# เจลพอลีแซ็กคาไรด์จากเปลือกผลทุเรียน
# สำหรับป้องกันการติดเชื้อแบคทีเรียก่อโรคเต้านมอักเสบในโคนม

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POLYSACCHARIDE GEL FROM DURIAN RINDS
FOR PREVENTING BACTERIAL MASTITIS IN DAIRY COWS

Mr. Raktham Maktrirat

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedicinal Chemistry
Department of Biochemistry
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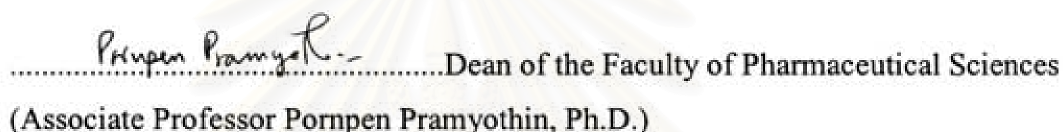
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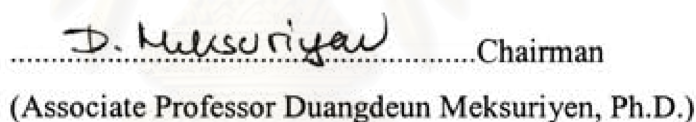
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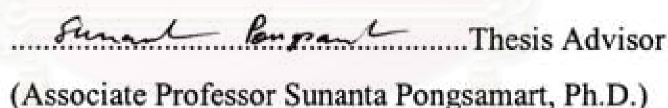
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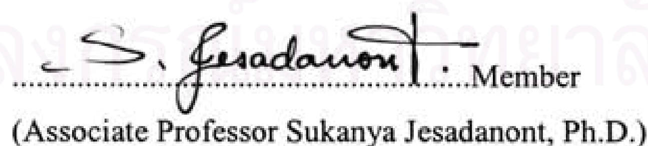
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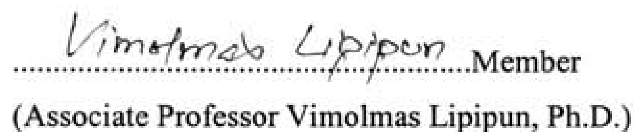
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รักธรรม เมฆไตรรัตน์: เจลพอลิแซ็กคาไรด์จากเปลือกผลทุเรียนสำหรับป้องกันการติดเชื้อแบคทีเรียก่อโรคเต้านมอักเสบในโคนม (POLYSACCHARIDE GEL FROM DURIAN RINDS FOR PREVENTING BACTERIAL MASTITIS IN DAIRY COWS) อาจารย์ที่ปรึกษา ร.ศ. ดร. สุนันท์ พงษ์สามารถ, อาจารย์ที่ปรึกษาร่วม ผ.ศ. ดร. กิตติศักดิ์ อัจฉริยะขจร, 149 หน้า

การใช้สารต้านแบคทีเรียที่ไม่มีพิษซึ่งได้จากผลิตภัณฑ์ธรรมชาติจากพืชคาดว่าจะสามารถนำมาใช้ในฟาร์มโคนมแทนการใช้ยาปฏิชีวนะซึ่งมีข้อจำกัดและมีสารตกค้างจากการใช้ที่มากเกินพอดี เป้าหมายของงานวิจัยนี้ เพื่อค้นหาสารต้านแบคทีเรียชนิดใหม่ ที่สามารถต้านเชื้อแบคทีเรียก่อโรคเต้านมอักเสบได้ ได้ศึกษาฤทธิ์ฆ่าเชื้อแบคทีเรียก่อโรคเต้านมอักเสบและประสิทธิผลในการป้องกันเต้านมอักเสบในโคนมของน้ำยาจุ่มเต้าที่เตรียมจากสารสกัดเจลพอลิแซ็กคาไรด์ของเปลือกทุเรียน ตำรับของน้ำยาจุ่มเต้าประกอบด้วยเจลพอลิแซ็กคาไรด์ 2.5 เปอร์เซ็นต์ (w/v) ในสารละลายริงค์เกอร์ ร่วมกับไกลเซอรีนและโพรไพลีน ไกลคอลอย่างละ 10 เปอร์เซ็นต์ การประเมินระยะเวลาในการฆ่าเชื้อแบคทีเรียที่แยกจากแม่โคป่วยด้วยโรคเต้านมอักเสบของน้ำยาจุ่มเต้าเตรียมจากเจลพอลิแซ็กคาไรด์โดยทดสอบฤทธิ์ในห้องปฏิบัติการ แสดงให้เห็นถึงความสามารถในการฆ่าเชื้อก่อโรคเต้านมอักเสบทุกสายพันธุ์ที่ทดสอบได้แก่ *Staphylococcus aureus, S. chromogenes, S. simulans, Streptococcus agalactiae, S dysagalactiae, S. uberis, S acidominimus, S. bovis, S. porcinus, Escherichia coli, Klebsiella* sp. และ *Pseudomonas* sp. ทำให้จำนวนเซลล์ของแบคทีเรีย Streptococci ที่ทดสอบลดลงมากกว่า 90 เปอร์เซ็นต์ภายในเวลาหนึ่งนาที ประสิทธิผลในการป้องกันเต้านมอักเสบของน้ำยาจุ่มเต้าเตรียมจากเจลพอลิแซ็กคาไรด์ ทดสอบด้วยวิธีท้าทายด้วยเชื้อ (experimental challenge) และสัมผัสเชื้อตามธรรมชาติ (natural exposure) พบว่าสามารถลดอัตราของการติดเชื้อใหม่เข้าสู่เต้านมจากเชื้อ *S. aurues* และ *S. agalactiae* ได้ 100 เปอร์เซ็นต์ จากวิธีการทดสอบแบบท้าทายด้วยเชื้อ ในขณะที่ผลทดสอบประสิทธิภาพการป้องกันการติดเชื้อตามธรรมชาติพบว่า อัตราการติดเชื้อใหม่ในแม่โคกลุ่มที่ใช้น้ำยาจุ่มเต้าเตรียมจากเจลพอลิแซ็กคาไรด์ไม่แตกต่างกับกลุ่มที่ใช้น้ำยาจุ่มเต้าชนิดไอโอโดฟอร์ (กลุ่มควบคุม) และมีประสิทธิภาพในการป้องกันการติดเชื้อแบคทีเรียติดต่อร้ายแรง (contagious bacteria) ตลอดระยะเวลาที่ทำการศึกษาไม่พบอาการระคายเคืองที่ผิวหนังเต้านม ผลการทดลองชี้ให้เห็นว่าน้ำยาจุ่มเต้าเตรียมจากเจลพอลิแซ็กคาไรด์จากเปลือกทุเรียนมีศักยภาพในการป้องกันการติดเชื้อเต้านมอักเสบใหม่ในแม่โค ยังได้ทดสอบผลของเจลพอลิแซ็กคาไรด์ต่อภูมิคุ้มกัน และทดสอบความเป็นพิษต่อเซลล์เม็ดเลือดขาวจากเต้านมโคโดยใช้วิธี XTT reduction method และโดยวิธีวัดการติดสีโพรปิเดียม ไอโอไดด์ ด้วยเครื่องโฟลไซโตมิเตอร์ ผลการทดลองไม่พบการเกิดพิษต่อเซลล์เมื่อใช้เจลพอลิแซ็กคาไรด์ 1 เปอร์เซ็นต์ แต่เจลพอลิแซ็กคาไรด์ 2.5 เปอร์เซ็นต์มีพิษต่อเซลล์ปานกลาง การทดสอบฤทธิ์กระตุ้นการทำงานระบบภูมิคุ้มกันของเต้านม โดยได้ทดสอบฤทธิ์กระตุ้นการเก็บกินของเม็ดเลือดขาวด้วยวิธี NBT reduction assay ผลการทดลองชี้ให้เห็นว่าเจลพอลิแซ็กคาไรด์ 1 เปอร์เซ็นต์ มีฤทธิ์กระตุ้นการเก็บกินของเม็ดเลือดขาวจากเต้านมโคสูงสุด เมื่อเปรียบเทียบกับกลุ่มที่ให้เจลพอลิแซ็กคาไรด์ 0.5 และ 2.5 เปอร์เซ็นต์ และกลุ่มควบคุม ภาพถ่ายจากกล้องจุลทรรศน์อิเล็คตรอนชนิดส่องกราดของมาโครฟาจจากเต้านมภายหลังจากให้สัมผัสกับสารเจลพอลิแซ็กคาไรด์ พบการสร้างเท้าเทียม (pseudopodal formation) อย่างมากมายบนผิวของเซลล์ที่ได้สัมผัสกับเจลพอลิแซ็กคาไรด์ 1 เปอร์เซ็นต์เช่นเดียวกัน จึงเลือกความเข้มข้น 1 เปอร์เซ็นต์มาฉีดเข้าเต้านมของแม่โคระยะแห้งนม เพื่อศึกษาการกระตุ้นระบบภูมิคุ้มกันในแม่โค ผลการทดลองในการฉีดสาร เจลพอลิแซ็กคาไรด์เข้าเต้านมที่ขนาด 12.5, 25 และ 62.5 มิลลิลิตร พบการเพิ่มขึ้นชั่วขณะหนึ่งของเซลล์โซมาติคในสารคัดหลั่งจากเต้านม รวมกับเม็ดเลือดขาวชนิดที่มีการแสดงออกที่ผิวเซลล์ของโมเลกุล CD14 หรือ MHC class II จำนวนเม็ดเลือดขาวทั้งหมดเพิ่มสูงขึ้นอย่างมีนัยสำคัญในกระแสเลือดของแม่โคที่ได้รับสารสกัดในวันที่ 8 อย่างไรก็ดีสัดส่วนเม็ดเลือดขาวชนิดต่างๆ ไม่มีความแตกต่างกับในกลุ่มควบคุม และการฉีดสารสกัดเข้าเต้านมแม่โคในระยะแห้งนมสามารถกระตุ้นการส่งเสริมของระบบภูมิคุ้มกันแบบเฉพาะที่ (local immune response) ฤทธิ์การฆ่าเชื้อแบคทีเรียและกระตุ้นภูมิคุ้มกันของเจลพอลิแซ็กคาไรด์อาจมีผลดีในการช่วยลดความเสี่ยงของการติดเชื้อในระยะแห้งนมลงได้ ผลการทดลองบ่งชี้ว่าเจลพอลิแซ็กคาไรด์อาจนำมาใช้เป็นน้ำยาจุ่มเต้าทางเลือกใหม่ที่น่าสนใจสำหรับใช้เป็นประจำในฟาร์มโคนม เนื่องจากการใช้สารเคมีในฟาร์มปศุสัตว์กำลังจะถูกจำกัดการใช้มากขึ้นในอนาคตอันใกล้นี้

ภาควิชา.........................ชีวเคมี.........................ลายมือชื่อนิสิต.........................

สาขาวิชา.........................ชีวเวชเคมี.........................ลายมือชื่ออาจารย์ที่ปรึกษา.........................

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## # 4676617533; MAJOR BIOMEDICINAL CHEMISTRY
KEYWORD: *Durio zibethinus* / POLYSACCHARIDE GEL / ANTIBACTERIA / IMMUNOMODULATOR

RAKTHAM MAKTRIRAT: POLYSACCHARIDE GEL FROM DURIAN RINDS FOR PREVENTING BACTERIAL MASTITIS IN DAIRY COWS :ASSOC. PROF. SUNANTA PONGSAMART, Ph.D., THESIS CO-ADVISOR; ASST. PROF. KITTISAK AJARIYAKHAJORN, Ph.D 149 pp.

The uses of natural non-toxic antibacterial agents from plant source are expected to replace the limited uses and contaminated residues of excessive antibiotics uses in dairy farm. This research aimed to find a novel antibacterial agent against bacterial isolates from mastitic cows. Bactericidal activity against mastitis-causing bacteria and mastitis preventing efficacy of teat antiseptic of polysaccharide gel (PG) from fruit-rinds of durian (*Durio zibethinus* Murr.) were investigated. PG teat dip was formulated as a teat sanitizer by using 2.5% PG (w/v) in Ringer's solution together with 10% glycerin and 10% propylene glycol. The PG teat dip was tested for bactericidal activity against nine field bacterial isolates causing mastitis by *in vitro* time-kill analysis. The PG teat dip illustrated good killing effect to all tested isolates including *Staphylococcus aureus*, *S. chromogenes*, *S. simulans*, *Streptococcus agalactiae*, *S. dysagalactiae*, *S. acidominimus*, *S. uberis*, *S. bovis*, *S. porcinus*, *Escherichia coli*, *Klebsiella* sp. and *Pseudomonas* sp. The reduction in cfu/ml values of tested Streptococci more than 90% was observed within 1 minute. The efficacy of PG teat dip was evaluated for mastitis prevention *in vivo* by experimental challenge and natural exposure field trials. The PG teat dip reduced percentage incidence density for new intramammary infection (IMI) by 100% ($p < 0.05$) with both *S. aureus* and *S. agalactiae* as demonstrated by using the experimental challenge protocol. The results of natural exposure study showed that the incidence rates between PG teat dip and iodophor (positive control) group was not different ($p > 0.05$). Soft teat skin and no irritation of teat end were also observed during the study period. PG teat dip effectively prevented mastitis infection against contagious bacteria was observed by natural exposure study *in vivo*. The results indicated that the antiseptic teat dip of PG from durian rinds has potential to prevent bovine IMI in lactating cows. Immunomodulatory activity of PG was also evaluated. Cytotoxic effect of PG was tested by XTT reduction method together with flow cytometric procedure with propidium iodide on bovine mammary leukocytes, no cytotoxic effect was observed with 1% w/v PG, however, at 2.5% w/v PG showed moderate cytotoxic effect. The immunostimulating potential of PG on bovine mammary gland was assessed by *in vitro* phagocytosis assay using nitroblue tetrazolium (NBT) reduction. The result indicated that at 1% w/v PG caused the highest phagocytic activation of bovine mammary leukocytes compared with 0.5, 2.5% w/v PG treated groups and its control. The scanning electron micrograph of macrophages after exposured with PG also showed a similar result, pseudopodal formation of macrophage was high with 1% w/v PG. Therefore 1% PG was used for *in vivo* study, intramammary infusion of 1% PG in non-lactating cows was performed to evaluated the immune system stimulation. The results showed that a single intramammary infusion of three different doses at 12.5, 25 and 62.5 ml of 1% PG caused a transient elevation of the somatic cell count by increasing number of CD14 and MHC class II-positive leucocytes in udder secretions. The total white blood cells count was significantly increased at day 8 post-infusion; however, differential counts of peripheral blood samples were not significantly different throughout the study in comparison with normal control cows. Intramammary infusion of PG into the bovine udder potentially enhanced some local immune responses in non-lactating cows. Bactericidal and immunostimulating activity of PG may benefit in reduction the risk of udder infections during the dry period. The results suggested that PG may be use as an alternative attractive teat antiseptic for routine use in dairy farm, since using of antibiotic and chemical agents in livestock will be restricted in near future.

