fresh cassava pulp (FCPu) together with *ad libitum* FG with approximately 70 ppm HCN (dry matter basis) by calculated.

SEM = standard error of mean.

[a, b] Means within a row with different superscripts are significantly different (P<0.05).

In particular, CP intakes of the cows fed FCPu were higher than those fed the control diets. In contrast to CP intake, no remarkable changes were found for milk yield and milk compositions among the treatments (Table 5.3). The amount of dietary FCPu did not affect live weight of the cows over the course of the trial; however, meanwhile live weight was lost at -250, -192 and -154 g/d in the cows fed the control diets, 3.5 and 7.0 kg/h/d FCPu, respectively. Many studies recommended to reduce the level of substitution of cassava with high HCN in concentrates diet in order to avoid low nutrient intake and milk production of ruminants (Suranindyah and Astuti, 2012; Ukanwoko and Ibeawuchi, 2014).

5.5.3 Milk production and milk composition

Milk production and milk composition were not affected by treatments (Table 5.3). Unchanged milk yield was also reported in the studies of Wanapat et al., 2000a, b; Nguyen et al., 2002; Khampa et al., 2006; Punthanara et al., 2009; Petlum et al., 2012; Lunsin et al., 2012. However, supplementing FCPu for lactating dairy cows were significantly improved milk yield, similar to supplementing cassava hay of Koakhunthod et al., 2001; Kiyothong and Wanapat, 2004. The milk yields increased linearly up to 400 g/day cassava hay (882, 979, 1164, 1327 and 1532 g/day respectively) with a slight reduction (1381 g/day) at 500 g/day of cassava hay in lactating goats diets (Dung et al., 2010). Furthermore, dairy cows fed concentrates containing cassava hay and 2.5% sunflower oil increased milk yield (Chantaprasarn and Wanapat, 2008). There is no evidence to show that the long-term use of the cassava hay would lead to any such conditional risks to decrease in milk production. Supplementation with cassava hay could markedly reduce the requirement of concentrate use and improve the yield and composition of dairy cattle milk (Wanapat et al, 1997, 2000a, 2000b). However, dairy cows on cassava root meal-based diets

have been shown to require higher mineral supplementation, particularly sulfur, than those fed on cereal-based diets (Lisovets and Lipyenchik 1982).

In contrast, the substitution of 30% of wheat bran with dried fermented cassava peel in the diet decreased milk yield and milk composition of lactating goats (Suranindyah and Astuti, 2012). Indeed, the decrease in milk production reported in some previous studies was associated with a depression in DMI and diet digestibility due to disturbances in rumen function caused by a high level of dietary NDF or HCN intake (i.e., >2 mg/kgBW). Discrepancies among studies on the effect of cassava supplementation on milk yield of dairy cows might be due to type of cassava, portion of plant, added level, chemical composition, HCN content and different duration of the experiment. Punthanara et al. (2009) concluded that supplementing cassava hay in the ration at 0, 1, 2 and 3 kg/h/d, which that average HCN contents were approximately 0, 112.29, 224.58 and 336.87 ppm, respectively, caused no harm effect, which may contribute to explain why milk yield of dairy cows fed cassava-containing or supplementing diet was similar to those fed high dietary fiber and HCN-comprising diet in the current study.

In the current study, supplementing FCPu to dairy cows had no effect on milk composition which agreed with previous studies when cows were fed cassava leaf meal (Liem et al., 2000) and cassava leaf silage (Modesto et al., 2009). Santos et al. (2009) reported that the replacement of cassava foliage silage for corn silage had no effect on milk concentrations and yields of fat, protein and lactose. Similarly, there were no interactions between CH and rice bran oil in terms of milk yield and milk composition when lactating dairy cows were supplemented with CH and rice bran oil (Lunsin et al., 2012). Numerous observations from laboratory and field studies indicate that the LPs does not induce adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products (FAO/WHO, 2005).

In contrast, lactating goats fed diets supplemented with CH (Dung et al., 2010) had greater milk fat, protein and TS compared to the control. A recent research also reported reduction of TS and lactose when lactating goats were supplemented cassava peel with 30% DM cassava leaf meal (Ukanwoko and Ibeawuchi, 2014); however, no effect was found on milk fat and protein. The decrease in milk fat in some previous studies was affected by lower DMI, NE_{LP} intake, nutrient digestibility and particularly fiber (Khunkaew, 2009).

Feeding high HCN are typically associated with a production of SCN^- exposure in milk (Wanapat et al. 2000a; Punthanara et al., 2009). These possible changes rely on the high level of HCN in cassava to alter SCN^-. In the present study, significant increases in SCN^- were observed (7.67 ppm for control, 8.20 ppm for 3.5 kg/h/d FCPu and 8.68 ppm for 7.0 kg/h/d FCPu). Similarly, lactating cows fed cassava hay (Wanapat et al., 2000; Buaphan et al., 2003; Punthanara et al., 2009) and cassava chip (Srinetra et al., 2001) increased milk SCN^- concentration. Petlum et al. (2012) showed that the increasing of the total HCN intake by increasing level of ECF supplementation significantly increased milk SCN^- concentration. Other studies have revealed that the SCN^- concentration in milk vary with breed (Pruitt and Reiter, 1985), differences in flocks (Medina et al., 1989) and species and type of feed (Wolfson and Sumner, 1993).

A gradual increase of enzyme activity was observed, being 4.37 U/mL for control after the samples were taken and continued to increase throughout with increasing FCPu, reaching values of 4.66 and 4.92 U/mL for 3.5 kg/h/d and 7.0 kg/h/d FCPu respectively. The highest activity was observed at 7.0 kg/h/d FCPu addition while the activity of control and 3.5 kg/h/d was similar. In contrast, dairy cows fed treatments diet supplemented with cassava hay (1, 2, 3 kg/h/d), the enzyme activity was not affected by treatments (Punthatnara et al., 2009). The average concentrations

of LP in raw milk of cows supplemented with different levels of cassava hay reported by Punthanara et al. (2009) were higher than found in the present study (10.6, 10.9, 11.3 and 11.6 U/mL respectively. The LP activity in raw milk in the present study was higher than that of Fonteh et al. (2002) who reported LP activity in cows with an overall mean of 2.3±1.0 U/mL. However, the result from the present study covered within the normal range of LP activity in raw cow's milk of between 1.2 and 19.4 U/mL (Priutt, 2003; Wolfson and Sumner, 1993). Zapico et al. (1991) suggested that milk SCN^- is used for the LPs. According to Reiter and Härnulv, (1984), Björck, (1978) and Dahlerg et al., (1984) reported that the level of SCN^- required in activating the LPs is 10-15 ppm to achieve an optimal antibacterial activity of the LPs in milk. The LP activity in milk in the present study was measured at 1.44 U/mL, which was sufficient to act as a catalyst for LPs activity (Marshall et al., 1986). Therefore, supplementations of 3.5 and 7.0 kg/d FCPu (35 and 70 ppm HCN) were sufficient to produce the LP activity (mean 8.2 U/ml) in raw milk. Previous research has demonstrated that variations in LP activity were probably dependent on regulating factors (Fragoso et al., 2009) including animal feed (FAO, 1993), the individual animals (Fonteh et al., 2002), breed and season (Fonteh, 2006), number or stages of cow's lactation (primiparous or postpartum) as well as the health of the dairy cow and may also be influenced by estrogen (Kern et al.1963).

A significant decrease in SCC was detected in 7.0 kg/h/d FCPu ($78x10^3$ cells/mL), with 3.5 kg/h/d FCPu equally decreasing SCC compared with the control and 7.0 kg/h/d FCPu. Similarly, Petlum et al. (2012) reported that SCC was statistically significantly decreased by increasing level of ensiled cassava foliage supplementation ($P<0.01$), and also suggested that the decrease in SCC could imply to a reduction in mastitis in lactating cows. Mastitis is an inflammation of udder tissue, usually caused by bacterial infection which contributes to decreased milk production

and subsequent economic losses in the dairy cattle industry. LP is a milk protein with antimicrobial function. LP has been suggested to play an important role in the prevention of mastitis which exhibit against a broad range of Gram-positive/negative bacterial species. Somatic cells are part of the body's self defense mechanism in milk from cow to indicate that the cow is probably affected by subclinical mastitis. Cows from the SUT farm are confirmed as healthy animals and obtained dairy standard farm as certified by Department of Livestock Development, Thailand. It is unexpected that the results derived from the samples prior to supplementing FCPu conformed quite closely to those affected by mastitis. Increased concentrations of LP and SCN^- are found in milk from mastitic cows as compared to milk from healthy animals (FAO/WHO, 2005). *In vivo* experiments indicated that the system of LP plays an important role in protecting the lactating mammary gland from *Streptococcus uberis* infection in cows (Marshall, Cole and Bramley, 1986). Additionally, since the level of LP in milk increases on mastitic infection (Shakeel-ur-Rehman and Farkye, 2003), the LP activity may be used as a possible index for mastitis. Similarly, Isobe et al. (2011) strongly suggested that there were high correlation between LP activity and SCC in bovine milk. Given to the milk with the high SCC ($\geq 500x10^3$ cells/mL) in group of LP activity was assessed to confirm the possibility that indicative SCC can be expected from LP activity. The LPs has been shown to be both bactericidal or bacteriostatic *in vitro* against numerous microorganisms that cause udder infections, e.g. *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S.uberis* and *P. aeruginosa* (Mickelson, 1966; Reiter et al., 1976; Marshall et al., 1986; Sandholm et al., 1988). However, some trial data illustrate that the LPs in mastitic milk is not as effective as in milk from healthy cows because of a higher concentration of reductive agents and higher catalase enzyme activity presented in mastitic milk (Sandholm et al., 1988).

Therefore, SCC is associated with mastitis in ruminants; mastitis is an inflammatory condition of the udder in bovine and other species, caused by bacterial infection. The innate immune function plays an important role in preventing bacterial infections. The innate immune system includes numerous antimicrobial peptides (AMPs), which exert antimicrobial activity against a broad range of Gram-positive and Gram-negative bacteria, viruses, protozoa and fungi (Kamen and Tangpricha, 2010). Therefore, Cathelicidin (Cath) is one of an AMP with broad-spectrum antimicrobial properties (Scott et al., 2011). Tomasinsig et al. (2010) reported that cathelicidin-5 (BMAP-27), -6 (BMAP-28), and -7 mRNAs are expressed in bovine mammary epithelial cells. The expression of bovine Cath-1 and Cath-4 mRNAs has also been reported in cultured mammary epithelial cells in response to *Escherichia coli* and *Staphylococcus aureus* (Ibeagha-Awemu et al., 2010). The Cath-1, -2, -3, -4 and -5 proteins have been identified in the skim milk of mastitic cows (Skerlavaj et al., 1996; Risso et al., 1998; Boehmer et al., 2008; Danielsen et al., 2010; Smolenski et al., 2011). These results indicate that bovine cathelicidins play an important role in the innate immune response in the mammary gland. In goats, the Cath-1 and -3 proteins have been identified in mastitic skim milk (Olumee-Shabon et al., 2013). It has also been reported that Cath-2, -3 and -4 exert strong antimicrobial activities against *E. coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Shamova et al., 1999; 2009). In previous study, the expression of Cath-1, -2, -3, -6 and -7 mRNAs was observed in the mammary tissues of healthy and mastitic goat udders (Zhang et al., 2014a). Immunoreactive Cath-2 did not localize to the epithelial cells of the teat skin, teat cistern or mammary alveoli, but localized to leukocytes collected from the blood and milk of the goats. The Cath-2 concentration in milk increased significantly after an intramammary infusion of LPS compared with the concentration before infusion. These results suggested that Cath-2 was expressed and synthesized in

leukocytes and might be secreted into the milk of goats (Zhang et al., 2014a), but there is no direct evidence to corroborate this. Therefore, the objective of this study was to clarify whether Cath-2 is secreted by goat leukocytes.