Department................Biochemistry............ Student's signature..............................
Field of study....Biomedicinal Chemistry.... Advisor's signature..............................
Academic year..................2007.................. Co-advisor's signature..............................

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| ATCC | American Type Culture Collection |
| bLf | bovine lactoferrin |
| $^{o}C$ | degree celsius (centigrade) |
| CNS | Coagulase-negative staphylococci |
| $CO_2$ | carbondioxide |
| DMSO | dimethylsulfoxide |
| DMEM | dulbecco’s modified eagle’s medium |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| *et al.* | Et alii, and Others. |
| FBS | fetal bovine serum |
| g | gram (s) |
| h | hour (s) |
| $H_2O_2$ | hydrogen peroxide |
| HRP | horseradish peroxidase |
| Ig | immunoglobulin |
| IMI | intramammary infection |
| KOH | potassium hydroxide |
| LPS | lipopolysaccharide |
| mg | milligram (s) |
| MHC | major histocompatibility complex |
| min. | minute (s) |
| ml | millilitre (s) |

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| | |
|---|---|
| mM | millimolar |
| NaCl | sodium chloride |
| NBT | nitro blue tetrazolium |
| NO | nitric oxide |
| OD | optical density |
| PBS | phosphate buffer saline solution |
| PG | Polysaccharide gel |
| pH | the negative logarithm of hydrogen ion concentration |
| PI | propidium iodide |
| PMN | Polymorphonuclear neutrophil |
| PMS | Phenazine methosulfate |
| RG | Rhamnogalacturonan |
| RNase | ribonuclease |
| ROS | Reactive oxygen species |
| SCC | somatic cell count |
| S.E. | standard error of mean |
| SPSS | Statistical package for social sciences |
| TMB | 3,3´,5,5´- tetramethylbenzidine |
| XTT | 3,3,-[(phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzensulfonic acid hydrate |
| μg | microgram (s) |
| μl | microlitre (s) |
| % | percentage |

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# CHAPTER I

# INTRODUCTION

Bovine mastitis, an inflammatory reaction of mammary gland, is the most common infectious disease affecting dairy cows worldwide. This kind of disease is a great cause of economic losses due to reduction in milk production, a major cause of premature culling, entailing massive antibiotic use and increased cost of veterinary services and labor wage (Blosser, 1979). Moreover, mastitis is also a serious public health problem due to the presence of antiseptics and antibiotics contaminated residues and a cause of food-borne illness from milk containing mastitis-causing pathogens.

The prevention and treatment of mastitis represent a serious burden to producers and a primary concern of the dairy industry. Postmilking teat antisepsis (teat dipping) application as the primary method for preventing new intramammary infection (IMI) is well documented (Dodd *et al.*, 1969). In the marketplace, many different products with various active ingredients are available for teat dipping products whose several chemical agents have shown the risk of milk contaminated residues (Ruegg and Tabone, 2000), teat skin irritation (Jeng and Severin, 1998) and causes of bacterial resistance. Using of antibiotics are the only proven method for treatment of mastitis, however antibiotic therapy of established mammary infection is only moderately efficacious and require prolonged milk withdrawal due to residue in milk. Many efforts have been tried to developed management tools for alternative approaches to mastitis control.

World Health Organization has recommended all member countries to actively promote native medicines of their respective country (Kamb, 2003). Fortunately, antibacterial activity has been studied in water-soluble polysaccharide gel from fruit-rinds of durian (*Durio zibethinus* Murr.) (Pongsamart *et al.*, 2005). Durian is the most famous fruit and economical importance in Thailand. Several hundred thousand tons of durian-rind wastes need to scavenge every year. Durian polysaccharide gel exhibits biological activity of different kinds. The polysaccharide gel isolated from durian fruit-rinds was previously characterized to

be a pectic substance containing polygalacturonic acid of acidic and neutral chains branched with side chains of neutral sugars such as glucose, rhamnose, galactose, fructose and arabinose (Hokputsa *et al.*, 2004). More investigations have also shown that water-soluble polysaccharide gel has bactericidal activities against several strains of Gram positive and negative bacteria and most of mastitis bacterial isolates from cows (Lipipun *et al.*, 2002). The immunological defense system stimulation of black tiger shrimp after feeding with diet containing durian polysaccharide gel was also recently reported (Pholdaeng *et al*., 2004). *In vivo* study demonstrated that the dressing films prepared from the durian polysaccharide gel enhance wound healing in pig and dog skin (Nakchat, 2002 and Siripoksupkul, 2004). Toxicity test of the polysaccharide gel has been reported, a high oral dose (2g/kg) did not induce severe toxicity in male mice and rats (Pongsamart, *et. al*., 2001). No toxic effect has been observed in subacute treatment in male mice and subchronic studies in male and female mice confirmed the consumptive safety of durian polysaccharide gel (Pongsamart *et al*., 2002).

Thailand is a major producers and world exporter of fresh and preserved durians. Durian season start from April to September, the most durian cultivated areas are the eastern and northern Thailand. Massive amounts of the durian waste lead to environmental problems. It has been estimated that up to 4.14 x $10^5$ tons of durian rinds is produced in nature each year (Center of agricultural information, 2004). In the interest of the environment, attempts have recently been made to derive this agricultural waste to produce a value added material that useful as pharmaceutical excipient for pharmaceutical, cosmetic and food industries (Pongsamart *et al*., 1989).

Since using of chemical agents and antibiotics in livestock will be soon restricted, the biodegradable polysaccharide gel from durian rinds seems to be a promising alternative attractive remedy for routine use in livestock. Antibacterial and gelling properties of durian polysaccharide gel make it suitable for using as a postmilking teat antiseptic preparation and also its immunostimulatory activity is valuable for using as an immunomodulating agent for mastitis control.

The purpose of this study was to examine; firstly, to determine the potential of durian polysaccharide gel as a natural antibacterial agent for developing a postmilking teat antiseptic, bactericidal efficacy of the teat antiseptic prepared from durian polysaccharide gel against bacterial isolates from matitic dairy cows by using *In vitro* time-kill analysis and *In vivo* study using both experimental challenge and natural exposure field trials. Secondly, to determine the immunomodulatory potential of durian polysaccharide gel on bovine mammary gland immunity by studying the cytotoxic reaction, scanning electron microscopy, *in vitro* phagocytosis assay, and also the effect of intramammary infusion of durian polysaccharide gel in mammary gland of non-lactating dairy cows. The results may provide preliminary information on the immunomodulatory effect of PG in protection of intramammary infection.

# CHAPTER II

# LITERATURE REVIEWS

## 1. Bovine mastitis

### 1.1 Definition and classification

Mastitis means breast inflammation, the word is derived from "mastos" and "itis" in Greek means breast and inflammation, respectively. Bovine mastitis is an inflammatory response of the bovine mammary gland to either infectious or non-infectious agents. Infectious agent involved with several organisms as bacteria, mycoplasma, yeasts and algae. (Watts, 1988; Quinn *et al*., 2000; Radostits *et al*., 2000). Mastitis could also be the result of non-infectious inflammation due to chemical, physical or mechanical trauma.

The bovine mastitis is usually classified in three modes: (1) contagious and environmental mastitis depending on the suspected site of origin and method of transmission: (2) per-acute, acute, sub-acute or chronic depending on the duration length of infections and perhaps the appearance onset of clinical signs: (3) clinical or subclinical mastitis. In this scheme, the clinical mastitis involves visible abnormalities in udder such as reddening, swelling, pain, and high temperature together with a change in appearance of milk such as flakes, clots, serum-like or bloody secretions. In contrast, subclinical mastitis is infections, which occur without obvious external changes characterized but udder pathogen presence has resulted in subclinical form, and laboratory techniques such as measurement of somatic cell count (SCC) and microbiological culture are needed to detect inflammation and infection causes.

## 1.2 Impaction of mastitis

### 1.2.1 Milkborne disease

Mastitis-causing bacteria and foodborne pathogens are proved interrelated. Several surveys have detected foodborne pathogens in bulk tank milk. Those studies have shown clearly that prevalence rate of foodborne pathogens including *Staphylococcus aureus* (Tondo *et al.* 2000), *Campylobacter jejuni*, Shiga-toxin producing *Escherichia coli* (STEC) (Jayarao and Henning, 2001), *L. monocytogenes* (Muraoka *et al.*, 2003), and *Salmonella spp.* (Van Kessel *et al.*, 2004) in milk vary considerably. Moreover, consumption of contaminated raw milk, faulty pasteurized milk or dairy products adulterated with contaminated raw milk was linked directly to cases of human foodborne disease. For example, a high proportion of human infections caused by *C. jejuni* occurred through ingestion of non-pasteurized milk, and faulty pasteurized milk contaminated with this foodborne pathogen (Evans *et al.*, 1996; Fashey *et al.*, 1995). The incidence of cheese-related foodborne disease of *L. monocytogenes* has been reported. Causative factors were post-pasteurization contamination, faulty pasteurization equipment or procedures, and use of raw unpasteurized milk (Johnson *et al.*, 1991). Outbreaks of human salmonellosis have also been linked to ingestion of raw milk contaminated

### 1.2.2 Antibiotics residue in milk

The potential for antibiotic residues in milk associated with treatment of dairy cows with mastitis is a significant public health concern. This situation causes development of allergic type responses to milk containing antibiotics and increased bacterial resistance to antibiotics. Reugg and Tabone, 2000 recently reported that dairy herds with a high incidence of mastitis are more likely to treat more cows with antibiotics, thus increasing the potential occurrence of antibiotic residues in milk. Effective mastitis control strategies including prudent use of antibiotics will help dairy producers achieve these important goals.

**1.2.3 Economic losses**

Mastitis is a great cause of economic losses that vary with the severity of the case and the response of the farmer. The mastitis cost are due to reduction in milk production and waste of milk unsafe for consumption; a major cause of premature culling, entailing massive antibiotic use and increased cost of veterinary services and labor wage (Table 1). An average cost for a typical case is about £131 per case (Berry *et al*., 2004). The national cost would be approximately £105 million. Subclinical mastitis has no direct costs, the infected udder reduce up to 5% production milk for every additional 100,000 cells/ml in the milk (Hamann, 2002). This adds up to a cost two to three times that of the overt disease making the United Kingdom and United State losses from mastitis in dairy cows about £300 and $2,000 million/year, respectively. (Philpot, 1984)

**Table 1.** An average cost of clinical mastitis in a cow per year (Berry *et al*., 2004)

| Source of loss | Dollar loss per cow | Percent of total |
|---|---|---|
| Reduced milk production | 121.00 | 66.0 |
| Discarded milk | 10.45 | 5.7 |
| Early cow replacement costs | 41.73 | 22.6 |
| Extra labor | 1.14 | 0.1 |
| Drug | 7.36 | 4.1 |
| Veterinary service | 2.72 | 1.5 |
| Total | 184.40 | 100 |

**1.3 Mastitis-causing microorganisms**

The microorganisms that most frequently cause mastitis can be grouped in four categories of pathogens as the following: 1. contagious group, 2. environmental group, 3. opportunistic group and 4. others.

### 1.3.1 Contagious microorganisms

Contagious pathogens are transmitted from fomites contaminated by infected cows to herd mates during the milking process. The most important contagious mastitis-causing microoganisms includes *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus disagalactiae*, *Corynebacterium bovis* and *Mycoplasma bovis* (Fox and Gay, 1993).

*Staphylococcus aureus* and *Streptococcus agalactiae* are the common contagious mastitis pathogens with a prevalence ranging from 7% to 40 % and ranging from 1% to 8%, respectively (Eberhart *et al*., 1987; Smith *et al*., 1995). The major reservoir for these pathogens is the infected udder and infections are spread among cows or between quarters during the milking process by contaminated milking equipment, milker's hands, and cloths used to dry udder more than one cow. Infections tend to be chronic and subclinical with periodic clinical episodes. *S agalactiae* is sensitive to penicillin and can be eradicated from individual dairy herds, whereas *S. aureus* is protected from fibrotic aereas of scar tissue to impede the distribution of antibiotics within the affected quarter after infusion.

*Streptococcus dysgalactiae* is generally characterized as an environmental pathogen, but also may have characteristics of a contagious organism and appears to spread from cow to cow (Smith *et al.*, 1995). The infections by this organism can occur in a herd when no other udder infections and less prevalent than another member.

*Mycoplasma* species are contagious pathogens that are rare in some areas but not uncommon in others. *Mycoplasma bovis* is the most common species and probably causes the most severe problems. Some characteristics of mycoplasma mastitis include sudden onset, rapid spread in the herd, marked reduction in milk production, and resistance to treatment. Veterinary assistance is recommended for the diagnosis and control of mycoplasma mastitis, because special bacteriological culture procedures are necessary for diagnosis (Fox and Gay, 1993).