Here, we examined whether Cath-2 is secreted by leukocytes. Different concentrations (10^5 to 10^8 cells/mL) of blood leukocytes were cultured for 0 to 48 h with or without lipopolysaccharide (LPS) under *in vitro* assay at Hiroshima University in Japan which published in Srisaikham et al. (2015) (DOI: 10.1111/asj.12438). After culture, the concentrations of Cath-2 in the conditioned media were measured. Blood was collected from male goats 0 to 24 h after the intravenous injection of *Escherichia coli* O111 : B4 LPS. The plasma Cath-2 concentrations were determined and the blood leukocytes immunostained with anticathelicidin-2 antibody to calculate the proportion of Cath-2-positive cells in the total leukocytes. The concentrations of Cath-2 in the media increased significantly when higher concentrations of leukocytes cultured, whereas the addition of LPS to the media caused no further increase. The plasma Cath-2 concentrations did not increase with time after LPS infusion. Therefore, after the leukocytes were cultured at 10^8 cells/mL for 0 to 48 h, the Cath-2 concentration increased dramatically, and the concentrations at 1 h and later were significantly higher than at 0 h. These results suggest that Cath-2 is secreted by leukocytes. However, the cell number of 10^8 was 1000-fold greater than that of 10^5, nevertheless was only an 8-fold increase in cathelicidin-2 concentration. Secreted Cath-2 may be degraded during long culture time or be affected by some feed-back regulation. To examine whether Cath-2 secretion was accelerated by LPS, leukocytes were cultured with LPS. However, the addition of LPS had no effect on the Cath-2 concentration, suggesting that Cath-2 is secreted spontaneously, without LPS stimulation. It has been reported that the Cath-2 concentration and SCC in milk increase significantly after the intramammary infusion of LPS compared with their

concentrations before infusion (Zhang et al., 2014a). Because the SCC predominantly refers to leukocytes, this increase in Cath-2 may be attributable to the increased SCC, but cannot be attributed to the increased secretion of Cath-2. This is also supported by the present results that increased concentration of leukocytes cultured *in vitro* showed higher Cath-2 concentration in conditioned medium. Blood leukocytes were immunostained with an anti-Cath-2 antibody and the Cath-2-positive leukocytes proportion in the total blood leukocytes was calculated. The proportion of Cath-2-positive cells in the total leukocytes was significantly reduced 1 h after LPS injection compared with that at 0 h, but increased again at 6 h and thereafter. Most cathelicidin-2 positive cells were neutrophil. These results suggest that the infusion of LPS had little effect on the secretion of Cath-2 by leukocytes, whereas Cath-2-containing leukocytes may be recruited from the blood vessels by LPS to the tissues showing inflammation, reducing the proportion of Cath-2-positive leukocytes in the blood. Although activity of defensin, another antimicrobial peptide, is inhibited under physiological salt conditions (150 mmol/L NaCl), the resistance of Cath genes was reported in the physiological or even higher NaCl concentration (Nagaoka et al., 2000; Travis et al., 2000; Kościuczuk et al., 2014). Therefore, even if NaCl concentration increases in the blood after LPS injection, cathelicidin must be stable; this supports the above mentioned discussion that Cath-2-containing leukocytes may be recruited from the blood vessels by LPS to the tissues showing inflammation. The recovery of the proportion of Cath-2-positive leukocytes at 6 h after LPS injection may be due to leukocytes replenishing Cath-2 in their cytoplasm. These results suggest that Cath-2 is secreted by leukocytes even without LPS stimulation, whereas LPS may be required for Cath-2-containing leukocytes to be recruited from the blood to tissues showing inflammation.

Table 5.3 Effects of the treatment diets on milk production, milk composition, thiocyanate concentration, lactoperoxidase activity, somatic cell count of the experimental dairy cows.

Item	Control	35 ppm HCN	70 ppm HCN	SEM	*P*-value
Yield					
Milk, kg/d	12.87	13.61	14.28	1.11	0.672
3.5% FCM[1], kg/d	12.89	13.45	14.28	0.86	0.627
Fat, g/d	452	467	500	33	0.615
Protein, g/d	408	438	461	33	0.496
Lactose, g/d	604	642	683	59	0.465
Solid-not-fat, g/d	1002	1176	1244	86	0.463
Total solid, g/d	1553	1644	1744	99	0.569
Composition (g/100 g of raw milk)					
Fat	3.51	3.44	3.50	0.15	0.932
Protein	3.17	3.22	3.23	0.05	0.726
Lactose	4.69	4.72	4.78	0.07	0.156
Solid-not-fat	8.56	8.64	8.71	0.12	0.221
Total solid	12.07	12.08	12.21	0.21	0.888
Thiocyanate (ppm)	7.67^{c}	8.20^{b}	8.68^{a}	0.14	<0.001
LP activity (U/mL)	4.37^{b}	4.66^{ab}	4.92^{a}	0.11	0.011
Somatic cell count (cellx10^{3})	352^{a}	104^{ab}	78^{b}	87.31	0.073

[1]FCM = fat-corrected milk; 3.5% FCM = (0.432 x kg of milk) + (16.216 x kg of milk fat).

5.5.4 Net energy intake

Estimates of the distribution of NE_{LM}, NE_{LOSS}, NE_{LL} and NE_{LR} of cows fed with 0%, 3.5 and 7.0 kg/h/d FCPu remained unchanged among the treatments, while NE_{LP} intake increased with increasing FCPu (Table 5.4). Although total NE_{LP} intake of cows fed with 3.5 kg/h/d FCPu was higher than other treatments. In generally, there will be some loss on the excretion of energy via urine, faeces, gases and heat (Suksombat, 1999).

All groups of cows had a considerable supply of NE_{LP} but the milk yields were lower than would have been predicted from NE_{LP} intakes. The respective intakes of 16.28, 16.92 and 17.40 Mcal daily by the control, 35 and 70 ppm HCN, in theory, should have been able to produce approximately 15.2, 14.8 and 15.2 kg milk/d. The lower milk yield than that would be expected from NE_{LP} available can be attributable to the probable underestimates of NE_{LM} for dairy cows in the tropics. Since the dairy cows in the tropics were fed lower quality feeds than those cows in the United States, the use of the equation suggested by the NRC (2001) might be inappropriate. AAC (1990) recommended that dairy cattle consuming feeds containing energy lower than 10 MJ ME/kg DM (2.39 Mcal ME/kgDM) needed more energy for maintenance. The present study used a net energy maintenance value of 0.080 Mcal/kg $BW^{0.75}$ for predicting NE_{LM}. Similarly with the hypothesis by AAC (1990) assumed that the average net energy values of milk and live weight change are unaffected by the quality of feeds as in a case of NE_{LM}. However, Suksombat and Junpanichcharoen (2005) suggested that, in the tropics, the average net energy for maintenance value of 0.106 Mcal/kg $BW^{0.75}$ would be more appropriate than the value of 0.08 Mcal/kg $BW^{0.75}$ recommended by NRC (2001). Similarly, the work of Suksombat and

Mernkrathoke (2005), the calculated average net energy for maintenance value was 0.083 Mcal/kg $BW^{0.75}$ which was 3.7% higher than that of NRC (2001) recommendation. Before a conclusion can be reached, further research is needed.

Table 5.4 Estimates of the distribution of net energy intake.

Intake	Control	35 ppm HCN	70 ppm HCN	SEM	*P*-value
NE_{LP} intake[1] (Mcal/d)	16.28[c]	16.92[b]	17.40[a]	0.03	<0.001
NE_{LM}[2] (Mcal/d)	7.00	7.05	7.02	0.31	0.993
NE_{LOSS}[3] (Mcal/d)	-0.64	-0.49	-0.40	0.09	0.510
NE_{LL}[4] (Mcal/d)	8.86	9.25	9.85	0.55	0.594
NE_{LR}[5]	15.22	15.81	16.47	0.94	0.745
Efficiency[6]	0.94	0.93	0.95	0.01	0.266

[1]NE_{LP} = net energy for lactation at production level.

[2]NE_{LM} = net energy requirement for maintenance = $0.08 \times LW^{0.75}$.

[3]NE_{LOSS} = net energy requirement for loss = reserve energy × (0.64/0.75) reserve energy = see NRC (2001).

[4]NE_{LL} = net energy requirement for lactation = milk yield (kg/d) × (0.0929 × %fat + 0.0547 × %CP + 0.0395 × %lactose).

[5]NE_{LR} = net energy retention.

[6]Efficiency = NE_{LR}/NE_{LP} intake.

SEM = standard error of mean.

[a, b]Means within a row with different superscripts are significantly different (P<0.05).

Table 5.5 The estimated supply of rumen degradable protein and rumen undegradable protein.

Intake	Control	35 ppm HCN	70 ppm HCN	SEM	*P*-value
RDP_{req} intake[1] (g/head/d)	1200[b]	1225[a]	1230[a]	7.95	0.032
RDP_{sup}[2] (g/head/d)	1081[c]	1123[b]	1155[a]	1.86	<0.001
Deficit/surplus (g/head/d)	-120[a]	-103[a]	-75[b]	7.79	0.002
RUP_{req}[3] (g/head/d)	954	984	1032	106.56	0.873
RUP_{sup}[4] (g/head/d)	761[b]	735[a]	706[a]	6.25	<0.001
Deficit/surplus (g/head/d)	-193	-249	-326	106.46	0.965

[1]RDPreq = rumen degradable protein requirement = 0.15294 × TDN actual.

[2]RDPsup = rumen degradable protein supply = total DM fed × 1,000 × diet CP × CP_RDP.

[3]RUPreq = rumen undegradable protein requirement = total CPReq - (MPBact + MP Endo)/diet RUPDigest.

[4]RUPsup = rumen undegradable protein supply = CP Total - RDPsup.

[a, b] Means within a row with different superscripts are significantly different (P<0.05).

5.5.5 Rumen degradable protein and rumen undegradable protein

The protein degradability values of each treatment diets were determined by nylon bag technique, the estimated supplies of RDP and RUP to the cows were calculated (Table 5.5; NRC, 2001). Cows with supplementing 0, 3.5 and 7.0 kg/h/d FCPu received inadequate RUP. RDP_{sup} and its efficiency of dairy cows fed 7 kg/h/d FCPu were higher than that of cows fed the control diet and 3.5 kg/h/d FCPu, it possible that the diets contain sufficient degradable nitrogen (N) to meet requirement of the microbes. If urea, molasses or non-structural carbohydrate (e.g. cassava waste, molasses and soybean meal (SBM) which are rapidly degraded to yield ammonia are used in the rumen. RDP_{sup} has been to support the synthesis of protein from microbes. The microbial yield increases substantially with increasing protein outflow rate

entering to small intestine, then be absorbed for utilisation (Oldham, 1984). The RDP_{sup} is enough to meet adequate RDP requirement. Therefore, feeds containing a high bypass protein or low protein degradability from cotton seed meal, green bean meal or heat treat soybean are needed to increase RUP supply. In order to achieve such protein requirement, this factor affecting the voluntary intake of dairy cows (Egan and Moir, 1965). Digestible RUP is rich in essential amino acids (Oldham, 1984), if absorbed amino acids are in short supply, the excess of energy yielding nutrients will then be either stored as fat or oxidized. If excess nutrients were to be stored as fat, then milk production might be less than optimal, but the efficiency of use of energy for milk plus tissue deposition would be little affected (ARC, 1980). If excess nutrients were to be oxidized, then it might be expected that the efficiency of utilization of energy for milk production plus tissue deposition would fall (Oldham, 1984). In the present study, the excess energy intake of cows may be stored as fat, then milk production might be less than would have been expected from energy intake.

5.5.6 Effect of temperatures and HCN levels on total bacterial count and coliform count (Exp. 2)

To ensure this *in vitro* screening methodology was relevant to *in vivo* results, we used three (0, 35 and 70 ppm HCN) levels of FCPu in the diets that had been used previously in activating LP studies with raw milk samples where positive results had been reported. However, cassava hay was used in the study by Punthanara et al. (2009), who allocated 16 multiparous cows in early-to-mid lactation (25 kg/h/d ruzi-GS) and at a rate of 6 kg/h/d concentrate with and without cassava hay supplementation (based on the milk yield record at a 2 : 1 ration). SCN^- concentration

in milk (6.99, 13.57, 14.89 and 15.90 ppm) increased with increasing cassava hay supplementation (0, 1, 2 and 3 kg/h/) with subsequent decrease in the standard plate, coliform, psychrotrophic and thermophilic. An increase in the concentration of SCN^- in milk was also reported in the studies of cassava hay (Wanapat et al., 2000); cassava chips (Buaphan, 2003) and ensiled cassava foliage (Petlum et al., 2012). Moreover, dairy cattle supplemented with cassava hay (0, 0.5, 1.0 and 1.5 kg/d) increased methylene blue reduction time from 2.65 h to 2.77, 3.18 and 4.05 h respectively (Srinetra, 2001). In using the same treatment pattern, SCN^- concentration and LP activity were all increased with supplemental cassava plants, regardless of the level of roughage in the diet. In fact, cassava contains cyanogenic glycosides in a form of linamarin (95%) and lotaustralin (5%). Thus, FCPu was considered as a supplement in this *in vivo* study.