*Corynebacterium bovis* is considered a minor pathogen. The main reservoir appears to be infected udders or teat ducts. However, *C. bovis* is the less pathogenic bacteria, the quarters infected with *C. bovis* are more susceptible to *S. agalactiae* and environment streptococci. Infections by *C. bovis* cause only moderate inflammation with SCC exceeding those of uninfected glands by only threefold. Infections are infrequently the major cause of elevated bulk tank SCC, clinical mastitis, marked compositional changes, or dramatic decreases in milk production. (Barkema *et al*., 1997).

**1.3.2 Environmental microorganisms**

The primary groups of environmental mastitis-causing bacteria are Gram-negative bacteria including *Escherichia coli*, *Klebsiella species*, *Enterobacter species*, *Serratia species, Pseudomonas species* and *Proteus species* and also species of streptococci group other than *S. agalactiae* such as *S. uberis*, *S. bovi, S. porcinus* and *S. acidominimus***.** The prevalence of environmental mastitis is usually less than 5%, thus environmental mastitis often has very little effect on the bulk tank SCC. More over a high percentage of such infection become clinical and the milk is withheld from the bulk tank. Environmental bacteria are abundant in the surrounding in which the cow lives, including manure, soil, bedding, feedstuffs, water and plant materials. The incident of clinical case may increase during winter months when animals are confined, but may also increase during summer months when exposure to the organisms is high. Because bacteria are so widespread in the cows' surrounding, eradication is not possible on a practical basis (Jones, 1990; Todhunter *et al*., 1991).

**1.3.3 Opportunistic microorganisms**

Opportunistic microorganisms are the most prevalent microorganisms isolated from infected quarters but they cause only mild inflammation in udder tissues. They live on the surface of the udder and teats in large numbers and are consequently a constant source of inflammatory infection. Other less common microorganisms also cause mastitis include fungi, yeast, and algae. This group of bacteria includes over 20 species of staphylococci other than

*Staphylococcus aureus*. They may commonly referred to Coagulase-negative staphylococci (CNS) including *S. chromogenes, S. hyics, S. epidermidis, S. simulans* and *S. waarneri* are normal teat skin flora, while *S. xylosus* and *S. sciuri* appear to arise from the environment. These bacteria are of special interest because they are the most frequently isolated microorganisms in every herd; however, infections are usually mild. Clinical symptoms are rare, and when they occur, such cases are mild, and local changes to the udder are limited to clots and flakes in milk (Smith *et al*., 1985).

#### 1.3.4 Other microorganisms

A wild variety of other microorganisms may also cause mastitis including *Pseudomonas* species, *Arcanoacterium pyogenes*, *Norcardia* species, *Mycobacterium* species, and various bacilli, yeasts, molds, and algae. Infections with some of them are often due to poor treament procedures when infusing antibiotics. Occurrence of infection is usually low, but outbreaks may occur when condition develop that increase exposure to them.

### 1.4 Epidemiology of bovine mastitis

#### 1.4.1 Epidemiological triad of mastitis

Mastitis is the result of complex interactions between the triad of the mastitis-causing bacteria, the cows and the environment (Figure 1). The balance of risk factors and protective factors that determine health status depend on the interaction of the components of the epidemiologic triad. Recognizing the different components of this triad is important because they are the source of opportunities to reduce mastitis at multiple points in the transmission cycle.

Over 140 different microorganisms involved bovine mastitis, and they live on the cows and in her environment. Thus, mastitis is the result of the interaction between cows, her environment and microorganisms

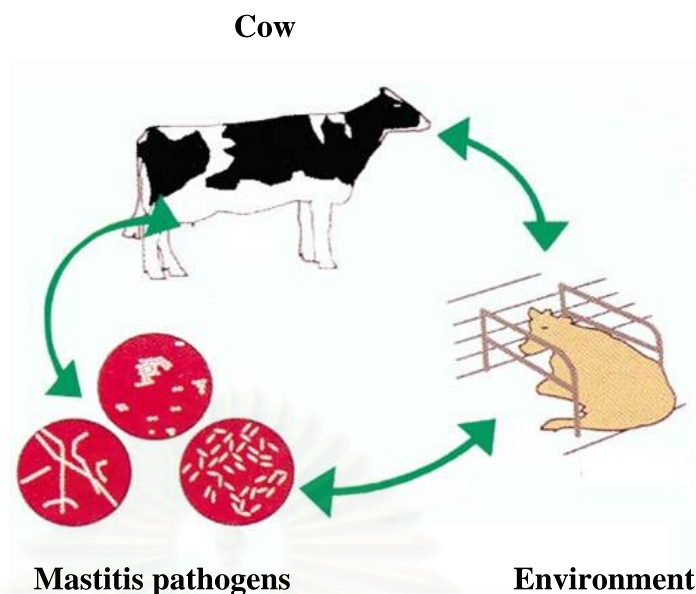


**Figure 1.** A schematic representation of the epidemiologic triad of bovine mastitis.

### 1.4.2 Incidence of mastitis

The likely impact on the UK dairy herd is that some 880,000 clinical cases occur in the 2.2 million cows annually. On average 25% of cows in the herd suffer clinical mastitis, with an average incidence of 40 cases per 100 cows per year. Treatment may be unsuccessful resulting in a recurrence rate of up to 60%. The reported incidence of clinical mastitis may be an underestimate as the person milking may only detect 80% of all cases showing clinical signs (Dodd *et al.*, 1969). The most common method is to count the somatic cells in milk. The count in a healthy udder quarter of the cow should be fewer than 100,000 cells/ml (Hamann, 2002). A level >200,000 cells/ml indicates infection (Smith *et al.*, 2001). In a typical farm 3-4% quarters is the usual prevalence of subclinical mastitis. The farm should target fewer than 10% of cows with an elevated leucocyte content in milk.

Microbiological examination of mastitic milk is done from aseptically taken quarter milk sample. A summary of examinations done in Thailand milk inspection laboratories in 2002-2003 is presented in Table 2.

**Table 2.** Subclinical investigation in smallholder dairyfarms of Chiang Mai province during 2002-2003 (Boonyayatra and Chaisri, 2004)

| | Farm number | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Average dairy milking cows (mean ±SD) | 6.4±0.5 | 9.5±3.0 | 8.4±2.4 | 9.0±2.5 |
| Incidence of subclinical mastitis | 2.90 | 2.15 | 3.88 | 4.80 |
| Prevalence of subclinical mastitis (%) | 0-32.3 | 14.3-50.0 | 36.4-71.4 | 36.4-83.3 |
| Intramammary infection pathogens (%)* | | | | |
| *Staphylococcus aureus* | 0 | 6.4 | 0 | 0 |
| Coagulase-negative Staphylococci | 17.7 | 27.0 | 18.8 | 13.1 |
| *Streptococcus agalactiae* | 0 | 0 | 0 | 0 |
| Environmental Streptococci | 0 | 32.4 | 11.3 | 49.5 |
| *Klebsiella spp.* | 0 | 0 | 1.25 | 0 |
| *Enterobacter spp.* | 0 | 2.7 | 5.0 | 3.0 |
| *Escherichia coli* | 0 | 0 | 0 | 0 |
| *Pseudomonas spp.* | 0 | 0 | 2.5 | 2.0 |
| *Corynebacterium bovis* | 5.9 | 0 | 6.3 | 5.1 |
| Yeasts | 29.4 | 2.7 | 0 | 0 |
| No bacterial growth | 47.1 | 29.7 | 55.0 | 27.3 |

* Percentage of pathogens isolated from all subclinical mastitic milk samples from each farm

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**2. Postmilking teat sanitation**

**2.1 History**

The new intramammary infection (IMI) is related to the number of mastitis-causing pathogens on teat ends. Disinfecting teats with a germicidal agent immediately after milking is necessary, in order to kills most of the pathogens on teats and reduces the chance of pathogen to get into the udder. The first advocated teat dipping since 1916 such as a dilute pine oil solution was used to reduce spread of *Streptococcus agalactiae*. However, the practice was not adopted widely, because products were ineffective and supportive data were not conclusive (Moak, 1916). In the late 1950's, postmilking teat dipping became a product of interest in the practice by demonstrating reduction of staphylococcal populations on milking machine liners after using of germicidal teat dips (Newbould and Barnum, 1960). Subsequently, the teat dipping was evaluated in two field trials in England (Dodd *et al.*, 1969). The hygiene programs reduced infection rates, and teat dipping was a highly effective component of the programs. The postmilking teat dipping application is now accepted, correctly used will reduce incidence of new IMI at least 50 to 90%. The value of teat dipping was confirmed and summarized in Table 3.

**2.2 Application of postmilking teat sanitizer**

Postmilking teat sanitizer may be applied by dipping or spraying. A recent survey indicated that 63% of dairy farmers dipped teats and 7% sprayed (Hoard's Dairyman Continuing Market Study, 1982). Application of teat sanitizer by dipping requires immersion of each teat covered with sanitizer at least the lower half teat. Application of postmilking teat sanitizer by spraying devices included a hand-held reservoir with pressure plunger and an electric pump unit with drop hoses located strategically in the milking parlor. Spray and dip application were equally effective in reducing incidence for IMI of *S. agalactiae* in a side by side comparison under experimental challenge procedures (Pankey *et al.*, 1983a).

## 2.3 **Product classifications**

In the marketplace, many different products with various active ingredients are available for teat dipping products. Information is presented on five classes of postmilking teat sanitizers including iodophors, quaternary ammonium compounds, chlorhexidines, sodium hypochlorites and dodecyl benzene sulfonic acid (DDBSA).

### 2.3.1 Iodophor

Iodine in the form of a tincture or iodophor, long has been recognized as an effective antiseptic and disinfectant. An iodophor is a combination of either iodine ($I_2$) or iodine ions (I) together with carrier molecule such as poloxamers, quaternary ammonium compounds, ethoxylated detergents, and polyvinyl-pyrrolidone (Gershenfeld, 1977). In iodophor solutions, a portion of the $I_2$ molecules unbound to the complexing agent (free $I_2$) in equilibrium are the active germicidal forms. Iodophor formulations fast destroy microbes through an oxidation-reduction mechanism (Prince *et al.*, 1978). However, low concentrations of iodine are considered relatively nontoxic, although the use in accordance with label directions, some irritation can develop (Windholz, 1976). Iodophor possesses a broad spectrum of antimicrobial activity against vegetative bacteria, fungi, viruses, and even bacterial spores (King *et al.*, 1981). Iodophor teat dips have provided effective control of new IMI by *S. aureus* and *S. agalactiae,* the two most frequently isolated mastitis pathogens. Protection against environmental pathogens is equivocal as with most other germicidal formulations. Elemental iodine is difficult to use alone because of its several properties such as poorly soluble in water, alcoholic solutions are irritating, stain and unpleasant acrid odor. These problems were reduced substantially by combining iodine with a solubilizing agent to form iodophor compounds. However, color is another advantage of iodophors for on-farm use because an iodophor teat dip is visible on teats, antimicrobial activity is reduced when iodinate with a variety of organic matter on teat skin. Efficacy of iodophor teat sanitizers has been extensively documented in *S. aureus* and *S. agalactiae*; while, protection against environmental pathogens is equivocal as with most other antiseptics (Table 3).

**2.3.2 Quaternary ammonium compounds**

The general formula of quaternary ammonium halide teat dips compose of the following; emollients, wetting agents, water dispersible food grade coloring agents, pH buffers, thickening agent and water. A typical alkyl dimethyI benzyl ammonium chloride is supplied to manufactures as a 50% solution in ethyl alcohol; whereas, a typical alkyl dimethyl benzyl ammonium bromide is supplied commercially in a 50% solution of isopropyl alcohol in water. Emollients replace some of the natural skin oil and moisture lost during the milking process and may aid in forming a protective coating on the skin. The suitable emollients are ethoxylated lanolin, glycerine, isopropyl myristate, isopropyl palmitage, other similar esters, polypropylene glycol, other glycol derivatives, vegetable oil, petroleum fractions, high molecular weight alcohol, allantoin etc. A thickening agent serves to produce a more heavy-bodied, retentive film that renders on teat skin. Some thickening agents form non-brittle films when dry, and some aid in moisture retention to assist skin conditioning. Available thickening agents include gum arabic, gum tragacanth, gum karaya, and many other related compounds. The mode of antimicrobial activity for quaternary ammonium compounds has not been proved definitively. Proposed mechanisms include denaturation of cell protein, inhibition of enzyme activity, and disruption of cell membrane permeability (Scharff, 1960). Quaternary ammonium compounds, at recommended use dilution, are safe and relatively nontoxic (Petrocci, 1977). Quaternary teat dips are non-corrosive to equipment. These compounds are degraded readily in the environment. Quaternary teat dips are effective germicides and contain no harsh alkalies or acids. Emollients help maintain teats soft and pliable. Color indicator shows when teats have been dipped and is removed easily by premilking udder wash. Activity is not lost under proper storage conditions. Properly formulated, quaternary teat dips have good activity in presence of milk soil loads encountered in normal dairy practices. Quaternary ammonium compounds do not volatilize readily and persist on the skin, possibly lending protection during milking interval. Proper formulation is necessary for effectiveness; many emollients, surfactants, viscosity modifiers, and dyes can interfere with germicidal properties of quaternary ammonium compounds. *Serratia*

*marcescens* isolated from clinical mastitis cases resistant with quaternary complex teat dip (Van Damme, 1982) and other pathogen in Table 3 have been reported.

### 2.3.3 Chlorhexidine

Chlorhexidine, [1, 6-di-(4 chlorophenyldiguanido) hexane], a colorless, odorless base, is one of the most active of the germicidal biguanide compounds. Concentrations from 0.2 to 1% have been tested in teat dip formulations but under practical conditions 0.5% is used most frequently (Table 3). Chlorhexidine is adsorbed rapidly onto the surface of bacterial cells (Longworth, 1971). Adsorption is enhanced with increasing pH, probably because of increased ionization on the cell surface. At low chlorhexidine concentration (0.01%) adsorption is followed by rapid and irreversible loss of cytoplasmic contents. Electron micrographs indicated that cytoplasm had coagulated, possibly from precipitation of proteins and nucleic acids. Death of bacterial cells may be from precipitation of cellular macromolecules (Longworth, 1971). In laboratory tests and in human clinical use, chlorhexidine was relatively nontoxic to unbroken skin and mucous membranes (Longworth, 1971). However, skin irritation resulted from use of chlorhexidine teat dips (Schultze *et al*., 1970), emollients are incorporated in commercial products to minimize irritation. The advantages of chlorhexidine include: (1) broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria (Hicks *et al.,* 1981); (2) germicidal activity reduction by organic material is less than some other common germicides; (3) persistence of activity on teat skin is greater than some other germicides (Godinho *et al*., 1980). Chlorhexidine teat dips were tested in various protocols and effective in reducing new IMI is summarized in Table 3.