For TBC, there was no temperature x HCN levels interaction at 0 through 12 h of incubation (Table 5.6). At 6, 8, 10 and 12 h, TBC was not affected by temperature; however, the reaction proceeds only in the forward direction. Although there were some small differences in TBC between 25°C and 30°C, TBC was significantly increased with increasing incubation time. TBC was highest at 25°C and 30°C for the control, and lowest for 35 and 70 ppm HCN at 25°C . This is in accordance with the findings of Dennis and Ramet (1989) who reported that the bactericidal effect of the LPs against *L. monocytogenes* depends on initial inoculum concentration, culture medium and storage temperature. Härnulv and Kandasamy (1982) revealed that the keeping quality of raw uncooled milk can be substantially extended by the LP-activated system by activity against microbes to varying extents (Siragusa and Johnson, 1989). When the LPs was activated, it extended the raw milk quality for

buffalo milk to keep within criteria at 30°C for both farm (Kumar and Mathur, 1989a) and field (Kumar and Mathur, 1989b). Seifu et al. (2004b) also exhibited that the LPs was bacteriostatic against *S. aureus* ATCC 25923 in South African Indigenous goat milk at 30°C; whereas, *S. aureus* ATCC 25923 is against by bactericidal in Saanen goat milk. Apart from controlling psychrotrophic spoilage bacteria and also preservation of milk by the LPs, it has been widely investigated for its potential to control mesophilic spoilage bacteria, particularly in bovine milk (Kamau and Kroger, 1984; Reiter and Härnulv, 1982; Zajac et al., 1983), and buffalo milk (Chakraborty et al., 1986; Thakar and Dave, 1986). For bacteria that survive the initial bactericidal effect of the LPs, there is an extended lag phase or recovery period. Seifu et al. (2005) clarified that the length of this lag period of time is highly temperature dependent, being much longer at cold during storage than at high temperatures (Björck et al., 1979; Kamau and Kroger, 1984; Zajac et al., 1983). The most bacteria resume the regular growth after recovery. The length of the antibacterial activity achieved by the LPs activation is inversely related to the during storage temperature of the milk (IDF, 1988). The antibacterial effect of the LPs lasts for 7 to 8, 11 to 12, 16 to 17 and 24 to 26 h, as milk is stored at 30, 25, 20 and 15°C, respectively (IDF, 1988).

TBC revealed that the difference in elevated HCN levels was influential in the microbiological properties (TBC at 70 ppm HCN were less than at 35 ppm HCN). TBC decreased linearly with increasing HCN level at 0, 2, 4, 6, 10 and 12 h, such that the highest level (70 ppm HCN) differed from the intermediate levels (35 ppm HCN). As a result, supplementation of FCPu in treatment diet decreased TBC. This is supposed that the increasing milk SCN^- concentration could enhance the efficiency of antimicrobial activity of LPs. Björck et al. (1975) concluded that the resulted LPs

activation by adding SCN^- concentration with H_2O_2 in raw milk reduced bacteria flora and prevented psychrotrophic bacteria growth. Reiter et al. (1976) reported that the *E. coli* was decreased by the bactericidal effect of the LPs whilst rising of SCN^- concentration rose. Kamau et al. (1990) showed that the number of *L. monocytogenes* and *S. aureus* decreased after SCN^- : H_2O_2 addition in milk. Haddadin et al (1996) reported that with the addition of SCN^- : H_2O_2 in raw milk at the lowest level of 15 : 10 ppm stored at 30°C showed inhibited microorganism growth for 9 to 12 h, which is twice the duration. Similar result was also found in the study of Fonteh et al (2005) who conducted the activity of the LPs in raw milk with the addition of 20 ppm SCN^- : 20 ppm H_2O_2 at room temperature (21-23°C), the lactic acid increment could be slowed to remain at acceptable levels for up to 12 h. With increased bacterial contamination, the presence of the bacteria converts more lactose (sugars) into lactic acid and increased acidity lowers the pH level. These two values are indicators of the quality of milk (Chaiprasop, 2001).

There is no response in CC due to HCN addition, the CC presented only at 2 and 4 h both 25°C and 30°C (Table 5.7). After 0 to 12 h, CC was temperature dependent ($P \leq 0.001$). At all incubation times, the CC was higher for 30°C than 25°C. CC responses to 25°C in the pattern of linear manner (only linear for 8, 10 and 12 h) and 30°C (0 and 6 h). Basically, the decreasing rate of CC growth slows down as the incubation proceeds, and if the incubation time is too long, then the measured growth activity of the number of CC is falsely low. CC response to HCN levels in linear (0, 4 and 6 h) and linear and quadratic (8, 10 and 12 h), such that both incubation times highest FCPu level decreased CC compared to the control. The reaction continues to decrease linearly, so there is a significant accumulation of LPs reaction between 4 and

8 h. The results in the present study agreed with Heuvelink et al. (1998), who reported LPs against verotoxigenic *E. coli* O157 : H7. Similarly, Zapico et al. (1995) demonstrated that *E. coli* were low after activation of the LPs in goat milk compared to the control during storage of goat milk at 8^{o}C for 5 days. The antimicrobial activity of LPs showed a bacteriostatic effect against *E. coli* in Saanen and South African Indigenous goat milk at 30^{o}C (Seifu et al., 2004b). However, the different types of bacteria show a varying degree of sensitivity to the LPs. In general, the effect of the antimicrobial activity of the LPs against *E. coli* reported in previously studied by Thomas and Aune (1978) seems to be related to the oxidation of bacterial sulphydryls. Discrepancies among studies on effect of inhibitory effect of the LPs on the difference in sensitivity of bacteria types might be due to be explained by the differences in cell wall structure and their different barrier properties of the experiment (de Wit and van Hooydonk, 1996). Marshall and Reiter (1980) reported that the inner membrane of Gram negative bacteria appears to be more extensively damaged by LP-treatment than is that of Gram-positive species. Indeed, Shin et al. (2001) concluded that *E. coli* are related to the inhibitory effect of the LPs of dehydrogenases in the respiratory chain of *E. coli*.

Table 5.6 Effect of temperature (25ºC vs 30ºC) and HCN levels of FCPu in lactating dairy cows' diets (0, 35 and 70 ppm HCN) on total bacterial count (TBC) in raw milk at incubation time (0, 2, 4, 6, 8, 10 and 12 h) (Exp. 2) (*N* = 8).

Temperature	Dose (ppm HCN)	Number of TBC of HCN in lactating dairy cows' diets (0, 35 and 70 ppm HCN)						
		Time (h)						
		0	2	4	6	8	10	12
25ºC	**Mean**	**$1.70x10^{5B}$**	**$2.53x10^{5B}$**	**$3.47x10^{5B}$**	**$4.78x10^{5B}$**	**$4.49x10^{5B}$**	**$7.36x10^{5A}$**	**$1.15x10^{6A}$**
	0	$2.02x10^{5a}$	$3.09x10^{5a}$	$4.15x10^{5a}$	$6.43x10^{5a}$	$7.20x10^{5a}$	$1.17x10^{6a}$	$1.70x10^{6a}$
	35	$1.66x10^{5b}$	$2.33x10^{5b}$	$3.19x10^{5b}$	$4.00x10^{5b}$	$3.08x10^{5b}$	$5.40x10^{5b}$	$9.66x10^{5b}$
	70	$1.42x10^{5b}$	$2.16x10^{5b}$	$3.08x10^{5b}$	$3.92x10^{5b}$	$3.18x10^{5b}$	$4.98x10^{4b}$	$7.91x10^{5b}$
	Contrast	*l*	*l*	*l*	*l*	*l, q*	*l*	*l*
30ºC	**Mean**	**$2.60x10^{5A}$**	**$3.33x10^{5A}$**	**$4.11x10^{5A}$**	**$5.33x10^{5A}$**	**$5.11x10^{5A}$**	**$7.80x10^{5A}$**	**$1.29x10^{6A}$**
	0	$3.03x10^{5a}$	$3.72x10^{5}$	$4.63x10^{5a}$	$6.91x10^{5a}$	$7.68x10^{5a}$	$1.22x10^{6a}$	$1.75x10^{6}$
	35	$2.48x10^{5b}$	$3.20x10^{5}$	$3.70x10^{5b}$	$4.41x10^{5b}$	$3.61x10^{5b}$	$5.84x10^{5b}$	$1.18x10^{6}$
	70	$2.29x10^{5b}$	$3.06x10^{5}$	$4.00x10^{5ab}$	$4.68x10^{5b}$	$4.02x10^{5b}$	$5.96x10^{5b}$	$9.29x10^{5}$
	Contrast	*l*	*l*	*l, q*	*l*	*l, q*	*l*	*ns*
Dose	**Mean***	**$2.15x10^{5F}$**	**$2.93x10^{5EF}$**	**$3.79x10^{5DE}$**	**$5.05x10^{5C}$**	**$4.80x10^{5CD}$**	**$7.68x10^{5B}$**	**$1.22x10^{6A}$**
	0	$2.53x10^{5a}$	$3.41x10^{5a}$	$4.39x10^{5a}$	$6.67x10^{5a}$	$7.44x10^{5a}$	$1.20x10^{6a}$	$1.73x10^{6a}$
	35	$2.07x10^{5b}$	$2.76x10^{5b}$	$3.44x10^{5b}$	$4.19x10^{5b}$	$3.35x10^{5b}$	$5.62x10^{5b}$	$1.07x10^{6b}$
	70	$1.86x10^{5b}$	$2.61x10^{5b}$	$3.54x10^{5b}$	$4.30x10^{5b}$	$3.60x10^{5b}$	$5.47x10^{5b}$	$8.60x10^{5b}$
	Contrast	*l*	*l*	*l*	*l*	*l, q*	*l*	*l*
SEM								
Temperature		0.633	0.392	0.403	0.368	0.581	0.241	0.360
Dose		0.775	0.480	0.493	0.451	0.712	0.296	0.441
Temperature x Dose		1.096	0.679	0.698	0.638	1.007	0.418	0.624
P value								
Temperature		<0.001	<0.001	0.001	0.295	0.022	0.560	0.410
Dose		<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Temperature x Dose		0.656	0.783	0.547	0.960	0.840	0.975	0.912

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C] Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C] Mean within the same column for the main effects of temperature or dose having different superscript letters are different at P<0.05.

Table 5.7 Effect of temperature (25ºC vs 30ºC) and HCN levels of FCPu in lactating dairy cows' diets (0, 35 and 70 ppm HCN) on coliform count (CC) in raw milk at incubation time (0, 2, 4, 6, 8, 10 and 12 h) (Exp. 2) (*N* = 8).

Temperature	Dose (ppm HCN)	Number of CC of HCN in lactating dairy cows' diets (0, 35 and 70 ppm HCN)						
		Time (h)						
		0	2	4	6	8	10	12
25ºC	**Mean**	**$2.09x10^{3B}$**	**$2.93x10^{3B}$**	**$2.22x10^{4B}$**	**$3.43x10^{4B}$**	**$2.59x10^{5B}$**	**$4.57x10^{5B}$**	**$1.65x10^{6B}$**
	0	$2.54x10^{3}$	$3.09x10^{3}$	$2.73x10^{4}$	$5.06x10^{4}$	$3.17x10^{5a}$	$5.28x10^{5a}$	$2.88x10^{6a}$
	35	$2.28x10^{3}$	$2.91x10^{3}$	$2.01x10^{4}$	$2.73x10^{4}$	$2.34x10^{5b}$	$4.23x10^{5b}$	$1.05x10^{6b}$
	70	$1.46x10^{3}$	$2.77x10^{3}$	$1.94x10^{4}$	$2.52x10^{4}$	$2.26x10^{5b}$	$4.21x10^{5b}$	$1.02x10^{6b}$
	Contrast	*ns*	*ns*	*ns*	*ns*	*l*	*l*	*l*
30ºC	**Mean**	**$4.56x10^{3A}$**	**$6.77x10^{3A}$**	**$4.99x10^{4A}$**	**$7.65x10^{4A}$**	**$5.79x10^{5A}$**	**$1.02x10^{6A}$**	**$3.69x10^{6A}$**
	0	$5.62x10^{3a}$	$7.45x10^{3}$	$6.09x10^{4}$	$1.12x10^{5}$	$7.02x10^{5a}$	$1.17x10^{6a}$	$6.38x10^{6a}$
	35	$4.63x10^{3a}$	$6.62x10^{3}$	$4.59x10^{4}$	$6.18x10^{4}$	$5.30x10^{5b}$	$9.61x10^{5b}$	$2.39x10^{6ab}$
	70	$3.40x10^{3b}$	$6.24x10^{3}$	$4.28x10^{4}$	$5.62x10^{4}$	$5.03x10^{5b}$	$9.37x10^{5b}$	$2.28x10^{6b}$
	Contrast	*l*	*ns*	*ns*	*l*	*l, q*	*l, q*	*l, q*
Dose	**Mean***	**$3.32x10^{3D}$**	**$4.85x10^{3D}$**	**$3.61x10^{4D}$**	**$5.54x10^{4D}$**	**$4.19x10^{5C}$**	**$7.40x10^{5B}$**	**$2.67x10^{6A}$**
	0	$4.08x10^{3a}$	$5.27x10^{3}$	$4.41x10^{4a}$	$8.11x10^{4a}$	$5.10x10^{5a}$	$8.49x10^{5a}$	$4.63x10^{6a}$
	35	$3.46x10^{3a}$	$4.77x10^{3}$	$3.30x10^{4b}$	$4.45x10^{4b}$	$3.82x10^{5b}$	$6.92x10^{5b}$	$1.72x10^{6b}$
	70	$2.43x10^{3b}$	$4.50x10^{3}$	$3.11x10^{4b}$	$4.07x10^{4b}$	$3.65x10^{5b}$	$6.79x10^{5b}$	$1.65x10^{6b}$
	Contrast	*l*	*ns*	*l*	*l*	*l, q*	*l, q*	*l, q*
SEM								
Temperature		0.269	0.316	0.252	0.275	0.463	0.206	0.253
Dose		0.330	0.387	0.308	0.337	0.566	0.252	0.310
Temperature x Dose		0.466	0.547	0.436	0.476	0.801	0.356	0.438
P value								
Temperature		<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Dose		0.004	0.370	0.001	0.019	<0.001	<0.001	<0.001
Temperature x Dose		0.468	0.708	0.483	0.557	0.086	0.188	0.022

[a, b, c] Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C] Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C] Mean within the same column for the main effects of temperature or dose having different superscript letters are different at P<0.05.

5.6 Conclusion

The present study results indicated that the between of 3.5 and 7.0 kg/h/d of FCPu can be used in the concentrate for lactating dairy cows to provided SCN^- concentration, LP activity in raw milk and decreased TBC and CC.

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CHAPTER VI

THE UTILISATION OF FRESH CASSAVA PEEL IN DAIRY COW'S DIET ON PRODUCTIVE PERFORMANCE AND MICROORGANISM GROWTH IN RAW MILK

6.1 Abstract

The objective of this study was to determine the effects of fresh cassava peel (FCPe) as HCN source on productive performance, microorganism growth in raw milk of lactating dairy cows. Twenty four Holstein Friesian crossbred lactating dairy cows were assigned into a completely randomized block design. All cows were fed approximately 6.5 kg/d of 21% CP concentrate and *ad libitum* grass silage (GS). Treatments were: the control concentrate for the 1st group, the 2nd group received the control concentrate supplemented with 400 g/d FCPe (75 ppm HCN) and the 3rd group received the control concentrate supplemented with 800 g/d FCPe (150 ppm HCN). The results showed that FCPe supplementation had no effects on total DMI, LWC, milk yield and the milk composition. The results showed that 400 and 800 g/h/d FCPe enhanced the efficiency of LP activity in raw milk to reduce TBC and CC; therefore 400 g/h/d FCPe can be used in the concentrate for lactating dairy cows.

Keywords : Fresh cassava peel, Cyanide, Raw milk, Lactating dairy cows

6.2 Introduction

Fresh cassava peel (FCPe) an agro-industrial by-product of starch production is widely used as the main source of feedstuff for ruminant animals in Thailand because the cost of other feedstuffs is often high. Increases in the cost of feeds inevitably cause increases in the cost of milk production. However, FCPe residue obtained before the extraction of starch from cassava roots is low crude protein (CP) content, high of toxic cyanogenic glucosides (CG), fibre and ash content (Tewe and Lyayi, 1989). The reported nutritive values of cassava peel were 62.5-71.0% nitrogen-free extract (NFE), 1.65-2.96 Mcal/kg GE, 1.03 Mcal/kg DE (Nwokoro and Ekhosuehi, 2005). FCPe offers an alternative to feedstuffs high-starch grains and can be used as both energy source in the form of silage and feedstuff. In addition, CG a substance found in FCPe tissues disintegrated to hydrocyanic acid after tissue was destroyed. Cyanide (HCN) toxin is transformed to the non-toxic thiocyanate (SCN^-) by rhodanese action in the liver and kidneys of animals (Drakhshan et al., 2004). SCN^- is eliminated mainly via the urine, milk, tears and saliva (Soto-Blanco and Górniak, 2003) which it is useful for maintaining the quality of raw milk. Therefore, lactoperoxidase system (LPs) appeared probable that antibacterial activity is catalyzed by the LP enzyme which is naturally present in milk leading to the oxidation of SCN^- by H_2O_2 to attain either $OSCN^-$ and hypothiocyanous acid (HOSCN) (Shin et al., 2001) and the oxidation of I to yield hypoiodite (OI) and hypoiodous acid (HOI; Bosch et al., 2000). Both of $OSCN^-$ and HOSCN have reduced agent property leading to significant effect against bacterial metabolism and growth. As a result, the bacteriological quality of raw milk is safeguarded by LPs where cooling facilities are often unavailable in rural areas. Without cooling equipment, milk products during storage begin to deteriorate through the process of acidification. LPs is an alternative

option for dairy cattle farmers to preserve raw milk quality instead of the use of expensive artificial cooling systems.

The present study is to evaluate the effectiveness of extending raw milk quality during storage by increasing milk SCN^- via feeding different levels of FCPe in dairy cow's diet.

6.3 Objective

The objective of this experiment was to investigate the utilisation of FCPe in dairy cow's diet on productive performance and microorganism growth in raw milk as an effective treatment to extend raw milk quality during storage.

6.4 Materials and methods

Experiment 1 (Exp. 1) Dairy cows were statistically analyzed using a randomized complete block design (RCBD) and treatment means were compared using Duncan's new multiple range test (DMRT).

Experiment 2 (Exp. 2) TBC and CC were conducted as a 2x3 factorial in RCBD with 6 treatments combination arranged as a factorial (2 different temperatures x 3 HCN levels) with eight replicates per run. In both experiments, the runs were conducted on the same 3 HCN levels from FCPe and separate temperature and times of incubation were used.

6.4.1 Fresh cassava peel collection

FCPe samples were collected from Korat Flour Industry CO., LTD., Nakhon Ratchasima in the Northeastern region of Thailand. FCPe were divided into 3 parts : the first part was placed in airtight plastic bag immersed immediately in ice slurry until analyzed for cyanide content by Pyridine Pyrazorone method (O'Brien, 1991) at

Cassava and Starch Technology Research Unit (CSTRU), Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Bangkok, Thailand. The second part; after naturally sundried for 4 or 5 d on a concrete floor, FCPe samples were ground through 1 mm screen and subject to proximate analysis, detergent analysis and *in sacco* disappearance trial (obtained from nylon bag technique (Ørskov and McDonald, 1979).

6.4.2 Animals, experimental design and treatments

Dairy cattle and feeding managements

All experimental procedures were conducted following the Ethical Principles and Guidelines for the Use of Animals issued by National Research Council of Thailand. Twenty four Holstein Friesian crossbred lactating dairy cows, averaging 87 ± 31 days in milk (DIM), 13.4±2.9 kg of milk and 397±52 kg body weight (BW), were blocked by lactation first and then stratified randomly balanced based on DIM, milk yield and BW into three groups of 8 cows each. All cows were fed approximately 6.5 kg/d of 21% CP concentrate. The treatments were : the control concentrate together with *ad libitum* grass silage (GS); control concentrate supplemented with 400 g/d FCPe together with *ad libitum* GS; and control concentrate supplemented with 800 g/d FCPe together with *ad libitum* GS. All cows had free access to clean water, were individually housed in a free-stall unit and individually fed according to treatments. The experiment lasted for 44 days with the first 2 periods (14 d) was the adjustment period, followed by 30 days (6 periods of 5 d) of the measurement period.

6.4.3 Laboratory analyses

Measurements, sample collection and chemical analysis

Concentrate and roughage was ground through a 2 mm screen for *in sacco*

ruminal disappearance determination. Approximately 5 g of 2 mm ground samples were placed into 8x11 cm nylon bags with 47 μm pore size. Samples of feeds were suspended in the rumen of each of 5 fistulated non-lactating dairy cow for 0 (pre-feeding), 2, 4, 6, 12, 24, 48, (concentrate) and 72 h (roughage) and then removed and washed in water and then dried at 65^{o}C for 48 h. After weighing each bag individually, the residues were subjected to DM, CP determination. The degradability value was obtained by subjecting nutrient losses at arbitrary of time using NEWAY EXCEL (Chen, 1996).

Feeds offered and residues were weighed for two consecutive days of each period. Feed samples were taken and dried at 60^{o}C for 48 hours. At the end of the experimental period, feed samples were composited and subsamples were taken for further chemical analysis. Samples were ground through a 1 mm screen and subjected to proximate analysis. Crude protein content was determined by Kjeldahl method (procedure 928.08, AOAC, 1998). Ash content was determined by burning in a muffle furnace at 550^{o}C for 3 h in a muffle furnace (procedure 942.05; AOAC, 1995). The ether extract was determined by using petroleum ether in a Soxtec System (procedure 948.15, AOAC, 1998). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined using the method described by Van Soest et al. (1991), adapted for Fiber Analyzer. Chemical analysis was expressed on the basis of final DM. Energy values were evaluated using equations recommended by the NRC (2001).

Milk production and compositions

Cows were milked twice daily at 05.00 and 15.00 h, and milk yields were recorded daily for each cow. The milk samples from both the evening and morning milking were collected on two consecutive days of each period and stored at 4^{o}C until

analyzed for SCN^- concentration (Codex Alimentarius Commission (CAC/GL 13-1991b), LP activity (Isobe et al., 2009b), somatic cell count (SCC) using Fossomatic 5000 basic; milk fat (%), milk protein (%), lactose (%), solid not fat (%) and total solid (%) using Foss MilkoScan^{TM} FT2 infrared automatic analyser (Fourier Transform Infrared Spectroscopy) (Foss Analytical; DK-3400 Hillerøed, Denmark) at The Center for Scientific and Technological Equipment Building, SUT. The second portion of milk samples were examined for microbiological properties including TBC (NA agar) and CC (EMB agar) (AOAC, 2000) and then incubated at 0, 3, 6, 9 and 12 h before and after LP activation. All cows were weighed at the start and end of the experiment. (See Chapter III)

6.4.4 Statistical analysis

Measured data of intake, milk production, milk composition and body weight change were analyzed by ANOVA for RCBD using the statistical analysis system (SAS, 1996) (except TBC and CC were analyzed by ANOVA for 2x3 factorial in RCBD). Significant differences among treatment were assessed by DMRT. A significant level of $P<0.05$ was used (Steel and Torrie, 1985).

6.4.5 Experimental location

The experiment was conducted at SUT's Cattle Farm, The Center for Scientific and Technological Equipment Building 10, SUT, Nakhon Ratchasima.

6.4.6 Experimental period

This experiment was carried out during February 2015 to May 2015 (The average of temperature =30.19±1.25^oC)

6.5 Result and discussion

6.5.1 Feed chemical composition

Chemical compositions of the control concentrate, FCPe and GS) are presented in Table 6.1. HCN content increased as the level of FCPe in the treatment diets increased. FCPe has higher DM than that reported by Adegbola (1980) (25.2 and 13.5% respectively). FCPe was low in protein (1.2%) and high in ash (23.4%). Nwokoro and Ekhosuehi (2005), Devendra (1977) and Adegbola (1980) found that cassava peel had 4.3, 4.8 and 6.5% CP respectively while ashes were 1.0, 4.2 and 6.5% respectively. Chumpawadee and Soychuta (2009) reported that cassava peel had high ash content, because of sand and soil from ground contaminated to processing production. CF of FCPe is close to Nwokoro and Ekhosuehi (2005) and Adegbola (1980) reports (10.8, 12.0 and 10.0% respectively). The nutrient values of cassava peel are dependent on climatology, soil fertility, species, an age of harvest and processing etc. (Khang et al, 2000).

FCPe was used only as a source of HCN due to it contains high amounts of HCN content (670.14±1.30 mg/kg dry solid). Tewe and Lyayi (1989) reported that hydrocyanic acid content of fresh peel was 364.2 to 814.7 ppm. In ruminants, a cyanide concentration of 0 to 150 ppm (DM basis) is safe level, 1000 ppm or above in forage (DM basis) is dangerous and then usually will cause death (Larson, 2006). Sandage and Davis (1964) reported that the cyanide contents of 0 to 750 ppm (DM basis) were low toxic and able to cause the death of animals with high toxic were 750-1000 ppm or above. Therefore, the results of this study found that chemical composition of FCPe was in the range reported by other researchers. The variations of

chemical composition and energy values of feeds reflect the differences in breeds, harvesting processes, soil types, fertilizer applications, weather, season etc.

Table 6.1 Chemical compositions of feeds.

Item	Concentrate[1]	Fresh cassava pulp	Fresh Grass
	---------- % of DM ----------		
Dry matter	90.65	25.81	28.54
Ash	9.16	23.44	8.63
Crude protein	20.48	1.19	6.51
Ether extract	2.90	1.91	1.83
Crude fiber	13.67	11.26	36.28
Neutral detergent fiber	44.03	70.60	61.51
Acid detergent fiber	16.43	16.37	57.06
Acid detergent lignin	7.10	7.02	5.58
Neutral detergent insoluble N	1.29	0.33	0.17
Acid detergent insoluble N	0.45	0.24	0.11
TDN_{1x} (%)[2]	61.82	36.50	55.98
DE_{1x} (Mcal/kg)[3]	2.95	1.63	2.53
DE_p (Mcal/kg)[4]	2.88	1.96	2.55
ME_p (Mcal/kg)[5]	2.45	1.53	2.12
NE_{lp} (Mcal/kg)[6]	1.54	0.89	1.30
Cyanide content (mg/kg dry solid)	-	670.14±1.30	-

[1]Contained (as DM basis) : Control (0%; without FCPe) = 18% Cassava, 10% Rice bran A, 6% Molasses, 20% Palm kernel meal, 12% Soybean meal, 12% Bush bean, 17% Cassava ethanol, 2.5% Urea, 1.6% Dicalcium phosphate, 0.4 % Premix, 0.02% Covatak 570 and 0.5% fat powder. The second and third group (supplementing FCPe at 400 and 800 g/h/d respectively).