### 2.3.4 Sodium Hypochlorite

Teat dips containing hypochlorite usually are prepared by dilution of commercial laundry bleaches to a final concentration of 4% sodium hypochlorite (Natzek *et al*., 1972). Emollients are not included in hypochlorite teat dips because of associated formulation problems (Bramley, 1981). Hypochlorite is a strong oxidizing agent and is highly reactive with proteins. It reacts quickly and

destructively with structural and enzymatic proteins of the bacterial cell. In aqueous solutions the hypochlorous ion is in equilibrium with undissociated hypochlorous acid, which is believed to be the germicidal molecular species (Trueman, 1971). Generally, hypochlorite solutions have low toxicity (Trueman, 1971). However, at the high concentrations in teat dips, teat irritation, usually mild and transitory, has been observed (Pankey and Philpot, 1975). These dips also may cause chapping of milking worker's hands. Final concentration of sodium hydroxide must be less than 0.05% for satisfactory use as a teat dip (Natzke, 1970). A strong unpleasant odor and bleaching action when spilled on clothing and inactivated by organic material (Dychdala, 1977); however, the hypochlorite teat dips are proven efficacy and low cost. Hypochlorite teat dips reduced microbial populations on teat skin (Pankey and Philpot, 1975) and reduced new IMI under conditions of experimental and natural exposure (Table 3).

### 2.3.5 Dodecyl Benzene Sulfonic Acid (DBSA)

The DDBSA teat dips contain an acid-anionic surfactant as the active ingredient. The mechanism of action is not understood completely; the three most commonly cited hypotheses include general denaturation of proteins; inactivation of essential enzymes and disruption of cell membranes resulting in permeability alterations. (Yamada, 1979). An extensive review of acute, subacute, and chronic toxicity of anionic surfacrants was published; however, median lethal dose ($LD_{50}$) in excess of 1 g/kg indicating of its low toxicity (Potokar, 1980). The teat dips containing DDBSA are generally to extended residual antimicrobial activity after dipping and substantial tolerance to organic matter but incompatible with quaternary ammonium udder washed. The DDBSA and other acid anionic surfactants possess excellent antimicrobial activity against vegetative bacteria and yeasts, except bacterial spores are fairly resistant to these compounds. The effective bactericidal activity against Gram-negative bacteria limited at pH above 3.5 but effectively killed *S. aureus* and *S. agalactiae* up to pH 5 (Pankey *et al*., 1983b).

**Table 3.** Summary of peer-reviewed research on efficacy of postmilking teat disinfectants published during 1980 to 2003.

| Active ingredient(s) & concentration | Type of study | Significant efficacy against | Reference |
|---|---|---|---|
| Iodine (0.05%) | Experimental challenge | *S. aureus* (P < 0.01) | Pankey et al, 1983 |
| Iodine (0.1%) | Experimental challenge | *S. agalactiae* (P < 0.005) | Boddie et al, 1990 |
| Iodine (0.1%) | Experimental challenge | *S. aureus* (P < 0.01) | Pankey et al, 1983 |
| Iodine (0.1%) | Natural exposure Positive control compared to Bovadine (1% iodine) | Not significantly different from positive control | Bay et al, 1983 |
| Iodine (0.1%) Glycerin (0.75%) | Experimental challenge | *S. aureus* (P < 0.001) *S. agalactiae* (P < 0.05) | Boddie et al, 1993 |
| Iodine (0.175%) | Experimental challenge | *S. aureus* (P < 0.001) | Boddie et al, 1993 |
| Iodine (0.18%) Collagen protein (15%) | Experimental challenge | *S. aureus* (P < 0.001) *S. agalactiae* (P < 0.025) | Boddie et al, 1989 |
| Iodine (0.25%) | Natural exposure | *S. aureus* (P < 0.05) *S. agalactiae* (P < 0.05) *C. bovis* (P < .05) *Staphylococcus* spp. (P < 0.05) | Oliver et al, 1991 |
| Iodine (0.25%) | Natural exposure Positive control compared to Bovadine (1% iodine) | Not significantly different from positive control | Bay et al, 1983 |
| Iodine (0.25%) | Experimental challenge | *S. aureus* (P < 0.05) | Pankey et al, 1983 |
| Iodine (0.3%) | Experimental challenge | *S. aureus* (P < 0.01) | Pankey et al, 1983 |
| Iodine (0.5%) | Natural exposure | *S. aureus* (P < 0.001) *S. agalactiae* (.05 < P < 0.10) *C. bovis* (P < 0.001) | Nickson et al, 1986 |
| Iodine (0.5%) | Experimental challenge | *S. agalactiae* (P < 0.005) | Boddie et al, 1990 |
| Iodine (0.5%) | Experimental challenge | *S. aureus* (P < 0.025) | Pankey et al, 1983 |
| Iodine (1%) | Natural exposure | *S. aureus* (P < 0.05) *S. agalactiae* (P < 0.001) Other streptococci (P < 0.001) | Nickson et al, 1986 |
| Iodine (1%) | Natural exposure | *S. aureus* (P = 0.03) *Streptococcus* spp. (P = 0.01) *Staphylococcus* spp. (P < 0.001) *C. bovis* (P < 0.001) | Eberhart et al, 1983 |
| Iodine (1%), 10% emollients (glycerin, lanolin & polyvinyl pyrolidine) | Natural exposure Positive control compared to Bovadine (1% iodine, 10% glycerin) | Not significantly different from positive control for streptococci & major pathogens. More coliforms (P < 0.05) & fewer *Staphylococus* spp (P < 0.05) than positive control | Golderg et al, 1994 |
| Iodine (1%) Glycerin (10%) | Experimental challenge | *S. aureus* (P < 0.001) *S. agalactiae* (P < 0.1) | Boddie et al, 1997 |
| Titratable iodine (10%) Glycerin (1%) Lanolin (0.5%) Aloe vera (0.5%) | Experimental challenge | *S. aureus* (P < 0.001) *S. agalactiae* (P < 0.005) | Boddie et al, 1997 |

**Table 3 (cont).** Summary of peer-reviewed research on efficacy of postmilking teat disinfectants published during 1980 to 2003.

| Active ingredient(s) & concentration | Type of study | Significant efficacy against | Reference |
|---|---|---|---|
| Titratable iodine (1%)<br>Glycerin (2%) | Experimental challenge | *S. aureus* ($P < 0.1$)<br>*S. agalactiae* ($P < 0.05$) | Boddie et al, 1997 |
| Iodine (0.5%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.1$) | Boddie et al, 2000 |
| Iodine (1%)<br>Glycerin (10%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.005$) | Foret et al, 2003 |
| Chlorhexidine gluconate (0.5%)<br>Glycerin (6%) | Experimental challenge | *S. aureus* ($P \leq 0.01$) | Hicks et al, 1981 |
| Chlorhexidine gluconate (0.55%) | Experimental challenge | *S. aureus* ($P < 0.01$)<br>*S. agalactiae* ($P < 0.01$) | Pankey et al, 1983 |
| Chlorhexidine gluconate (0.4%)<br>Glycerine (10%) | Natural exposure<br>Positive control compared to Nolvasan (0.5%chlorhexidine & 4.9% glycerin) | Not significantly different from positive control for *S. aureus*, *Streptococcus* spp, and coliforms | Westfall et al, 1987 |
| Chlorhexidine gluconate (0.35%) | Natural exposure | *S. uberis* ($P < 0.01$)<br>*C. bovis* ($P < 0.01$)<br>*Staphylococcus* spp ($P < 0.05$) | Oliver et al, 1990 |
| Chlorhexidine gluconate (0.5%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.005$) | Boddie et al, 1990 |
| Chlorhexidine gluconate (0.55%) | Natural exposure<br>Positive control compared to FS103<br>(1% iodine) | CNS ($P \leq$ 0 01)<br>*Escherichia coli* ($P \leq 0.08$)<br>Gram-positive bacilli ($P \leq 0.05$) | Hogan et al, 1995 |
| Chlorhexidine gluconate (0.5%)<br>Glycerin (4%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.05$) | Boddie et al, 1997 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Experimental challenge | *S. aureus* ($P < 0.01$)<br>*S. agalactiae* ($P < 0.01$) | Barnum et al, 1982 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Natural exposure | *S. aureus* ($P < 0.005$) | Fisher et al, 1983 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Natural exposure | *S. aureus* ($P < 0.05$) | Pankey et al, 1984 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Experimental challenge | *S. aureus* ($P < 0.05$)<br>*S. agalactiae* ($P < 0.01$) | Pankey et al, 1984 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Natural exposure | *S. agalactiae* ($P < 0.005$) | Pankey et al, 1985 |
| Linear dodecyl benzene sulfonic acid (1.94%) | Natural exposure<br>Positive control compared to Udder Guard (0.5%chlorhexidine & 4.9% glycerin) | Significantly ($P < 0.05$) more effective than positive control for *S. aureus* | Pankey et al, 1985 |
| Linear dodecyl benzene sulfonic acid (1.94%) plus .55% iodophor | Experimental challenge | *S. aureus* ($P < 0.05$)<br>*S. agalactiae* ($P < 0.025$) | Pankey et al, 1985 |
| Phenol | Natural exposure | *S. aureus* ($P < 0.05$)<br>*S. uberis* ($P < .05$)<br>*Staph.* species ($P < 0.005$)<br>*C. bovis* ($P < 0.005$) | Oliver et al, 1989 |

**Table 3 (cont).** Summary of peer-reviewed research on efficacy of postmilking teat disinfectants published during 1980 to 2003.

| Active ingredient(s) & concentration | Type of study | Significant efficacy against | Reference |
|---|---|---|---|
| Quaternary ammonium (0.5%) | Natural exposure | *S. aureus* ($P < 0.01$)<br>*C. bovis* ($P < 0.01$) | Stewart et al, 1982 |
| Quaternary ammonium (0.5%) | Experimental challenge | *S. agalactiae* ($P \leq 0.025$) | Pankey et al, 1983 |
| Sodium chlorite (0.64%)<br>Lactic acid (2.64%) | Natural exposure | *S. aureus* ($P < 0.01$)<br>*S. dysgalactiae* ($P < 0.025$)<br>Major pathogens ($P < 0.01$) | Oliver et al, 1989 |
| Sodium chlorite (0.64%)<br>Lactic acid (2.64%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.1$) | Drechler et al, 1990 |
| Sodium chlorite (0.64%)<br>Lactic acid (2.64%) | Natural exposure<br>Positive control compared to Bovadine<br>(1% iodine, 10% glycerin) | Not significantly different from<br>positive control for environmental pathogens | Drechler et al, 1990 |
| Sodium chlorite (0.64%)<br>Lactic acid (2.64%) | Natural exposure<br>Positive control compared to iodine (0.5%) | Significantly ($P = 0.06$) more effective than positive control against all pathogens | Poutrel et al, 1990 |
| Sodium chlorite (0.64%)<br>Lactic acid (2.64%) | Experimental challenge | *S. aureus* ($P \leq 0.001$)<br>*S agalactiae* ($P \leq 0.1$) | Boddie et al, 1994 |
| Sodium chlorite (0.64%)<br>Lactic acid (3%) | Experimental challenge | *S. aureus* ($P \leq 0.001$)<br>*S. agalactiae* ($P \leq 0.01$) | Boddie et al, 1994 |
| Sodium dichloro-s-triazene-trione (1.0%) | Experimental challenge | *S. aureus* ($P < 0.01$)<br>*S. agalactiae* ($P < 0.025$) | Pankey et al, 1983 |
| Sodium dichloro-s-triazene-trione (1.7%) | Experimental challenge | *S. agalactiae* ($P < 0.025$) | Pankey et al, 1983 |
| Sodium chlorite (0.32%)<br>2.5% glycerin<br>0.53% sodium<br>1.32 % dodecylbenzene sulfonic acid,<br>and lactic acid | Experimental challenge | *S. aureus* ($P < 0.05$)<br>*S. agalactiae* ($P < 0.05$) | Oura et al, 2002 |
| Sodium hypochlorite (0.6%) | Experimental challenge | *S. aureus* ($P < .05$) | Pankey et al, 1983 |
| Sodium hypochlorite (0.9%) | Experimental challenge | *S. aureus* ($P < .01$) | Pankey et al, 1983 |
| Phosphoric acid (1.67%)<br>Sodium chlorite (2.5%) | Experimental challenge | *S. aureus* ($P < .01$) | Boddie et al, 1998 |
| Lauricidin ® (0.25%),<br>Caprylic/capric acids (1.25%)<br>lactic acid (1.5%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.001$) | Boddie et al, 1988 |
| Lauricidin ® (1%),<br>Caprylic/capric acids (5%)<br>lactic acid (6%)<br>laulic acid (0.85%) | Experimental challenge | *S. aureus* ($P \leq 0.001$)<br>*S. agalactiae* ($P \leq 0.025$) | Boddie et al, 1992 |
| Lauryl sulfate, solubilized milk protein, and glycerin (4.8%) | Experimental challenge | *S. aureus* ($P < 0.01$)<br>*S. agalactiae* ($P < 0.005$) | Pankey et al, 1985 |
| Lactic acid (2.9%)<br>Sodium chlorite (0.7%) | Experimental challenge | *S. aureus* ($P < 0.001$)<br>*S. agalactiae* ($P < 0.001$) | Boddie et al, 2000 |

## 3. Mammary gland immunity

The mammary gland is protected by the defense mechanisms, including innate immunity and specific immunity. Innate immunity, a nonspecific responsiveness, is the predominant defense during the early stages of infection. Nonspecific responses are activated quickly at the site of augmented by repeated exposure to the same insult. Nonspecific responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophil and soluble factors. Conversely, the acquired immune system recognizes specific determinants of a pathogen that facilitate selective elimination. Recognition of pathogenic factors is mediated by antibody molecules and several lymphoid populations. In addition, Innate immunity are prevalent than acquired immunity in the mammary gland and modern research are radically modifying the prospects for the understanding of the interplay between the mammary innate immune system and mastitis-causing bacteria, this dissertation focus onto innate protective factors exclusively.