[2]Total digestible nutrients, TDN_{1X} (%) = tdNFC + tdCP + (tdFA x 2.25) + tdNDF-7 (NRC, 2001).

[3]Digestible energy, DE_{1X} (Mcal/kg) = [(tdNFC/100)x4.2] + [(tdNDF/100)x4.2] + [(tdCP/100)x5.6] + [(FA/100)x9.4]-0.3.

[4]DE_P (Mcal/kgDM) = DE_{1X} x Discount (NRC, 2001).

[5]Metabolisable energy, ME_p = [1.01 x (DE_p)-0.45] + [0.0046 x (EE-3)] (NRC, 2001).

[6]Net energy for lactation, NElp = ([0.703 x MEp (Mcal/kg)]-0.19) + ([(0.097 x MEp + 0.19)/97] x [EE-3]) (NRC, 2001).

Table 6.2 Effects of treatment diets on mean values for nutrient intake, milk production and milk composition and performance values of the experimental dairy cows (dry matter intake; DMI, crude protein intake; CPI; NE_{LP} intake), live weight (initial live weight; ILW, final live weight; FLW, and live weight change; LWC).

Item	Control	75 ppm HCN	150 ppm HCN	SEM	*P*-value
DM, kg/cow/d					
Concentrate	5.86	5.86	5.86	-	-
Grass silage	5.59	5.68	6.00	0.22	0.327
Fresh cassava peel	0	0.11	0.22	-	-
Total	11.45	11.65	12.08	0.22	0.113
CP, g/cow/d					
Concentrate	1200	1200	1200	-	-
Grass silage	364	370	391	14.20	0.326
Fresh cassava peel	0	1.31	2.62	-	-
Total	1564	1571	1594	14.17	0.276
NE_{LP}[1], Mcal/cow/d					
Concentrate	9.0	9.0	9.0	-	-
Grass silage	7.27	7.39	7.82	0.28	0.328
Fresh cassava peel	0	0.10	0.20	-	-
Total	16.27	16.49	17.01	0.28	0.165
Live weight change					
Initial live weight, kg	395	401	398	23.33	0.984
Final live weight, kg	380	382	383	21.51	0.995
Live weight change, g/d	-500	-629	-509	156.24	0.810

[1]NE_{LP} = net energy for lactation at the production level.

[2]control concentrate plus 400 g/h/d fresh cassava peel (FCPe) together with *ad libitum* GS with approximately 75 ppm HCN (dry matter basis) by calculated.

[3]control concentrate plus 800 g/h/d fresh cassava peel (FCPe) together with *ad libitum* GS with approximately 150 ppm HCN (dry matter basis) by calculated.

SEM = standard error of mean.

[a, b]Means within a row with different superscripts are significantly different ($P<0.05$).

6.5.2 Intake and live weight

The foremost consideration to supplement FCPe in dairy cow feeding is potential adverse effects of cyanogenic glycosides which yield the high HCN content from FCPe on feed intake and lactation performance. The intakes of DM in the current study were in agreement with literature report that showed lesser cytotoxic effects of HCN content and type of cassava supplement when total HCN lethality was less than 2 mg/kg BW of cow (Majak and Cheng 1987). Similarly, Kumar (1992) also reported that HCN can be lethal at 2 to 4 mg/kg BW in cattle and sheep. No significant differences were found for DM, CP and NE_{LP} intakes among groups (Table 6.2), however the cows supplemented with high FCPe tended to have higher total DM, CP and NE_{LP} intake than those fed the control diets. The effect of HCN content from cassava supplementation on feed intake has been variable among previous studies. Wanapat et al. (2000) reported that dairy cows' diet based on concentrate and milk yield (1 : 2 to 1 : 4) with level of HCN content supplemented increased from cassava hay at 0, 0.56, 1.13, 1.70 and 5.20 kgDM/h/d in treatment 1 to 5 respectively did not affect on overall intake. This result was supported by Sommart and Bunnakit (2004) who fed Brahman or Charolais-Brahman crossbred yearling beef cattle with concentrates containing 50% cassava chip, 50% cassava pulp or 50% cassava peel at a rate of 1.5% BW together with *ad libitum* rice straw and found no significant difference in body weight gain between the treatment groups although beef cattle on cassava peel consumed less DM than other cattle. In addition, Suranindyah and Astuti (2012) demonstrated that the substitution of 30% of wheat bran with dried fermented cassava peels in lactating Etawah Crossed bred goats had no effect on DM and organic matter intake. In contrast, Lounglawan et al. (2012) reported that DM and

NE_{LP} intake were decreased in the group supplemented with 40% of cassava peel in lactating dairy cows, while no negative effect were found on body weight change. Contrast result was also found in the recent study of, Hai and Preston. (2009) who reported that the cattle fed with dried cassava root peelings (DCRP) at 0, 0.25, 0.50 and 0.75 kgDM/100 kg LW decreased intake of the grass but linear increased overall DMI and decrease in the rumen degradability of grass DM with minimal effects on the degradability of DCRP. It is possible that DMI has a direct relatively effect on NE_{LP} intake depending on the utilisation efficiency of energy via energy losses of fecal-urine excretion, gas production and heat. Research on feeding concentrates containing cassava peel to lactating dairy cows is very limited.

Although, cows supplemented with 800 g/h/d FCPe a total DM, CP and NE_{LP} than those supplemented with 400 g/h/d FCPe and control respectively, no remarkable changes were found for milk yield and compositions among the treatments (Table 6.3). Final live weight (FLW, kg) and live weight change (LWC, g/d) were unaffected by treatments; however, meanwhile LWC was lost at -500, -629 and -509 g/d in the cows fed the control diets, 400 and 800 g/h/d FCPe, respectively. Anaeto et al. (2013) concluded that West African Dwarf fed diet with cassava leaf silage and cassava peels at 1.5% BW had significant greater weight gain compared with that control, while no mortality or effect on wither height, body length and heart girth were found as the animals were fed cassava peels.

6.5.3 Milk production and milk composition

Supplementation of FCPe at 400 and 800 g/h/d had no effect on milk yield and the milk composition (Table 6.3). Unchanged milk yield was also reported in the studies supplementing cassava products of Wanapat et al., 2000a, b; Nguyen et al.,

2003; Suksombat et al., 2006; Khampa et al., 2006; Punthanara et al., 2009; Lunsin, Wanapat and Rowlinson, 2012. However, the supplementing of FCPe for lactating dairy cows tended to increase milk yield being similar to supplementing cassava hay of Koakhunthod et al., 2001; Wanapat, 2001; Kiyothong and Wanapat, 2004 and lactating goats (Dung et al., 2010). Furthermore, dairy cows fed concentrates containing cassava hay and 2.5% sunflower oil increased milk yield (Chantaprasarn and Wanapat, 2008). A similar result was also found in the recent study of Ukanwoko and Ibeawuchi (2014) who reported that milk yield and composition were improved by feeding cassava peel-cassava leaf meal based diets to lactating does. In contrast, substitution of 30% of wheat bran with dried fermented cassava peel in the diet decreased milk yield and composition of lactating goats (Suranindyah and Astuti, 2012). Indeed, the decrease in milk production reported in some previous studies was related to a low DM, CP, dietary NDF and TDN consumptions due to their low digestibility's in the rumen. Discrepancies among studies on the effect of cassava supplement on milk yield of dairy cows might be due to the differences in types, a portion of the cassava plant, HCN level and the experimental duration. However, parity and size have been identified as strong factors influencing milk yield in lactating animals (Akpa et al., 2001).

In the current study, supplementing cassavas for ruminants had no effect on milk composition which was agreed with previous studies using cassava leaf meal (Liem et al., 2000; cassava leaf silage (Modesto et al., 2009), ensiled cassava foliage (Petlum et al., 2012). Santos et al. (2009) reported that the replacement of cassava foliage silage for corn silage had no effect on milk concentrations and yields of fat, protein and lactose. Similarly, there were no interactions between CH and rice bran

oil in terms of milk composition when lactating dairy cows were supplemented with CH and rice bran oil (Lunsin et al., 2012). Numerous observations from laboratory and field studies indicate that the LPs does not induce adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products (FAO/WHO, 2005). In contrast, supplementing CH to lactating goats diets (Dung et al., 2010) had greater milk fat, protein and TS compared to the control. A research also reported reduction of TS and lactose when lactating goats were supplemented with cassava peel and 30% DM cassava leaf meal (Ukanwoko and Ibeawuchi, 2014); however, no effect was found in milk fat and protein. The decrease in milk fat in some previous studies was affected by lower DMI, NE_{LP} intake, nutrient digestibility and particularly fiber (Khunkaew et al, 2009)

The supplementing FCPe diets resulted in a greater concentration of milk SCN^- representing respectively 9.19 and 9.67 ppm when the cows were fed 400 and 800 g/h/d FCPe diets compared to the control diet. The control was milk SCN^- concentration at 8.63 ppm. The results were in agreement with those studies (Wanapat et al., 2000; Srinetra et al., 2001; Buaphan et al., 2003; Petlum et al., 2012), who reported similar increases in the concentration of SCN^- in milk with increasing cassava product. Therefore, SCN^- concentration in milk vary with a great number of factors including breed (Pruitt and Reiter, 1985), differences in flocks and individual animals (Medina et al., 1989), species and type of feed (Wolfson and Sumner, 1993). The high level of HCN content fed diets resulted in marked alternations in the concentration of milk SCN^- relative to the level of supplemented FCPe. These possible changes rely on the high level of HCN in FCPe to alter SCN^-. Zapico et al. (1991) demonstrated that SCN^- concentration in the milk of cows receiving natural

pastures with clover can increase up to 15 ppm. Findings indicating an increase in milk SCN^- concentration were due to the detoxification of cyanide in cassava hay by enzyme rhodanese recorded by Reiter and Härnulv (1984) and Drakhshan Vaziri and Aminlari (2004).

Changes in the LP activity in the milk of dairy cows were determined by different levels of FCPe supplementation (0, 400 and 800 g/h/d). Significant differences were obtained between the LP activity levels determined of control and 800 g/h/d FCPe, whereas, the value of 5.25 U/mL (400 g/h/d) showed no significant difference from those 0 and 800 g/h/d. In contrast, dairy cows fed treatments diet supplemented with cassava hay (1, 2, 3 kg/h/d), the milk lactoperoxidase concentrations was not affected by treatments (Punthatnara et al., 2009). The average concentrations of LP in raw milk of cows supplemented with different levels of cassava hay reported by Punthanara et al. (2009) were higher than found in the present study (10.6, 10.9, 11.3 and 11.6 U/mL respectively). LP activity is ubiquitous in cow's milk, the concentration varies widely from between 1.2 up to 19.4 U/mL (Gothefors and Marklund, 1975). Each type of cow's milk contains LP activity different in an average to 1.4 U/mL (Stephens et al., 1979) or, milk sheep 0.14-2.38 U/mL (Medina et al., 1989), goat milk 1.55 U/mL (Zapico et al., 1990) and 4.45 U/mL (Saad de schoos et al., 1999), breast milk 0.06-0.97 U/mL (Reiter, 1985), the guinea pig milk has the most quantity is 22 U/mL (Stephens et al., 1979) and the colostrum has a very low volume but the rapid rise at 4-5 days after birth (Ryoba et al., 2004).

Table 6.3 Effects of the treatment diets on milk production, milk composition, thiocyanate concentration and lactoperoxidase activity of the experimental dairy cows.

Item	Control	75 ppm HCN	150 ppm HCN	SEM	*P*-value
Yield					
Milk, kg/d	11.32	11.82	12.53	0.83	0.593
3.5% FCM[1], kg/d	12.16	12.53	13.41	0.80	0.247
Fat, g/d	481	459	558	36	0.147
Protein, g/d	308	327	338	20	0.577
Lactose, g/d	486	520	545	50	0.628
Solid-not-fat, g/d	873	930	971	68	0.483
Total solid, g/d	1354	1389	1529	95	0.336
Composition (g/100 g of raw milk)					
Fat	4.25	3.88	4.45	0.18	0.095
Protein	2.72	2.77	2.70	0.06	0.679
Lactose	4.29	4.40	4.35	0.08	0.308
Solid-not-fat	7.71	7.87	7.75	0.08	0.223
Total solid	11.96	11.75	12.20	0.23	0.329
Thiocyanate (SCN^-)	8.63^b	9.19^b	9.67^a	0.16	0.001
LP activity (U/mL)	4.87^b	5.25^{ab}	5.66^a	0.16	0.008
SCC (cellx10^3)	311^a	99^{ab}	71^b	87.31	0.073

[1]FCM = fat-corrected milk; 3.5% FCM = (0.432 x kg of milk) + (16.216 x kg of milk fat).