### 3.1 Anatomical defenses

Mastitis occurs when bacteria get into the mammary gland via the teat canal. For this reason, the teat end is considered to be the first barrier of defense against invading pathogens. The teat end contains sphincter muscles that maintain tight closure between milkings and hinder bacterial penetration. Increased potency of these muscles is directly related to increased incidence of mastitis (Murphy and Stuart, 1953). The teat canal is lined with keratin, which is crucial to the maintenance of the barrier function of the teat end, damage or and removal of the keratin has been correlated to increased susceptibility to bacterial invasion and colonization (Bramley and Dodd, 1984; Capuco *et al.*, 1992). Teat keratin is a waxy material that is derived from stratified squamous epithelium. The keratin structure enables trapping of invading bacteria, thus hindering their migration into the gland cistern (Hibbitt *et al*, 1969). Within the keratin lining, antimicrobial agents have been identified. The esterified and nonesterified fatty acids present in teat keratin, such as myristic acid, palmitoleic acid, and linoleic acid, are bacteriostatic (Treece, 1966). Additionally, cationic protein in the canal

can bind electrostatically to mastitis pathogens, which alters the bacterial cell wall, thus rendering them more susceptible to osmotic pressure. The inability to maintain osmolarity causes lysis and death of the invading pathogens (Murphy and Stuart, 1953).

### 3.2 Cellular defenses

Bacterial pathogens passing through the teat end opening must escape the antibacterial agents in the defense mechanism of the mammary gland then developing disease. The activities of resident and newly recruited leukocytes during the early stages of pathogenesis play an important role in the establishment of intramammary infection (IMI). Milk somatic cell count (SCC) consist of several cell types, including neutrophils, macrophages, lymphocytes, and a smaller percentage of epithelial cells. In the healthy lactating mammary gland, total SCC are often $<10^5$ cell/ml of milk. During a bacterial IMI, however, total SCC can increase to $>10^6$ cell/ml of milk within just a few hours (Paape *et al*., 1981; Persson *et al*., 1992).

**Neutrophils** contribute in normal milk to the defense against mastitis is not clear; however, neutrophils are the predominant cell type found in mammary tissues and in mammary secretions during early inflammation and can constitute >90% of total mammary gland leukocytes (Sordillo *et al*., 1989). These non-specific cells travel from the blood to the mammary gland in response to a variety of inflammatory mediators, such as cytokines, complement, and prostaglandins (Baumann *et al*., 1994; Persson *et al*., 1993). The cell is described by a plasma membrane that has a number of functionally important receptors including L-selectin and $\beta$2-integrin adhesion molecules associated with the binding of PMN to endothelial cells which are important for migration into sites of infection (Zimmerman, 1992). Membrane receptors for the Fc component of the IgG2 and IgM classes of immunoglobulins and complement component C3b are necessary for mediating phagocytosis of invading bacteria (Paape *et al*., 1991). Apoptotic PMN expresses receptors marking for quick disposal by macrophages. The most prominent characteristic of the PMN is multilobulated nucleus (Figure 1b) that can line up its nuclear lobes in a thin line, permitting rapid migration between

endothelial cells. The isles of glycogen within the PMN cytoplasm make up 20% of the cell on a dry weight basis and numerous membrane bound granules that are used by the cell in killing bacteria. Bovine PMN kill bacteria by using both oxygen-dependent and oxygen-independent killing mechanisms. The most important antibacterial mechanism is the exclusive store of powerful oxygen-independent bactericidal compounds derived from myeloperoxidase, lysozyme, cationic protein and lactoferrin from three granules type, azurophilic, specific granules and third novel granules. The key components of oxygen-independent killing mechanism of PMN exert from a respiratory burst that produces hydroxyl and oxygen radicals (Selsted *et al*., 1993).

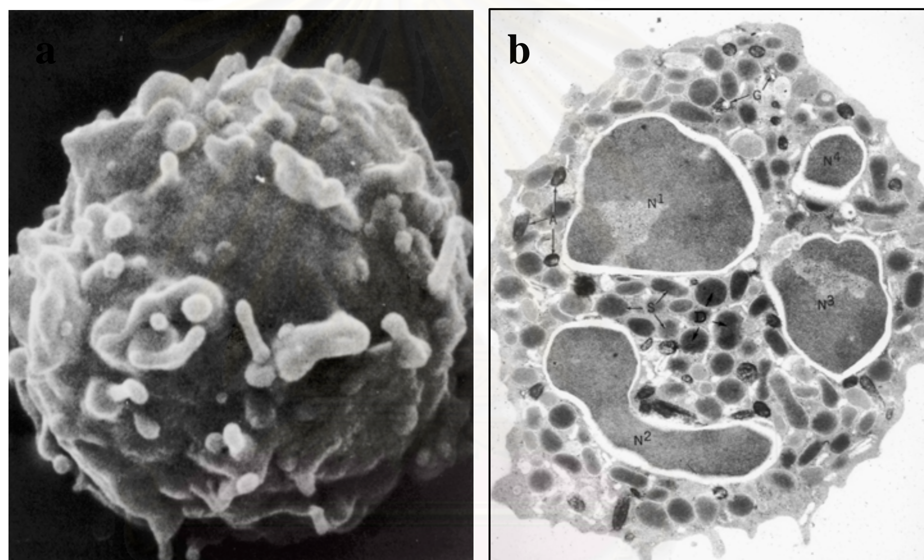


Figure 2. (a) Scanning electron micrograph and (b) Transmission electron micrograph of bovine polymorphonuclear neutrophil (PMN) leukocyte isolated from milk. (Paape *et al*., 1991)

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**Macrophages** in the mammary gland tissues are the mononuclear phagocytic system besides monocytes circulating in the blood. The monocytes circulate in the bloodstream for about 8 hrs before migrating into the tissues and differentiate into specific tissue macrophages. Differentiation of a monocyte into a tissue macrophage involves a number of changes including enlargement of the cell approximately five to ten fold, increasing of intracellular organelles in both number and complexity, increasing of levels of hydrolytic enzymes and secrete a

variety of soluble factors. Macrophages are the predominant cell type found in the milk and tissues of healthy lactating mammary glands (Sordillo *et al*., 1987). Macrophages are one of phagocytic cells member that are capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson, 1988). The phagocytic rate of macrophages can be increased substantially in the presence of opsonic antibody for specific pathogens. Because of the indiscriminate ingestion of fat, casein, and other milk components, mammary gland neutrophils and macrophages are less effective at phagocytosis than are blood leukocytes (Sordillo and Babiuk, 1991). In addition to their role in early nonspecific defenses, macrophages also play a key role in antigen processing and presentation (Politis *et al*., 1992). Antigens from ingested bacteria are processed within macrophages and appear on the membrane in association with major histocompatibility complex (MHC) class II antigens. These MHC class II antigens are polymorphic membrane molecules that are required by other host cells (lymphocytes) for the recognition of foreign antigens. Macrophages also present a variety of plasma membrane receptor as show in Table 4.

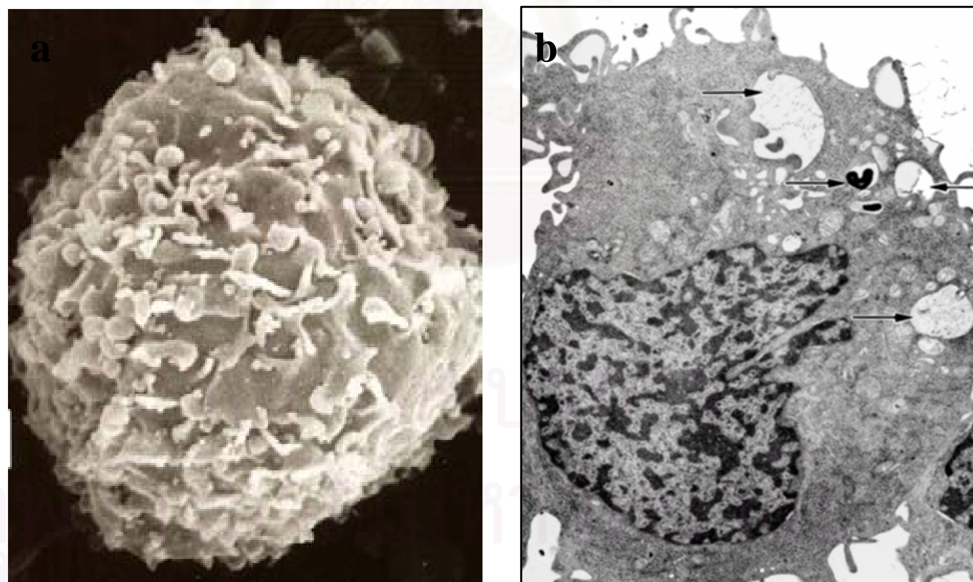


**Figure 3.** (a) Scanning electron micrograph and (b) Transmission electron micrograph of bovine macrophage isolated from milk. (Paape *et al*., 1991)

**Table 4.** Plasma membrane receptors on macrophages (Abbas *et al.*, 2000)

| Receptor | Ligand |
|---|---|
| Fc receptors (FcR) | |
| FcγRI | Monomers of IgG |
| FcγRII | Aggregates of IgG |
| FcγRIII | Aggregates of IgG |
| Complement receptors (CR) | |
| CR1 | C3b, C4b, iC3b |
| CR3 | iC3b, fibrinogen, factor X |
| Fibronectin receptor | Fibronectin oligomers |
| Mannose-fucose receptor | Oligosccharides |
| Receptors for growth factors and cytokines | |
| Interferons α and β (IFN-α, IFN-β) | |
| Interferons γ (IFN-γ) | |
| Interleukin-1 (IL-1) | |
| Monocyte colony-stimulating factor (MCSF) | |
| Tumor necrosis factor (TNF) | |

Both Neutrophils and Macrophages are capable of elimination in both exogenous antigens and endogenous matter. In the first step in phagocytosis, the cells are attracted by and move toward a variety of chemotaxis generated in an immune response. The next step is adherence of the antigen to the macrophage cell membrane. Adherence induces membrane protrusions, pseudopodia, to extend around the attached material. Fusion of the pseudopodia encloses the material within a membrane-bounded structure (phagosome) which then enters the endocytic processing pathway. The phagosome moves toward the cell interior and fused with a lysosome to form a phagolysosome. The foreign are destroyed by using oxygen-dependent and oxygen-independent killing pathway. (Table 5)

**Table 5.** The list of mediators in neutrophil and macrophage (Goldsby *et al*., 2001).

| Killing mechanism | Neutrophil | Macrophage |
|---|---|---|
| **Oxygen-independent killing mechanism** | Myeloperoxidase<br>Lysozyme<br>Cationic protein<br>Lactoferrin | Defensins<br>TNF α<br>Lysozyme<br>Hydrolytic enzymes |
| **Oxygen-dependent killing mechanism** | Reactive oxygen intermediates<br>$O_2^-$ (superoxide anion)<br>$OH^{\bullet}$ (hydroxyl radicals)<br>$H_2O_2$ (hydrogen peroxide)<br>$ClO^-$ (hypochloride anion)<br>Reactive nitrogen intermediates<br>NO (nitric oxide)<br>$NO_2$ ( nitrogen dioxide)<br>$HNO_2$ (nitrous acid)<br>$OONO^-$ (peroxynitrite) | |

**Oxygen-independent killing mechanism**, activated macrophages synthesize lysozyme and various hydrolytic enzymes whose degradative activities do not require oxygen. In addition, activated macrophages produce a group of antimicrobial and cytotoxic peptides, commonly known as defensins. These molecules are cysteine-rich cationic peptides containing 29-65 amino acid residues. These circularized defensin peptides have been shown to form ion-permeable channels in bacterial cell membranes. Defensins can kill a variety of bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*. Activated macrophages also secrete tumor necrosis factor α (TNF-α), a cytokine that has a variety of effects and is cytotoxic for some tumor cells.

**Oxygen-dependent killing mechanism**, activated phagocytes produce a number of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates that have potent antimicrobial activity. Ligand binding of Fc receptors (on neutrophils, monocytes or macrophages) and mannose receptors (on macrophages) increases their oxygen ($O_2$) uptake, called the respiratory burst.

These receptors activate a membrane-bound NADPH oxidase that reduces $O_2$ to $O_2^-$. Superoxide can be reduced to $OH^\bullet$ or dismutated to $H_2O_2$ by superoxide dismutase. $O_2^-$, $OH^\bullet$, and $H_2O_2$ are activated oxygen species that are extremely toxic to ingested microorganisms. As the lysosome fused with the phagosome, macrophages will induce hypochlorite ($ClO^-$) from $H_2O_2^-$ and chloride ions ($Cl^-$) through the action myeloperoxidase. Hypochlorite, the active agent of household bleach, is toxic to ingested microbes (Babior, 1984)

**3.3 Soluble defenses**

The nonspecific bacteriostatic components of mammary gland include Immunoglobulins, lactoferrin, complements, lysozyme, and the lactoperoxidase-thiocyanate-hydrogen peroxide system.

**Immunoglobulins** of bovine mammary gland including IgG1, IgG2, IgA, and IgM as the soluble components of humoral immune responses. These proteins are produced by antigen-activated B lymphocytes that subsequently proliferate and differentiate into antibody-secreting plasma cells. The concentration of each Ig class in mammary secretion varies depending on stage of lactation and infection status of the mammary gland. In healthy glands, the concentration of Ig is low during lactation but slowly increases during the non-lactating periods. The peak concentration occurs during colostrogenesis and inflammation (Sordillo *et al*, 1987). Research has shown that IgG1, IgG2, and IgM can act as bacterial opsonins that enhance phagocytosis of neutrophils and macrophages. These antibodies can bind bacterial pathogens directly or with the C3b component of complement (Howard *et al*, 1987). Neutrophils and macrophages can bind antibody-bacteria complexes and antibody-C3b-bacteria complexes via their Fc receptors and subsequently more effectively phagocytose the invading bacteria. In contrast, IgA does not bind complement or opsonize bacteria. Instead, IgA appears to contribute to agglutination, preventing bacterial colonization and toxin neutralization (Musoke *et al*, 1987).

**Lactoferrin** (Lf) is a protein which exerts several functions related to innate immunity. Lf was first known for its iron chelating properties, the basis of two of its activities, bacteriostasis and protection against oxygen radicals catalyzed by free iron (Legrand *et al*, 2004). Bacteria which have high iron requirements are susceptible to the bacteriostatic activity of Lf. The main source of Lf in milk is the mammary epithelial cells (MEC). Neutrophils, which contain Lf in their secondary and large granules, can account for about 5% of Lf found in milk during acute inflammation (Paape *et al*, 1981). Bovine milk contains very little 20-200 μg/ml of Lf; while, colostrum contains higher amounts 2–5 mg/ml of Lf. In the healthy mammary gland, the concentration of lactoferrin is low but increases during involution and inflammation. Besides antibacterial activity, Lf is endowed with immuno-modulating and anti-inflammatory properties. In particular, anti-inflammatory properties of Lf could come into play in normal milk. Human Lf was found to bind to the lipid A of bacterial lipopolysaccharides (LPS) with high affinity, resulting in the neutralization of LPS by preventing LPS from interacting with the main actors of LPS signaling, like LPS-binding protein (LBP), soluble CD14 (sCD14) and membrane CD14 (mCD14) (Legrand *et al*, 2005) Bovine Lf also is able to reduce the endotoxin-induced response of inflammatory cells (Mattsby *et al*, 1996). Lf may be able to operate in synergy with other defense components, such as complement or lysozyme. Bovine Lf has been shown to modulate complement activation: the binding of Lf to *S. agalactiae* activates the classical pathway of complement, resulting in the opsonization of the bacteria (Rainard *et al*, 1993). The activation of the classical pathway in full lactation milk can operate only after inflammatory exudation of plasma components, since only the alternative pathway is functional in milk. Interestingly, activation of the alternative pathway, resulting in increased deposition of C3, was demonstrated at the surface of *S. aureus* after incubation with bovine Lf (Kai *et al*, 1993).

**Complement** is a collection of proteins that is present in serum and milk, which functions in concert with a specific antibody to cause lysis of invading bacteria. Concentrations of complement are highest in colostrum, inflamed mammary glands, and during involution. In contrast, concentrations of complement are lowest during lactation. Therefore, because of its intermittent presence in milk, complement is thought to play only a minor bactericidal role in

the mammary gland (Reiter *et al*, 1978); however, complement-sensitive organisms, including some strains of *E. coli*, are killed by the alternative complement pathway.

**Lysozyme** is a bactericidal protein that is present in milk and that functions by cleaving peptidoglycans from the cell wall of Gram-positive bacteria as well as the outer membrane of Gram-negative bacteria. Lysozyme may enhance the binding of lactoferrin to bacterial cell walls (Schanbacher, and Smith, 1975). In porcine and human milks, lysozyme, in combination with complement and secretory IgA, exhibited significant bactericidal activity to *E. coli* in vitro. However, whether this mechanism is active in the bovine mammary gland is unknown, especially considering that a study demonstrated that lysozyme limits chemotaxis and toxic oxygen production by neutrophils (Gordon *et al*, 1979). Because ruminant milk contains only a small concentration of IgA and 300 times less lysozyme than human milk (Chandran *et al*, 1964), this system may offer little protection to the bovine mammary gland.

**Lactoperoxidase enzyme**, in the presence of thiocyanate and hydrogen peroxide, is bacteriostatic for Gram-positive bacteria such as *S. aureus* and streptococci and bactericidal for Gram-negative bacteria such as coliforms (Outteridge and Lee, 1988). However, several factors can vary the effectiveness of this system in the mammary gland epithelial cells. Lactoperoxidase is produced in small concentration by mammary gland. The levels of thiocyanate in the mammary gland are dependent on the plane of nutrition. The source of hydrogen peroxide in the mammary gland is generated by enzymatic constituents of milk and, if present, by streptococci (Hibbitt *et al*, 1992). The lactoperoxidase-thiocyanate-hydrogen peroxide system exerts its antibacterial properties through the production of hypothiocyanate, a reactive metabolite from the oxidation of thiocyanate (Roberts *et al*, 1992). Myeloperoxidase produced by neutrophils also catalyzes the same reaction and additionally catalyzes the oxidation of chloride, the product of which provides the bacteriocidal activity of this system.

## 4. Immunomodulation of bovine mammary gland

Antibiotics are the only proven method for prophylaxis and treatment of bovine mastitis in lactation and dry period, however antibiotic therapy of established mammary infection are only moderately efficacious and require prolonged milk withdrawal due to residue in milk (Daley and Hayes,1992). Mammary gland leukocytes that are the cellular defenses of the mammary gland are depressed during periparturient period (Cai *et al*., 1994), whereas, most of the antibiotics used for the treatment of mastitis further depress the activity of the PMNs defense (Hoeben *et al*., 1997). One possible approach to control mastitis involves manipulation of host defense mechanism. During immunosuppressive stages would greatly impact the ability of the animal to resist pathogenic infection.

### 4.1 Cytokine immunotherapy

Many studies have reported the effects of the use of recombinant cytokines with a view to prevent or cure mammary infections as summarized in Table 6. The stimulation of leukocytes with cytokines has been given particular attention. The granulocyte colony-stimulating factor (G-CSF), which targets the neutrophils, causes dramatic increases of neutrophil numbers in blood and milk, stimulates the phagocytic and bactericidal activities of neutrophils, expected to help the cow to combat mastitis (Kehrli, *et. al*., 1991). The granulocyte-macrophage CSF (GM-CSF), which targets both neutrophils and monocytes, stimulates the antibacterial efficiency of phagocytes, and affords some protection from subsequent *S. aureus* challenge (Daley, *et. al*., 1993). The effect of recombinant bovine IL-1β and IL-2 were also tested on mammary gland infections. These cytokines induce an influx of neutrophils into the milk after intramammary infusion in the lumen of the mammary gland. The studies demonstrated some preventive and curative effects on experimental infections, but their effective doses were not far from the toxic doses (Sordillo, *et. al*., 1991). Recombinant bovine interferon γ, which potentates the activities of T lymphocytes, macrophages and neutrophils, has been shown to modulate mammary gland neutrophil functions during the periparturient period (Sordillo, *et. al*., 1991).

**Table 6.** Summary of the efficacy of cytokines against bovine mastitis caused by *Staphylococcus aureus* or *Escherichia coli*.

| Cytokine[1] | Mastitis model | Observation | Reference |
|---|---|---|---|
| G-CSF | *S. aureus* | No effect | Kehrli, *et. al*., 1991 |
| | *S. aureus* | Reduction (47%) in new IMI | Nickerson *et. al*., 1989 |
| GM-CSF | *S. aureus* | Prevents new IMI | Daley, *et. al*., 1993 |
| Interferon γ | *E. coli* | Reduced rate, duration, and severity of disease | Sordillo, *et. al*., 1991 |
| IL-2 | *S. aureus* | Dry cow treatment | Daley, *et. al*., 1991 |
| IL-2 | *S. aureus* | Improved antibiotic efficacy | Daley, *et. al*., 1992 |
| IL-1 and IL-2 | *S. aureus* | Prevents new IMI | Daley, *et. al*., 1993 |

[1]G-CSF = Granulocyte colony-stimulating factor, GM-CSF = granulocyte-monocyte colonystimulating factor, IFN-g = interferon-g, and IL = interleukin.

**4.2 Mastitis vaccine**

The past decade has seen development of effective and economical R-mutant vaccine for Gram-negative mastitis (Hagan, *et. al*., 1992). Theses vaccines doubtless will prove beneficial on well managed dairies that have eradicated contagious mastitis pathogens. Development of vaccines for other mastitis pathogens has been noticeably slower. A commercially available *S. aureus* vaccine appears to reduce the frequency and severity of clinical episodes, but probably has minimal impact on the incidence or prevalence of infection (Watson, 1984). Little effort has focused on the development of vaccines for streptococcal mastitis phatogens. Immunization with *S. agalactiae* readily elicits detectable humeral immune responses, but serological recognition apparently confers no protection (Mackie, *et. al*., 1983). Unfortunately, no success has been observed in immunization program attempting to decrease the susceptibility of cows to mycoplasmal mastitis (Boothby, *et. al*., 1983) and *Clostridium perfingens* mastitis (Smith, 1983).

### 4.3 Natural product and herbal immunotherapy

Researchers have been tried to developed conventional plant and natural product for elimination of mastitis by immunomodulation of bovine mammary gland. The aqueous extract of *Ocimum sanctum* leaf belongs to a group of medicinal plant that grows in tropical region were studied in subclinical mastitic cows showed that neutrophil and lymphocyte counts increased with enhanced phagocytic activity (Reena, *et al*, 2005). The root extract of *Panax ginseng* treatment in cows with subclinical mastitis caused by *Staphylococcus aureus* has shown that numbers of *S. aureus*-infected quarters and milk SCC tended to decrease and also phagocytosis and oxidative burst activity of blood neutrophils were found in ginseng-treated cows (Hu, *et al*, 2001). The ginseng extracts also used to be adjuvant on the immune responses to immunisation against *Staphylococcus aureus* in dairy cattle (Hu, *et al*, 2003). The β1,3-Glucan, a polyglucose component of the cell wall of cereal and yeast, is a potent macrophage stimulant and can modulate both cellular and humoral immunity in several animal species. Studying on non-lactating ewes shows that intramammary infusion of β1,3-Glucan enhanced the numbers of macrophages and the proportion of CD14-positive leucocytes, as well as the proportions of certain lymphocyte subpopulations in udder secretions; whereas studying in dry cows shows a similar results (Inchaisri, *et al*, 2000). Moreover, β1,3-Glucan can reduce the numbers of staphylococcal infections in the ovine udder (Buddle, *et al*., 1988).

### 4.4 Probiotic therapy

Treatment of subclinical mastitis was tried to use intramammary infusions of *Lactobacillus* spp. while nontreated controls were not included in this study, so no statements can be made relative to the efficacy of probiotic therapy. However, cattle receiving intramammary antibiotic agents had a greater reduction in subclinical mastitis (73.7%) than *Lactobacillus* spp. infused cows (21.7%). Cows treated with *Lactobacillus* spp. also had higher somatic cell counts following treatment.

## 5. Durian fruit

### 5.1 biological data

The durian is a famous and widely known fruit in Southeast Asia as the King of Fruits. The typically fruit weighs one to three kilograms. Its shape ranges from oblong to round and green to brown in color. The hard outer husk is covered with sharp, prickly thorns, while the edible custard-like flesh within emits the strong, distinctive odor, which is regarded as either fragrant or overpowering and offensive. The taste of the flesh has been described as nutty and sweet.

The durian is the fruit of trees organized in the genus *Durio* belonging to the family Malvaceae, which is a large family includes hibiscus, okra, cotton, mallows and linden trees. Among the thirty known species of *Durio*, nine species have been identified to produce edible fruits including *D. zibethinus*, *D. dulcis*, *D. grandiflorus*, *D. graveolens*, *D. kutejensis*, *D. lowianus*, *D. macrantha*, *D. oxleyanus* and *D. testudinarum*. However, many species for which the fruit has never been collected or properly examined, and other species with edible fruit may exist. *D. zibethinus* is the only species commercially cultivated on a large scale and available outside of its native region. There are estimated to be 28 species in the genus *Durio* in Thailand. The list of eight *Durio* species and its cultivars are demonstrated in Table 7. The *D. zibethinus* Murr is the common durian that produces edible fruit of very high quality and most economical. Durian cultivation is everywhere extended in Thailand such as Nonthaburi province of central; Rayong, Chantaburi, Trat, Prachin Buri province of estern; Uttaradit province of northern Thailand. Monthong and Chanee cutivars are particularly dominant in those regions. Kanyao and Kradumthong cultivars are also widely grown.

**Table 7:** Descriptions of *Durio* species and cultivars

| Scientific name | Cultivar name |
|---|---|
| *D. zibethinus* | Chani, Kanyao, Monthong, Taptim, Watsak, Yammawa, Tosamsao, Taphapnam, Eaimen, Eaimai, Tonyai, Saoyai etc. |
| *D. malaccensis* | Don |
| *D. graveolens* | Kuatid |
| *D. kutejensid* | Rakkha |
| *D. monsoni* | Charian |
| *D. griffithii* | Nok-1 |
| *D. lowianus* | Nok-2 |
| *D. oxleyanus* | Khonyao |

### 5.2 Medicinal Uses

Durian fruit contains a high amount of sugar, vitamin C, potassium, and the serotoninergic amino acid tryptophan, and is a good source of carbohydrates, proteins, and fats (Heaton, 2006). In traditional medicine, the decoction of roots is used for diarrhea treatment. The fruit-rinds extracts used to treat blister, particularly follicular pharyngitis and the ash of the burned rinds dispersed in coconut oil use to be applied topically for mumps. The durian flesh is full of sulphur which used to kill intestinal parasites. In Malaya, a decoction of the leaves and roots is prescribed as a febrifuge. The leaf juice is applied on the head of a patient to reduce fever (Morton, 1987). The leaves are employed in medicinal baths for people with jaundice. Decoctions of the leaves and fruits are applied to swell and skin diseases. The seeds are believed to possess a toxic property that causes shortness of breath. The fruit is believed to have medicinal properties, restoring health to humans and domestic animals.