The LP activity in milk in the present study was found to be 1.44 U/mL, which was sufficient to act as a catalyst for LPs activity (Marshall et al., 1986). Therefore, dairy cows fed treatments diet supplemented with FCPu (Chapter III) at 3.5 and 7.0 kg/h/d were sufficient to produce adequate LP activity (mean 8.2 U/mL) in raw milk. Previous research has demonstrated that variations in LP activity were probably dependent on regulating factors (Fragoso et al., 2009) including animal feed (FAO, 1993), daily variations (Korhonen et al., 1977), individual animals (Fonteh et al., 2002), breeds and seasons (Fonteh, 2006), number or stages of cow's lactation

(primiparous or postpartum) as well as the health of the dairy cow and may also be influenced by estrogen (Kussendrager and van Hooijdonk 2000).

SCC was highest decreased in 800 g/h/d FCPe, with 400 g/h/d FCPe equally decreasing compared with the control and 800 g/h/d FCPe. Similarly, Petlum et al. (2012) reported that SCC was statistically significantly decreased by increasing level of ensiled cassava foliage supplementation and could decrease mastitis in lactating cows. Mastitis is an inflammation of udder tissue, usually caused by bacterial infection. The surveys of mastitis causing bacteria in New Zealand's milk found that 9-16% of the cows were infected by *S. agalactiae*, 2-3% by *S. dysgalactiae*, 0.5-3.3% by *S. uberis* and 24-40% by *S. aureus* (Brookbank, 1966; Elliott et al., (1976). Somatic cells are part of the body's self-defense mechanism in milk from cow indicating that the cow is probably affected by subclinical mastitis. Increased concentrations of LP and SCN^- are found in milk from mastitis cows as compared to milk from healthy animals (FAO/WHO, 2005). Isobe et al. (2011) strongly suggested that there were high correlation between LP activity and SCC in bovine milk. Given to the milk with the high SCC ($\geq 500 \times 10^3$ cells/mL) in group of LP activity was assessed to confirm the possibility that indicative SCC can be expected from LP activity. The LPs has been shown to be the bactericidal or bacteriostatic *in vitro* against numerous microorganisms that cause udder infections, e.g. *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *P. aeruginosa* (Reiter et al., 1976; Marshall et al., 1986). However, some trial data illustrate that the LPs in mastitic milk is not as effective as in milk from healthy cows because of a higher concentration of reductive agents and higher catalase enzyme activity present in mastitic milk (Sandholm et al., 1988).

6.5.4 Net energy intake and rumen degradable protein and rumen undegradable protein

Neither the distribution of net energy intake (Table 6.4) nor RDP and RUP intakes (Table 6.5) were affected by 0, 400 and 800 g/h/d FCPe. All groups of cows had the considerable supply of NE_{LP}, but the milk yields were lower than would have been predicted from NE_{LP} intakes. The respective intakes of 16.27, 16.49 and 17.01 Mcal daily by the control, 75 and 150 ppm HCN, in theory, should have been able to produce approximately 13.3, 14.4 and 13.9 kg milk/d. The lower milk yield than that would be expected from NE_{LP} available can be attributable to the probable underestimates of NE_{LM} for dairy cows in the tropics. Since the dairy cows in the tropics were fed lower quality feeds than those cows in the United States, the use of the equation suggested by the NRC (2001) might be inappropriate. AAC (1990) recommended that dairy cattle consuming feeds containing energy lower than 10 MJ ME/kg DM (2.39 Mcal ME/kgDM) needed more energy for maintenance. The present study used a net energy for maintenance value of 0.080 Mcal/kg $BW^{0.75}$ for predicting NE_{LM}. If the hypothesis by AAC (1990) is true, assumed that the average net energy values of milk and live weight change are unaffected by the quality of feeds as in case of NE_{LM}, the average net energy for maintenance value of 0.081 Mcal/kg $BW^{0.75}$ should be used in this study. This is approximately 1.3% higher than NRC (2001) recommendation. Suksombat and Junpanichcharoen (2005) suggested that, in the tropics, the average net energy maintenance value of 0.106 Mcal/kg $BW^{0.75}$ would be more appropriate than the value of 0.08 Mcal/kg $BW^{0.75}$ recommended by NRC (2001). Similarly, the work of Suksombat and Mernkrathoke (2005), the calculated average net energy maintenance value was 0.083 Mcal/kg $BW^{0.75}$ which was 3.7%

higher than that of NRC (2001) recommendation. Before a conclusion can be reached, further research is needed.

The protein degradability values of each treatment diets were determined by nylon bag technique, to estimated supplies of RDP and RUP to the cows (Table 6.5). Cows on all treatments received inadequate both RDP and RUP. Feeds of higher CP degradability, such as urea, are needed to increase RDP supply. Ruminants derive their protein supply mainly from microbial protein synthesized in the rumen and from ruminal undegradable protein (UDP) (ARC, 1980). Rumen microbes have an absolute requirement for NH_3-N, while microbes that digest non-structural carbohydrate can also utilize NH_3-N, but grow more efficiently when provided with a source of RDP from true protein (Russell et al., 1992). Therefore, feeds containing a true protein (e.g. SBM) are needed to increase RDP supply. However, optimum levels of RDP to provide the proper balance of NH_3-N and true protein are not well defined. With poorer-quality forages, which are common in the tropics, undegradable dietary protein generally stimulates milk yield. Therefore, feeds containing a high bypass protein or low protein degradability from cotton seed meal, green bean meal or heat treat soybean are needed to increase RUP supply.

Table 6.4 Estimates of the distribution of net energy intake.

Intake	Control	75 ppm HCN	150 ppm HCN	SEM	*P*-value
NE_{LP} intake[1] (Mcal/d)	16.27	16.49	17.01	0.28	0.165
NE_{LM}[2] (Mcal/d)	6.97	7.03	7.00	0.30	0.993
NE_{LG}[3] (Mcal/d)	-1.24	-1.56	-1.26	0.11	0.361
NE_{LL}[4] (Mcal/d)	8.25	8.32	9.42	0.57	0.292
NE_{LR}[5]	13.98	13.79	15.17	0.80	0.503
Efficiency[6]	0.86	0.84	0.89	0.02	0.301

[1]NE_{LP} = net energy for lactation at production level.

[2]NE_{LM} = net energy requirement for maintenance = $0.08 \times LW^{0.75}$.

[3]NE_{LG} = net energy requirement for gain = reserve energy × (0.64/0.75) reserve energy = see NRC (2001).

[4]NE_{LL} = net energy requirement for lactation = milk yield (kg/d) × (0.0929 × %fat + 0.0547 × %CP + 0.0395× %lactose).

[5]NE_{LR} = net energy retention.

[6]Efficiency = NE_{LR}/NE_{LP} intake.

SEM = standard error of mean.

[a, b]Means within a row with different superscripts are significantly different (P<0.05).

Table 6.5 The estimated supply of rumen degradable protein and rumen undegradable protein.

Intake	Control	75 ppm HCN	150 ppm HCN	SEM	*P*-value
RDP_{req} intake[1] (g/head/d)	1047	1060	1091	16.31	0.157
RDP_{sup}[2] (g/head/d)	872	875	883	5.10	0.256
Deficit/surplus (g/head/d)	-175	-185	-208	11.22	0.117
RUP_{req}[3] (g/head/d)	894	958	931	115.79	0.924
RUP_{sup}[4] (g/head/d)	692	696	711	9.07	0.294
Deficit/surplus (g/head/d)	-202	-262	-220	115.08	0.926

[1]RDPreq = rumen degradable protein requirement = 0.15294 × TDN actual.

[2]RDPsup = rumen degradable protein supply = total DM fed × 1,000 × diet CP × CP_RDP [3]RUPreq = rumen undegradable protein requirement = total CPReq - (MPBact + MP Endo)/diet RUPDigest.

[4]RUPsup = rumen undegradable protein supply = CP Total – RDPsup.

[a, b]Means within a row with different superscripts are significantly different (P<0.05).

6.5.5 Effect of temperatures and HCN levels on total bacterial count and coliform count (Exp. 2)

There were no temperatures x dose interactions at all incubation times. At 0, 2, 4 and 8 h of incubations, TBCs were higher at 30°C than at 25°C while at 6, 10 and 12 h of incubations; TBCs were similar between 30°C and 25°C. TBCs were decreased with increasing doses both for 25°C and 30°C. For 25°C, TBCs were linearly (at all incubation times) and quadratically (8 h incubation) reduced whereas for 30°C, TBCs were linearly (at all incubation times) and quadratically (4, 6, 8 and 10 h incubation) decreased. TBCs were increased with increasing incubation times regardless of temperature effects.

LPs enhanced thermal destruction of *L. monocytogenes* and *S. aureus* (Kamau et al., 1990b). *L. monocytogenes* is occurred the most rapid killing as samples was heated soon after LPs activation. The initial TBC for the controls has already exceeded this level at 6 h which is above allowable standards and therefore, unacceptable for use in milk production. Slightly higher findings were recorded by Björck et al., (1979); Patel and Sannabhadti (1993); Vivek-Sharma et al., (1999); Bennett (2000). Similarly, Kamau and Kroger, (1984); El-Agamy et al., (1993); Barabas (1995); Lin and Chow (2000). Another influence on the effectiveness of the stimulated LPs is the microbiological condition of the milk prior to treatment (Härnulv and Kandasamy, 1982). The optimum effectiveness for LPs activation is reported to occur if the system is not activated immediately but delayed slightly until after the multiplication of existing bacteria has begun and when the indigenous antibacterial system remains significantly effective (Reiter, 1978). Further, the concept which involves the activation of LPs in milk should be considered as the

fundamental method for inhibiting the growth of acid-producing micro-organisms in un-chilled raw milk (Swedish, 1975).

These declines in the TBC were related to the HCN levels ($P<0.001$). Thus, CC showed a similar pattern of treatments lower for both 75 and 150 ppm HCN compared to the control. For the LPs activated milk by HCN, TBC is inhibited and remains within acceptable standards for up to 6 h ($4.10x10^5$ and $4.16x10^5$ CFU/mL) and 8 h ($3.25x10^5$ and $3.46x10^5$ CFU/mL) at 75 and 150 ppm HCN respectively. In the current study, that supplementing HCN from cassava plants had effect on milk SCN^- concentration to increased and no effect on milk yields in dairy cows agreed with previous studies (Wanapat et al., 2000; Punthanara et al., 2009; Petlum et al., 2012 (also decreasing number of milk somatic cells)). Similarly, an inclusion of the content of cassava chips in total mixed rations in the diet of cows increased milk SCN^- concentration with the subsequent decrease in the total plate and CC in the milk (Buaphan, 2003). Accordingly Gregory et al., (1989) demonstrated that the addition of SCN^- and H_2O_2 can inhibit *L. monocytogenes* (Gram-positive bacteria) and aerobic bacteria at 20°C for 8.9 h and 30°C for up to 2.8 h. Kamau et al. (1990) showed that the number of *L. monocytogenes* and *S. aureus* decreased after SCN^- and H_2O_2 addition in milk. Activation of the LPs in the raw ewe and goat milk samples by using 20 : 20 ppm SCNK : H_2O_2 or 60 : 60 ppm SCNK : H_2O_2 was established to be an effective means of preservation against microbial growth stored at 20 or 30°C for at least 6 h (Gürsel et al., 1999). Similarly, Haddadin et al. (1996) reported that the possibility of extending the shelf-life of bovine, ovine and caprine milks, with the addition of 15 ppm SCN^- : 10 ppm H_2O_2 at 30°C inhibited microorganism growth for 9 to 12 h, which is twice the duration. LPs catalyzes, in the presence of (SCNK : H_2O_2) to yield hypothiocyanite (OSCNK) product, which is bactericidal for enteric

pathogens including multiple antibiotic resistant strains of *E. coli* (Naidu, 2000). These product compounds react with microbial sulfhydryl group to inhibit various cellular functions (Shin et al., 2001). Similar result was also found in the recent study of Fonteh et al. (2005) conducted that the activity of the LPs in raw milk with the addition of SCN^- and H_2O_2 at ratios of 20 : 20 ppm at room temperature (21-23^oC), the lactic acid increment could be slowed to remain at acceptable levels for up to 12 h. Otherwise, it is considered that the increasing of SCN^- concentration in the milk could enhance the antimicrobial effect of LPs. However, Codex Alimentarius Commission (1991) reported that raw milk contains various species of bacteria and that the resistance of those bacteria in raw milk using the LPs is based on microorganism's specific species and strains. LP inhibited mesophilic bacteria and some of the Gram-negative bacteria, including *Pseudomonades* and *E. coli* were more resistance than others bacterial species and strains. Accordingly Wolfson and Sumner (1993), reported that inhibitor reaction or phagocytosis of the LPs depends on the type and number of microorganisms, media conditions of microorganisms and incubation temperature.

There was no temperature x dose interactions at 0, 2, 4, 6, 8 and 10 h of incubations; however, at 12 h of incubation the response of CC to dose depended on temperature. CCs were higher at 30^oC than at 25^oC at all incubation times. For 25^oC, CCs at 0, 2, 4, 6 and 10 h of incubations were similar, i.e. no dose effect, however, at 8 and 12 h CCs were linearly reduced with increasing doses. For 30^oC, CC was unaffected by dose at 2 h incubation, whereas at 0, 4, 6, 8, 10 and 12 h of incubations, CCs were linearly decreased with increasing doses and at 8 and 12 h of incubations they were quadratically reduced by doses.

Table 6.6 Effect of temperature (25ºC vs 30ºC) and HCN levels in lactating dairy cows' diets (0, 75 and 150 ppm HCN) on total bacterial count (TBC) in raw milk at incubation time (0, 2, 4, 6, 8, 10 and 12 h) (Exp. 2) (*N* = 8).

Temperature	Dose (ppm HCN)	Number of TBC of HCN levels in lactating dairy cows' diets (0, 75 and 150 ppm HCN)						
		Time (h)						
		0	**2**	**4**	**6**	**8**	**10**	**12**
25ºC	**Mean**	**$1.66x10^{5B}$**	**$2.49x10^{5B}$**	**$3.43x10^{5B}$**	**$4.74x10^{5A}$**	**$4.45x10^{5B}$**	**$7.32x10^{5A}$**	**$1.15x10^{6A}$**
	0	$1.99x10^{5a}$	$3.07x10^{5a}$	$4.12x10^{5a}$	$6.41x10^{5a}$	$7.18x10^{5a}$	$1.17x10^{6a}$	$1.70x10^{6a}$
	75	$1.62x10^{5b}$	$2.28x10^{5b}$	$3.14x10^{5b}$	$3.94x10^{5b}$	$3.03x10^{5b}$	$5.35x10^{5b}$	$9.61x10^{5b}$
	150	$1.38x10^{5b}$	$2.11x10^{5b}$	$3.03x10^{5b}$	$3.87x10^{5b}$	$3.13x10^{5b}$	$4.94x10^{5b}$	$7.87x10^{5b}$
	Contrast	*l*	*l*	*l*	*l*	*l, q*	*l*	*l*
30ºC	**Mean**	**$2.41x10^{5A}$**	**$3.13x10^{5A}$**	**$4.01x10^{5A}$**	**$5.40x10^{5A}$**	**$5.08x10^{5A}$**	**$8.33x10^{5A}$**	**$1.34x10^{6A}$**
	0	$2.88x10^{5a}$	$3.52x10^{5a}$	$4.72x10^{5a}$	$7.51x10^{5a}$	$8.00x10^{5a}$	$1.36x10^{6a}$	$1.94x10^{6a}$
	75	$2.32x10^{5b}$	$3.04x10^{5ab}$	$3.54x10^{5b}$	$4.24x10^{5b}$	$3.46x10^{5b}$	$5.68x10^{5b}$	$1.17x10^{6b}$
	150	$2.05x10^{5b}$	$2.82x10^{5b}$	$3.76x10^{5b}$	$4.44x10^{5b}$	$3.78x10^{5b}$	$5.82x10^{5b}$	$9.05x10^{5b}$
	Contrast	*l*	*l*	*l, q*	*l, q*	*l, q*	*l, q*	*l*
Dose	**Mean***	**$2.04x10^{5E}$**	**$2.81x10^{5DE}$**	**$3.72x10^{5D}$**	**$4.77x10^{5C}$**	**$5.07x10^{5C}$**	**$7.82x10^{5B}$**	**$1.24x10^{6A}$**
	0	$2.44x10^{5a}$	$3.29x10^{5a}$	$4.42x10^{5a}$	$6.96x10^{5a}$	$7.58x10^{5a}$	$1.76x10^{6a}$	$1.82x10^{6a}$
	75	$1.97x10^{5b}$	$2.66x10^{5b}$	$3.34x10^{5b}$	$4.10x10^{5b}$	$3.25x10^{5b}$	$5.52x10^{5b}$	$1.06x10^{5b}$
	150	$1.72x10^{5c}$	$2.47x10^{5b}$	$3.40x10^{5b}$	$4.16x10^{5b}$	$3.46x10^{5b}$	$5.33x10^{5b}$	$8.46x10^{5b}$
	Contrast	*l*	*l*	*l, q*	*l, q*	*l, q*	*l, q*	*l*
SEM								
Temperature		0.204	0.383	0.381	0.365	0.609	0.259	0.361
Dose		0.250	0.469	0.467	0.447	0.746	0.317	0.442
Temperature x Dose		0.354	0.664	0.660	0.632	1.055	0.449	0.625
P value								
Temperature		<0.001	<0.001	0.002	0.209	0.025	0.390	0.253
Dose		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Temperature x Dose		0.596	0.744	0.735	0.812	0.837	0.851	0.953

[a, b, c]Means within the same column within temperature having different superscript letters are different at P<0.05.

[A, B, C]Means* within the same row within time having different superscript letters are different at P<0.05.

[A, B, C]Mean within the same column for the main effects of temperature or dose having different superscript letters are different at P<0.05.

CCs were increased with increasing doses after 8 h through 12 h of incubations. CC was not influenced by doses at 2 h of incubation regardless of temperature effect, however, CCs were linearly at (0, 4, 6, 8, 10 and 12 h of

incubations) and quadratically (8 and 12 h incubation) reduced with increasing doses. Eyassu et al. (2004) conducted research involving the use of the LPs to inhibit bacteria that cause disease in the milk of Saanen and indigenous goats. The results showed that LPs can inhibit *E. coli*, *L. monocytogenes* and *B. melitensis* at 30^{o}C for 6 h in the milk of both goat species. It was also found that the inhibition of the growth of microbiological organisms through the use of LPs activation can effectively destroy *S. aureus* and *E. coli* in the milk of two species of goat. Similarly Beumer et al. (1985) demonstrated that the bactericidal effect of the LPs against *C. jejuni* in milk which causes of human gastroenteritis.

Seifu et al. (2005) has been reported that the Gram-negative are inhibited by the LPs but also, depending on the medium conditions (pH, temperature, incubation time, cell density) may be killed provided that H_2O_2 is supplied exogenously (Björck et al., 1975).

The present experiment found that after HCN addition, CC decreased as the 0, 4, 6, 8, 10 and 12 h. During incubations, CC was linear and quadratic, with highest CC growth activity at the highest incubation times. Treatments of HCN in the pattern of linear (only linear for 0, 4, 6 and 10 h), with high CC at the low HCN. Levels on initial for mean CC at 0 h were below $1x10^4$ CFU/mL, whereas those mean at 2 and 4 h were gradually high, after during incubations for all intervals responded to be higher throughout the experiment. CC are elevated above acceptable levels which can cause bacterial contamination within the udder, outside the udder and from the surface of equipment used for milk handling and storage (Wallace, 2008), both quantitative and qualitative analyses can help pinpoint the causes. CC is used as an index of sanitation during the handling and processing of milk products. The lowest CC the present experiment was approximately 4 to 6 h for 75 and 150 ppm HCN but was

higher than acceptable levels. Punthanara et al. (2005) (*in vitro*); 2009 (*in vivo*)) also found CC decreased when SCN^- in milk increased.

Although LPs exerts a wide antimicrobial activity against bacteria (Reiter et al., 1976), viral (Poutois et al., 1990) and fungal pathogens (Popper and Knorr, 1997) *in vitro*, its antimicrobial properties are controlled by the reaction of SCN^- and H_2O_2 under LP catalysis and the resultant generation of short-lived intermediary oxidation hypothiocyanate ($OSCN^-$). The $OSCN^-$ is believed to be the most active antibacterial agent (Kussendrage and Hooijdank, 2000), however, it is rendered ineffective by processes involving heat treatment (Björck et al., 1975), while the by-products of the oxidation process (CO_2, NH^-_4, SO^-_4) are inert and harmless (Oram and Reiter, 1966; Hoogendoorn et al., 1977). Sulfhydryl (-SH) oxidation of the cytoplasmic membrane is considered to be the specific activity which LP utilises against bacteria. The degradation of the membrane damages the bacteria's ability to transport glucose and promotes the loss of potassium ions, amino acids and peptides from within the cell's structure (Aune and Thomas, 1978). However, the LPs are much targeted as it specifically exhibits this activity only toward bacterial membranes and has no effect on the mammalian cell membranes (Klebanoff and Rosen, 1979; Reiter, 1979). The activity of the system as described above has shown that the LPs can increase storage times and extend the shelf life of raw milk by delaying bacterial growth (Wolfson and Summer, 1993).

Indicating for CC which renders raw milk unacceptable for use in milk production processing is more than $1x10^4$ CFU/mL, (Ministry of Public Health, 1979). The study concludes the CC at 25°C showed that the control, 75 and 150 ppm HCN at 8 and 12 are significantly different, as well as for at 0, 4, 6, 8, 10 and 12 at

30°C. At 4 h, CC at 25°C and 30°C, this is higher than the acceptable standard for CC for use in milk production processing. CC showed lower in 75 and 150 ppm HCN than the control, but not reaches to an acceptability standard for up to 4 h both at 25°C and 30°C. It must be highlighted that the utilisation of LPs activation for the extension of the shelf-life of milk does not release producers or purchasers from the obligation of practicing good farming techniques and vigilance in terms of observing the general principles of hygiene for the handling and transportation of raw milk. Emphasis should be placed on its use for the purposes of retaining the quality of high-grade milk rather than improving the viability of low-grade or marginal milk. Equally, this technique for the preservation of milk quality can provide encouragement to the dairy farming industry in terms of minimizing wastage and reducing the costs of transportation while facilitating the potential for collection of milk from more remote operations.

Table 6.7 Effect of temperature (25ºC vs 30ºC) and HCN levels in lactating dairy cows' diets (0, 75 and 150 ppm HCN) on coliform count (CC) in raw milk at incubation time (0, 2, 4, 6, 8, 10 and 12 h) (Exp. 2) (*N* = 8).

Temperature	Dose (ppm HCN)	Number of CC of HCN levels in lactating dairy cows' diets (0, 75 and 150 ppm HCN)						
		Time (h)						
		0	**2**	**4**	**6**	**8**	**10**	**12**
25ºC	**Mean**	**$1.88x10^{3B}$**	**$2.66x10^{3B}$**	**$2.20x10^{4B}$**	**$3.41x10^{4B}$**	**$2.59x10^{5B}$**	**$4.57x10^{5B}$**	**$1.65x10^{6B}$**
	0	$2.29x10^{3}$	$2.85x10^{3}$	$2.71x10^{4}$	$5.03x10^{4}$	$3.17x10^{5a}$	$5.28x10^{5}$	$2.87x10^{6a}$
	75	$2.06x10^{3}$	$2.57x10^{3}$	$1.97x10^{4}$	$2.69x10^{4}$	$2.33x10^{5b}$	$4.21x10^{5}$	$1.04x10^{6b}$
	150	$1.29x10^{3}$	$2.56x10^{3}$	$1.92x10^{4}$	$2.50x10^{4}$	$2.26x10^{5b}$	$4.20x10^{5}$	$1.02x10^{6b}$
	Contrast	*ns*	*ns*	*ns*	*ns*	*l*	*ns*	*l*
30ºC	**Mean**	**$4.27x10^{3A}$**	**$6.41x10^{3A}$**	**$4.87x10^{4A}$**	**$7.49x10^{4A}$**	**$5.74x10^{5A}$**	**$1.00x10^{6A}$**	**$3.64x10^{6A}$**
	0	$5.31x10^{3a}$	$7.23x10^{3}$	$6.06x10^{4a}$	$1.11x10^{5a}$	$7.02x10^{5a}$	$1.16x10^{6a}$	$6.31x10^{6a}$
	75	$4.49x10^{3a}$	$6.21x10^{3}$	$4.55x10^{4ab}$	$6.14x10^{4b}$	$5.30x10^{5b}$	$9.60x10^{5b}$	$2.37x10^{6b}$
	150	$3.01x10^{3b}$	$5.78x10^{3}$	$4.00x10^{4b}$	$5.22x10^{4b}$	$4.89x10^{5b}$	$8.69x10^{5b}$	$2.15x10^{6b}$
	Contrast	*l*	*ns*	*l*	*l*	*l, q*	*l*	*l, q*
Dose	**Mean***	**$3.08x10^{3D}$**	**$4.53x10^{3D}$**	**$3.53x10^{4D}$**	**$5.45x10^{4D}$**	**$4.16x10^{5C}$**	**$7.28x10^{5B}$**	**$2.65x10^{6A}$**
	0	$3.80x10^{3a}$	$5.04x10^{3}$	$4.38x10^{4a}$	$8.08x10^{4a}$	$5.10x10^{5a}$	$8.49x10^{5a}$	$4.63x10^{6a}$
	75	$3.28x10^{3a}$	$4.38x10^{3}$	$3.26x10^{4b}$	$4.41x10^{4b}$	$3.82x10^{5b}$	$6.91x10^{5b}$	$1.72x10^{6b}$
	150	$2.15x10^{3b}$	$4.18x10^{3}$	$2.96x10^{4b}$	$3.86x10^{4b}$	$3.58x10^{5b}$	$6.45x10^{5b}$	$1.59x10^{6b}$
	Contrast	*l*	*ns*	*l*	*l*	*l, q*	*l*	*l, q*
SEM								
Temperature		0.247	0.326	0.249	0.274	0.457	0.223	0.253
Dose		0.302	0.399	0.305	0.335	0.560	0.273	0.310
Temperature x Dose		0.428	0.564	0.432	0.474	0.792	0.386	0.438
P value								
Temperature		<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001
Dose		0.001	0.291	0.005	0.015	<0.001	<0.001	<0.001
Temperature x Dose		0.323	0.582	0.342	0.501	0.052	0.057	0.017

[a, b, c]Means within the same column within temperature having different superscript letters are different at P<0.05; [A, B, C]Means* within the same row within time having different superscript letters are different at P<0.05; [A, B, C]Mean within the same column for the main effects of temperature or dose having different superscript letters are different at P<0.05.