### 5.2 Environmental concerns

Thailand is a major producers and world exporter of fresh and preserved durians. Durian season start from April to September, the most durian cultivated areas are the eastern and northern Thailand. Massive amounts of the durian waste lead to environmental problems. It has been estimated that up to 4.14 x $10^5$ tons of durian rinds is produced in nature each year (Center of agricultural information, 2004). In the interest of the environment, attempts have recently been made to derive this agricultural waste to produce a value added material that useful as pharmaceutical excipient for pharmaceutical, cosmetic and food industries (Pongsamart *et al*., 1989).

## 6. Polysaccharide gel from fruit-rinds of *Durio zibethinus* Murr.

### 6.1 Characterization

A process of polysaccharide gel (PG) isolation from fruit-rinds of *Durio zibethinus* was performed based on the method previously described by Pongsamart and Panmuang (1998). The molecular weight of crude PG is approximately 100-1300 kDa. The water soluble PG is composed of pectic polysaccharide as the principal component and starch as a contaminant. The sugar compositions of PG are (20.9%), rhamnose (4.8%), galactose (4.9%), xylose (0.4%), rhamnose (4.8%), arabinose (1.2%) and galacturonic acid (67.9%) (Hokputsa *et. al*., 2004). PG is separated into two main fractions, 'acidic chain fraction' and 'neutral chain fraction' by DEAE-Sepharose column. The main sugar in acidic chain is 86.2% of galacturonic acid which is 1, 4 linked polygalacturonic acid. Neutral chain consists of 34.9% of galacturonic acid containing other side chain neutral sugar contents more than acidic chain (Hokputsa *et al*., 2004).

### 6.2 Bioactivity and applications

An application as a pharmaceutical exipient of PG such as a tablet binder, tablet disintegrator and gelling agent have been well reported (Pongsamart and

Panmaung, 1998; Umprayn, *et. al*., 1990). Bioactivity of PG has been studied, promising antibacterial and immunostimulating activity are elucidated. For antibacterial investigations, the water-soluble polysaccharides have bactericidal activities against several strains of Gram positive and negative bacteria (Lipipun, *et. al*., 2002; Pongsamart *et al*., 2005) and most of mastitis bacterial isolates from cows (Lipipun *et al*., 2006). The attempts have recently been made to use PG as an active ingredient in pharmaceutical preparation. The following PG products have been prepared, antiseptic hand-gel and anti-acne gel (Paphattarapong, 2005; Pongwiwatana, 2005). For studying on immunostimulating activity of PG, the polysaccharide inhibits the heamolysis by complement fixation test (Hokputsa *et al*., 2004). The immunological defense system stimulation of black tiger shrimp after feeding with diet containing durian polysaccharide gel was also recently reported (Pholdaeng *et al.,* 2004). *In vivo* study demonstrated that the dressing films prepared from the durian polysaccharide gel enhance wound healing in pig and dog skin (Nakachat, *et. al*., 2001 and Siripoksupkul, *et. al*., 2004).

### 6.3 Safety of PG

The toxicity test of the polysaccharide gel has also been reported, acute toxicity with a high oral dose (2g/kg) did not induce severe toxicity in male mice and rats (Pongsamart, *et. al*., 2001). No toxic effect has been observed in subacute toxicity test in male mice (Pongsamart *et al*., 1989) and subchronic toxicity studies in male and female mice confirmed the consumptive safety of durian polysaccharide gel (Pongsamart *et al*., 2002).

## 7. Pectic polysaccharide

### 7.1 Molecular structure

Pectin is a common name given to a class of polysaccharides of plant origin. Pectic polysaccharides occur in the primary cell walls of all higher plants and contribute to a number of important functions. The pectic matrix resists compressive forces resting upon cell walls, determines porosity and contributes to ionic status (Basic *et al*, 1988).

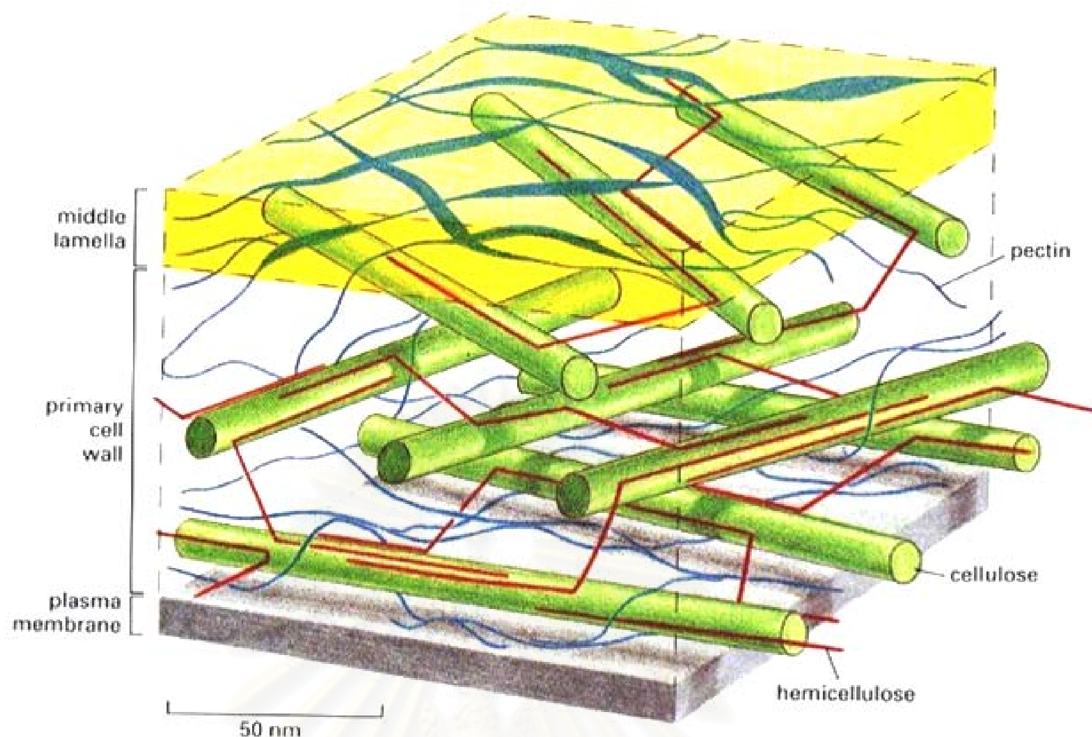


**Figure 4.** A schematic diagram of plant cell wall

Primary plant cell walls are a highly complex cooperative assembly of many different glycans and proteins. The composition of cell walls is highly variable, and some of the major features are shown in Figure 4. The main glycans found between the plasma membrane and the middle lamella are cellulose, the most abundant carbohydrate in plants, hemicellulose, and the pectic polysaccharide fibers (pectin) which are intertwined in the cellulose network in the tissue. It is often stated that the pectic aggregates within the cell wall are of the egg-box structure, where two pectic chains in a twofold helical conformation retain calcium ions between them like eggs in egg-box.

Pectic acids or pectins are common words for describing polymers containing galacturonic acid. Long sequences of polygalacturonans (homogalacturonans) can be found in the pectins. Studying the pectin polymer from different sources showed pectic polysaccharides possess immensely complex structures. The majority of pectic polysaccharides backbone is D-galacturonic acid and apart from the abundant uronic acid, neutral sugars such as L-rhamnose, D-

galactose, D-xylose, and L-arabinose are also major components of pectic polysaccharides. The main types of pectic matrix are homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). The major constituent is linear sequences of 1,4 linked-D-galactopyranosyluronic acid that forms the backbone. The pectin-backbone occasionally branched on where galacturonic acid is replaced by 1,2-linked L-rhamnose residues. The rhamnose units in the alternating core were frequently found as branch points, primarily on position 4, carrying side chains of various neutral sugars branch off including galactan and arabinan side chains. This type of pectin is called rhamnogalacturonan I (RG-I). The stretches consist of alternating galacturonic acid and rhamnose called “hairy or rhamified regions” and others with lower density of rhamnose called “smooth regions” and an average image of RG-I is given in Figure 5. In addition to the hairy region, it was found that the pectic polymer contained a so called smooth region only composed of $\alpha$-1,4-galacturonic acid residues. This smooth region can carry methyl ester groups and also be acetylated at positions 2 or 3 sugar residues. It has also been reported that the “homogalacturonans” can be substituted, often with single xylose residues. The position of these is schematically shown in the model of the pectic polymer as proposed by Perez et al in Figure 5 and 6. The another type of pectin is Rhamnogalacturonan II, the backbone of RG-II contains 1,4-linked α-D-Gal*p*A residues which is four different oligosaccharide chains attached via positions 3 or 4 of the uronic acid backbone. The most characteristic part of RG-II is the presence of the rare sugars 2-*O*-methylfucose, 2-*O*-methylxylose, apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and 3-deoxy-D-lyxo-2-heptulosaric acid (DHA) as proposed in Figure 5 and 7. Chemically, pectin is a linear polysaccharide containing from about 300 to 1,000 monosaccharide units. The molecular weight of pectin ranges from 50,000 to 150,000 Daltons.

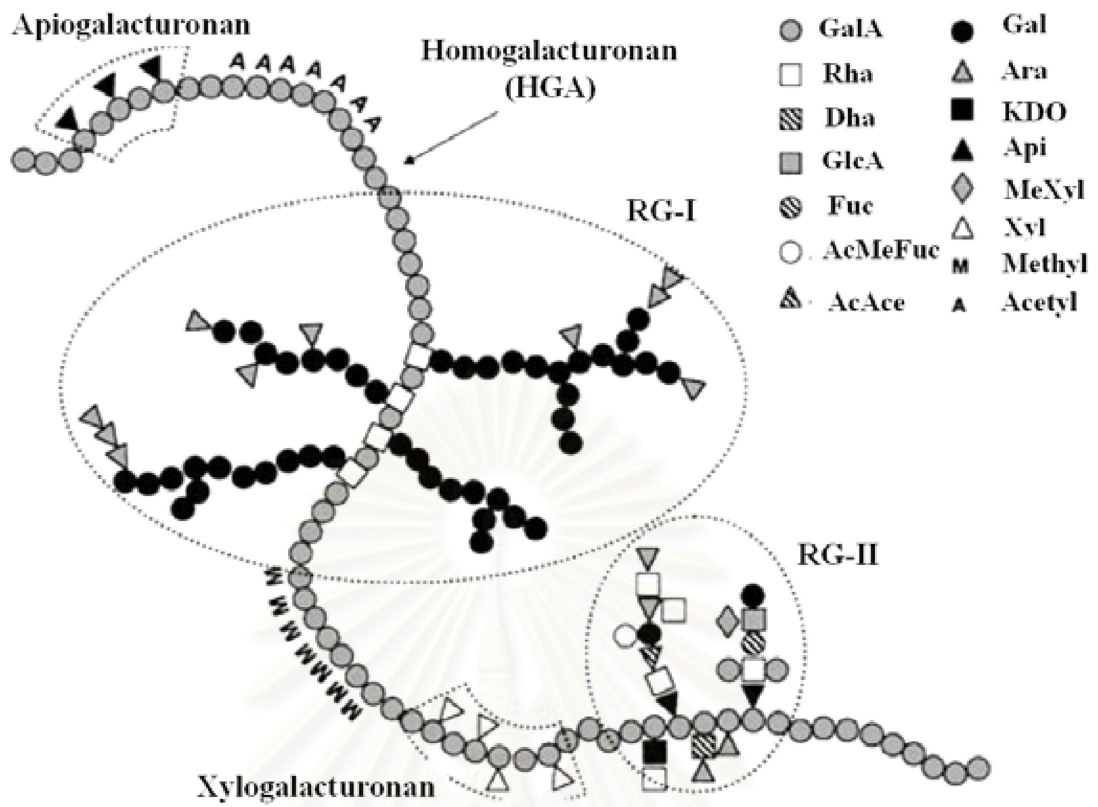


**Figure 5.** A schematic presentation of the primary structure of pectic polysachharide (Perez *et al*, 2003)

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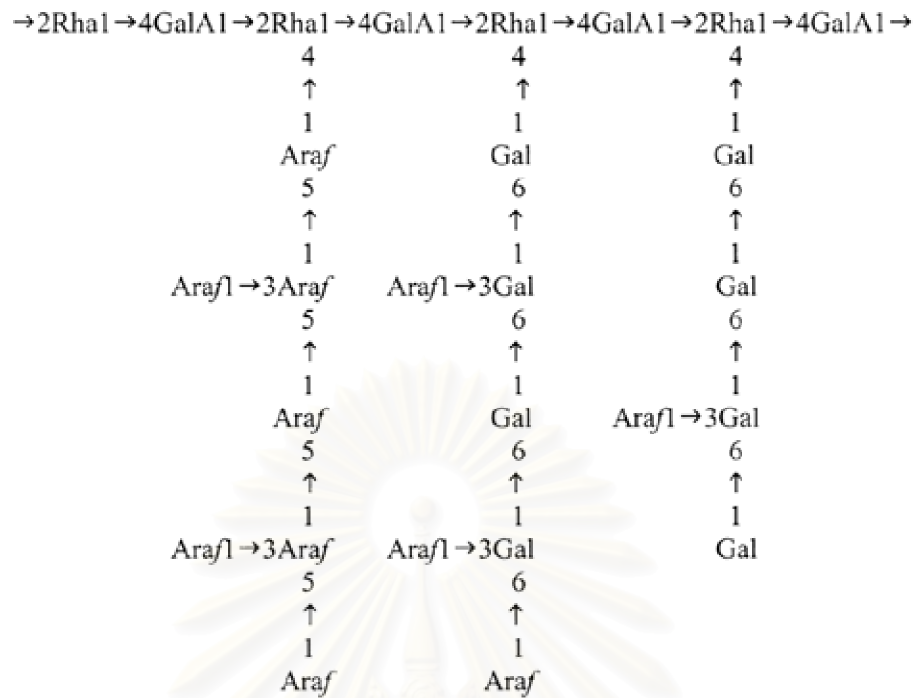