6.6 Conclusion

The present study results indicated that 400 and 800 g/h/d FCPe enhanced the LP activity in raw milk, thereby 400 g/h/d FCPe (75 ppm) can be used in the concentrate for lactating dairy cows.

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CHAPTER VII

OVERALL CONCLUSION AND IMPLICATION

7.1 Conclusion and implication

The study aimed to investigate whether supplementation of FCPu or FCPe in dairy cow's diet can enhance the efficiency of antibacterial activity of the LPs in raw milk, without negative effects on LWC, milk yield and milk composition. For these purposes, the study was carried out comprising 4 experiments that divided into 2 parts including *in vitro* and *in vivo* studies. The first two *in vitro* experiments were investigate to screen number of samples and treatments of the effects of activation of the LPs by adding NaSCN alone (Chapter III) and NaSCN : $2Na_2CO_3 3H_2O_2$ (Chapter IV) in raw cow's milk. The *in vitro* technique in analysis of the LP activity was practical research work in goats and dairy cows at Hiroshima University in Japan, during September 2013 - March 2014. The last two experiments, which were designed from the suitable rate of treatments of the above experiments, were conducted to evaluate the effect of SCN^- supplementation from FCPu (Chapter V) and fresh cassava peel (Chapter VI) in dairy cow’s diet on productive performances and microorganism growth in raw milk. Results from these studies can be summarized as follows :

Experiments I and II were conducted as a 2x4 factorial in completely randomized designs (CRD), which factor A was temperature (25^oC vs 30^oC) and factor B with different levels of NaSCN (0, 7, 14 and 21 ppm) in Experiment I, and

NaSCN : $2Na2CO_33H_2O_2$ (0 : 0, 7 : 15, 14 : 30 and 21 : 45 ppm) in Experiment II with 8 replicates per run using 0, 3, 6, 9 and 12 h incubation time *in vitro* assay. The part of *in vitro* study was divided into 2 experiments including, the first portion of the milk samples were analysed for SCN^- concentration, H_2O_2, LP activity and the milk composition, immediately. The second portion of the milk samples were diluted to examined for microbiological properties including TBC and CC before and after LP-activation.

The results from Experiment I showed that the SCN^- concentration and LP activity in raw milk increased with increasing NaSCN. The concentration of SCN^- at 25°C was higher than 30°C after activation of the LPs. The effect of LPs activation by the addition of NaSCN level on SCN^- concentration and LP activity depended upon the level (temperature x NaSCN level interactions, $P \leq 0.01$). The milk composition was not significantly affected after NaSCN addition. TBC and CC growth were inhibited at 14 ppm NaSCN both at 25°C and 30°C while the results from Experiment II showed that the SCN^- concentrations and LP activities in the raw milk increased with increasing the NaSCN : $2Na_2CO_33H_2O_2$ levels. The milk composition was not affected after NaSCN : $2Na_2CO_33H_2O_2$ addition. LP activated milk was an antimicrobial activity at 14 : 30 ppm NaSCN : $2Na_2CO_33H_2O_2$ both at 25°C and 30°C.

In Experiment III, 24 Holstein Friesian crossbred lactating dairy cows were assigned into completely randomized block design with 8 cows in each group. All cows were fed approximately 6 kg/d of 21% CP concentrate and *ad libitum* fresh grass (FG). Treatments were: the control concentrate for the 1st group, the 2nd group received the control concentrate supplemented with 3.5 kg/d of FCPu (35 ppm HCN) and the 3rd group received the control concentrate supplemented with 7.0 kg/d of

FCPu (70 ppm HCN). The 3.5 and 7.0 kg/d FCPu supplementation had no effect on live weight change (LWC), milk yield and the milk composition. The increasing of the total HCN intake by increasing level of FCPu supplementation significantly increased milk SCN^- concentration. A gradual increase of enzyme activity was observed, being 4.37 U/mL for control after the samples were taken and continued to increase throughout with increasing FCPu, reaching values of 4.66 and 4.92 U/mL for 3.5 kg/h/d and 7.0 kg/h/d FCPu respectively. A significant decrease in SCC, leukocytes and neutrophil cell were detected in 7.0 kg/h/d FCPu, with 3.5 kg/h/d FCPu equally decreasing leukocytes and neutrophil compared with 7.0 kg/h/d FCPu. Plasma cath-2 concentration was not affected by treatments. TBC and CC were lower in 3.5 and 7.0 kg/d FCPu compared to the control. This is believed to be due to an improvement in the efficiency of the antibacterial activity of LP in the raw milk. The results showed that at 3.5 and 7.0 kg/d FCPu supplementation increased the efficiency of antibacterial activity of the LPs in the raw milk. Therefore, 3.5 kg/d FCPu can be used in the concentrate for lactating dairy cows although SCN^- concentration and LP activity in raw milk of 7.0 kg/d FCPu cows were higher than other treatments.

In Experiment IV, 24 Holstein Friesian crossbred lactating dairy cows were assigned into completely randomized block design. All cows were fed approximately 5 kg/d of 21% CP concentrate and *ad libitum* grass silage (GS). Treatments were: the control concentrate for the 1st group, the 2nd group received the control concentrate supplemented with 400 g/d FCPe (75 ppm HCN) and the 3rd group received the control concentrate supplemented with 800 g/d FCPe (150 ppm HCN). The FCPe supplementation had no effect on total DMI, LWC, milk yield and the milk composition. Therefore, HCN content in both FCPu and FCPe were at a safe level

based on considerations of potentially toxic that cause death in cattle and they could be used as a supplement for dairy cow diets with no harm. The supplementing FCPe diets resulted in a greater concentration of milk SCN^- representing respectively 9.19 and 9.67 ppm when the cows were fed 400 and 800 g/h/d FCPe compared to the control. However, the significant differences were obtained between the LP activity levels determined of control and 800 g/h/d FCPe supplementation. The results showed that 400 and 800 g/h/d FCPe enhanced the efficiency of LP activity in raw milk to reduce TBC and CC; therefore 400 g/h/d FCPe can be used in the concentrate for lactating dairy cows.

Over the last decades researches have been done so far to preserve extend raw milk during storage by use of chemical and an alternative system of milk preservation based on the activation of a complex natural antibacterial enzyme in the milk. The most widely recommended industrial application of the LPs in food or milk production is in the dairy industry for the raw milk preservation during storage and/or transportation to processing plants. To date these challenge findings have not yet delivered a comprehensive approach to small holdings dairy farm in Thailand, the addition of NaSCN and H_2O_2 for raw milk preservation has not been allowed. So enhancing the efficiency of antibacterial of LPs activity by increasing milk SCN^- via FCPu and FCPe supplementation in dairy cows diet was the objective of this present work. The researches described in this thesis represent a contribution to these overall objectives. An attractive feature of the feeding approach in these experiments was that it was able to integrate into those small holdings dairy farm in several developing countries whereby there was a lengthy duration for inadequate transportation, poor handling systems and mechanical refrigeration which was either unavailable or

economically prohibitive. This is especially relevant to Thailand situation where cooling facilities are often unavailable in rural areas which are poorly resourced in terms of technology including the cooling equipment required to maintain milk quality. Thus, farmers will obtain knowledge information on this research regarding ways to avoid or reduce the use of chemical additives. The novelty of this work is the exploration a feeding method to solve this problem.

Based on the responses observed, the levels of NaSCN addition alone or NaSCN : $2Na_2CO_3 3H_2O_2$ used *in vitro* study showed different responses to inhibitory effect on microorganism growth depending on the temperature keeping, addition level, incubation time and also probably due to the microbiological condition of the milk prior to treatment that influence on the effectiveness of the stimulated LPs. The optimum effectiveness for activation of the LPs is reported to occur if the system is not activated immediately but delayed slightly until after the multiplication of existing bacteria has begun and when the indigenous antibacterial system remains significantly effective. Further, the concept which involves the activation of LPs in the milk should be considered as the fundamental method for inhibiting the growth of acid-producing micro-organisms in un-chilled raw milk. Finally, the activation of the LPs is considered to be a useful method for extending the shelf-life of cow's milk from Chapter III and Chapter IV.

According to these Chapter V and VI, 3.5 kg/h/d FCPu and 400 g/h/d FCPe were the recommended supplemental level in lactating cows. It is important to establish optimum inclusion rate of specific HCN because inclusion rate directly affects the cost: benefits ratio of feeding FCPu or FCPe. Therefore, FCPu or FCPe levels were the valuable supplements for dairy cattle diets because they had no effects

on live weight change, milk yield and the milk composition, thus may enhance the economic return for the farmers. However, the normal levels of milk SCN^- depend on the levels of SCN^- and its precursors in the animals' diet, including thioglycosides (glucosinolates) and cyanogenic glycosides.

For the purposes of interpreting this result against results to the other farms, efforts are required to be made to ensure that the farm management, lactation periods and/or feeding should be the identical to that of the SUT farm criteria, a prototype of medium holding farming system, particularly in the standard dairy farm. It must be highlighted that the utilisation of LPs activation for the extension of the shelf-life of milk does not release producers or purchasers from the obligation of practicing good farming techniques and vigilance in terms of observing the general principles of hygiene for the handling and transportation of raw milk. Emphasis should be placed on its use for the purposes of retaining the quality of high grade milk rather than improving the viability of low-grade or marginal milk. Equally, this technique for the preservation of milk quality can provide encouragement to the dairy farming industry in terms of minimising wastage and reducing the costs of transportation while facilitating the potential for collection of milk from more remote operations. The broader focus of this study is to provide additional rationale with respect to solutions for developing nations which dairy farming industries concentrated around small operations and why 'this strategy to maintain raw milk quality by use of the LPs is necessary for agriculture in developing countries. Based on the responses observed, further work is needed to determine this method to the other local plants for developing countries with might therefore be more effective in a combined system with the other biopreservative.

APPENDIX A

Reagents preparation

1. Tetra-methylbenzidine (TMB) solution

Mix TMB A and TMB B solution at 1 : 1

1.1 TMB A solution

- Citric acid H_2O 2.58 g
- Urea hydrogen peroxidase 0.25g
- $NaHPO_4H_2O$ 8.5 g
- ddH_2O 250 mL

1.2 TMB B solution

- 3, 3', 5, 5'- Tetramethylebenzidine 0.125 g is resolved in Dimethyl sulfoxide 10 mL.
- Add to 250 mL of citric acid. H_2O 2.58 g solution.

2. Lactoperoxidase (LPO) activity measurement

2.1 Sample preparation (milk)

- Centrifuge milk to remove fat 5 min at 12,000 rpm.

2.2 Reaction

- Mix 0.01 mL (10 µL) of skim milk and 0.2 mL TMB solution in 1.5 µL tube and indicate for 30 min at 37°C.
- Centrifuge tube for 1 min.
- Measure optical density of supernatant at 655 nm.

2.3 Preparation of standard

- 24 U of LPO (stock at -80 freezer).
- 18.9 U = 35 µL of 24 U and 15 µL of ddH_2O.
- 14.4 U = 35 µL of 18.9 U and 15 µL of ddH_2O.
- 10 U = 35 µL of 14.4 U and 15 µL of ddH_2O.
- 7 U = 35 µL of 10 U and 15 µL of ddH_2O.
- 4.9 U = 35 µL of 7 U and 15 µL of ddH_2O.
- 3.4 U = 35 µL of 4.9 U and 15 µL of ddH_2O.
- 2.4 U = 35 µL of 3.4 U and 15 µL of ddH_2O.

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