**Figure 6.** Structure of the ramified region in an a pectic polysaccharide, with rhamnogalacturonan I backbone substituted at position 4 of the rhamnose units with arabinan and arabinogalactan type II side chains

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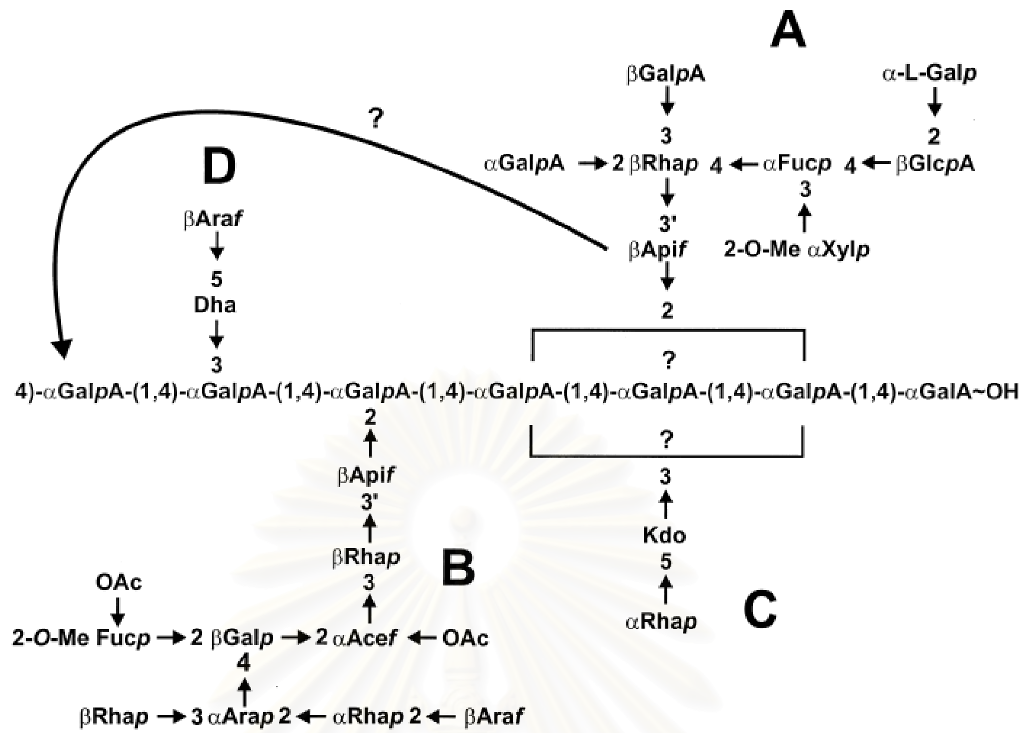


**Figure 7.** Rhamnogalacturonan II (RG II). Four structurally different oligosaccharide side chains (A–D) are linked to the RG-II backbone (Ridley *et al.*, 2001).

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### 7.2 Physical property

In nature, around 80% of carboxyl groups of galacturonic acid are esterified with methanol. This proportion is decreased more or less during pectin extraction. Pectins are classified as high or low-ester pectins, in short termed HM or LM-pectins, with more or less than half of all the galacturonic acid esterified, respectively. Pectin must be completely dissolved to ensure full utilization and to avoid heterogeneous gel formation. Complete dissolution requires dispersion without lumping; if pectin lumps are allowed to form they are extremely difficult to dissolve. Pectin, like any other gelling agent, will not dissolve in media where gelling conditions exist. It is recommended that HM-pectin is dissolved at solids below 20% and preferably in water. The most important factors which influence the gel formation are temperature, degree of esterification, pH, sugar and other solutes, and calcium ions. HM-pectins require a minimum amount of soluble solids and a pH within a pretty narrow range, around 3.0, in order to form gels. LM-pectins require the presence of a controlled amount of calcium or other divalent cations for gelation.. The degree of esterification of high ester pectin influences the gelling properties. This difference is reflected in terms of rapid set, medium set and slow set. Furthermore, the gel formation depens on the temperature. Gels form on cooling and melt when heating. Pectin solutions usually show relatively low viscosities compared to other plant gums and thickeners. Pectin with a high degree of esterification is more viscous in solution than otherwise comparable pectin of lower degree of esterification so the degree of esterification is important for gel application. Viscosity of a pectin solution may be determined for the purpose of obtaining a measure of the molecular weight of the pectin or for evaluating the thickening effect of the pectin. Calcium or other polyvalent ions increase the viscosity of pectin solutions and low ester pectin solutions may even gel if the calcium content exceeds a certain limit. Moreover, the viscosity of pectin solution is also a function of the temperature and pectin concentration as shown in the Figure 8. The viscosity increases exponentially with pectin concentration. However, pH also influences the viscosity of pectin solutions. In a calcium-free solution the viscosity drops when pH is increased. The pK-value of pectin is approximately 3.5. LM-pectins are higher pH-values than high-ester pectins. At low pH-values and elevated temperatures degradation

due to hydrolysis of glycosidic links is observed. De-esterification is also favoured by low pH. As the results, pectin becomes slower setting or gradually adapts low ester pectin characteristics. At near to neutral pH (5-6), HM-pectin is stable at room temperature only. As the temperature (or pH) increases, the polysaccharide chains are cleavage, so-called the β-elimination. It is very rapid loss of viscosity and gelling properties (Dumitriu, 1998).

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## CHAPTER III

## MATERIALS AND METHODS

### Materials

#### Chemicals

- Antibody of Goat anti-Bovine Lactoferrin-affinity purified (Bethyl, U.S.A.)
- Antibody of Goat anti-Bovine Lactoferrin-conjugated to HRP (Bethyl, U.S.A.)
- Antibody of Mouse anti-Human CD14 conjugated RPE-Cy5 (Serotec, England)
- Antibody of Mouse anti-Ovine CD45 conjugated to RPE (Serotec, England)
- Antibody of Mouse anti-Ovine MHC class II conjugated to FITC (Serotec, England)
- Bovine serum albumin (BSA), Sigma (St. Louis, MO, USA).
- Bovine Lactoferrin Calibrator (Bethyl, U.S.A.)
- Citrate agar (Oxoid, England)
- Bile-Esculin medium (Sigma, U.S.A)
- Citric acid (Carlo. ERBA., Germany)
- Calcium chloride (Merk, Germany)
- Dimethyls sulfoxide (DMSO) (Fisher Scientific, England)
- Di-sodium hydrogen phosphate anhydrous ($Na_2HPO_4$) (Fluka, Switzerland)
- Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Grand Island, U.S.A.)
- Eosin G (Merck, Germany)
- Ethyl alcohol USP (Merk, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Merck, Germany)
- Fetal bovine serum (FBS) (Seromed, Germany)
- Glycerin (Carlo. ERBA., Germany)
- Gram stain reagents (Chiang, Thailand)
- Hydrochloric acid (HCl) 36.5-38%, analytical grade (J.T. Baker, U.S.A.)
- Hydrogen peroxide ($H_2O_2$)

- McConkey agar (Merck, Germany)
- Magnesium sulfate ($MgSO_4$) (E. Merck, Germany)
- Methanol ($CH_3OH$), analytical grade (J.T. Baker, USA.)
- Mueller Hinton agar (Merk, Germany)
- Mueller Hinton broth (Merk, Germany)
- Nitro blue tetrazolium (NBT) (Sigma, U.S.A)
- Phenazine methosulfate (PMS) (Sigma, U.S.A.)
- Plate Count agar (Merk, Germany)
- Polyoxyethylenesorbitan Monooleate (Tween 20) (Sigma, U.S.A)
- Potassium chloride (Merk, Germany)
- Potassium hydrogenphopate ($KH_2PO_4$) (Merck, Germany)
- Potassium hydroxide (KOH) (Merck, Germany)
- Propidium iodide (PI) (Sigma, U.S.A)
- Propylene glycol (Dow, U.S.A.)
- Rabbit plasma (Becton Dickinson, U.S.A)
- Ribonuclease A (Sigma, U.S.A)
- Sodium bicarbonate ($Na_2HCO_3$) (Merck, Germany)
- Sodium hexametaphophate (Carlo. ERBA., Germany)
- Sodium hydrogencarbonate (Merck, Germany)
- Sodium chloride (Merk, Germany)
- Sulfur Indole Motility agar (Merck, Germany)
- Tetramethyl-p-phenylenediamine dihydrocholoride (Fluka, Switzerland)
- Triple Sugar Iron agar (Merck, Germany)
- Trishydroxymethylaminomethane (Tris) (GFS Chemicals, U.S.A)
- Trypan blue (Sigma, U.S.A.)
- Tryptic soy agar (Merk, Germany)
- Tryptic soy broth (Merk, Germany)
- Urea (Oxoid, England)
- Wright stain reagents (Chiang, Thailand)
- Zymosan A (from *Saccharomyces cerevisiae*) (Sigma, U.S.A)
- 3,3,-[(phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzensulfonic acid hydrate (XTT) (Sigma, U.S.A)
- 3,3´,5,5´- tetramethylbenzidine (TMB) substrate solution (Edogen, England)

**Equipments**

- Autoclave HA-3D (Hirayama manufacturing coorperation, Japan)
- Blender (Moulinex 327, Spain)
- Cellquest, version 1.2.2. (Becton Dickinson Immunocytometry System)
- Electronic Coulter Counter ZM® (Coulter Electronicics Limited, England)
- FACStar Plus flow cytometer (Becton Dickinson, U.S.A.)
- Hammer mill
- Hemocytometer counting chamber (Boeco, Germany)
- Hematometer Cell-DynR3500 (Abbott diagnostics, USA)
- Hot air oven (Mammert, Germany)
- Incubator Model 6 (Thelco)
- Laminar air flow (ISSCO, model BV2225, Thailand)
- pH meter (MP230, Mettler Toledo,LE413, ME51340 251, Switzerland)
- Rotary evaporator (Buchi R-200, Switzerland)
- Scanning electron microscope (JEOL JSM 5410 LV)
- Viscometer (Brookfield, Model LVDV-I+, Brookfield Engineering Laboratories INC., USA)
- Water bath

**Experimental animals**

Holstein Friesian cows were from the Faculty of Veterinary Science, Chulalongkorn University, Thailand. A permit to use animals was approved by both ethics committee of Faculty of Pharmaceutical Sciences (No. 229/2006) and Faculty of Veterinary Science (No. 0731043), Chulalongkorn University, Bangkok, Thailand.

## Methods

### 1. Extraction of polysaccharide gel (PG) from fruit rinds of durian

Waste of fresh durian fruit-rinds (*Durio zibethinus* Murr.) of Monthong cultivar harvesting from Chanthaburi province was collected from local supermarket. The durian fruit-rinds was washed, blended and dried in hot air oven at 50-60 °C for 45-48 hours or until constant weight. The Polysaccharide gel (PG) was isolated from dried fruit-rind with boil water (70-80 °C for 45 minutes) which was performed based on the method previously described by Pongsamart and Panmuang (1998). The aqueous extract of PG was concentrated under reduced pressure and precipitated by addition of acidified aqueous ethanol, filtered, dried and ground.

### 2. Formulation of PG teat dip

Pale beige powder of PG was dispersed in Ringer's solution (11.8 mM NaCl, 4.7 mM KCl, 2.52 mM $CaCl_2$, 1.18 mM $MgSO_4$, 25.01 mM $NaHCO_3$, 1.18 mM $KH_2PO_4$ in 1 lite distilled water) until uniform. Propylene glycol and glycerin were added into the mixture of PG with continuous stirring. The product was prepared under sterile condition and examined for the contaminated bacteria. Physical appearances, air bubbles, color, viscosity and pH of the postmilking teat dip preparation were determined after freshly prepared and after 5 months stand at ambient temperature.

**Formula of PG teat dip**

| **Ingredients** | | **Content** |
|---|---|---|
| PG | | 2.5 g |
| Propylene glycol | | 10.0 ml |
| Glycerin | | 10.0 ml |
| Ringer's solution | to make | 100.0 ml |

**3. Determination of the PG teat dip efficacy using time-killed analysis *In vitro***

**3.1 Bacterial strains and growth conditions**

The field bacterial isolates causing mastitis in dairy cattle provided by Faculty of Veterinary Science, Chulalongkorn University, Thailand were used. The test organism eomposed of the following: 1) mastitis-causing Streptococci including *S. agalactiae*, *S. uberis, S. dysagalactiae, S. bovis, S. acidominimus*, *S. porcinus*; 2) mastitis-causing Staphylococci including *S. aurues*, *S. chromogenes*, *S. simulans*; 3) mastitis-causing bacteria, Gram-negative, including *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas sp.*. One single colony from plated stock culture of tested bacteria was inoculated in 10 ml trypticase soy broth (TSB) at 37$^{o}$C for 24 hours. The bacterial culture was then streaked onto a Trypticase Soy Agar plate containing 5% sheep blood for confirmation a purity examination. A standard plate count was carried out in Plate Count Agar (PCA) to determine the required dilution to prepare an initial bacterial suspension containing ~1x10$^{8}$ cfu/ml.

**3.2 Time-killed analysis**

**3.2.1 *In vitro* time kill analysis in broad interval (hours)**

An *in vitro* modified time kill analysis was carried out to determine the time killing rates of nine tested bacterial isolates by applying on sterile Petri dish surface. The *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as standard controls. The Petri dishes containing 50 µl of bacterial suspension over 2 x 2 cm$^{2}$ area was covered with 100 µl of PG teat dip in treated group or Mueller Hinton Broth (MHB) in control group and incubated at 37°C. The cultures were removed by using sterile cotton swab after incubation for 0, 2, 4 and 8 hours, respectively. A number of viable colonies were determined by pour plate technique on PCA after serial ten fold dilutions in sterile 0.01M buffered saline (phosphate-buffered saline, PBS). The plates were incubated at 37°C for 24 hours, the visible colonies were counted and calculated as colony forming unit (cfu) per milliliter. Number of survival as a logarithm of the visible count was plotted against time, all time-kill experiments were conducted in duplicate.

### 3.2.2 *In vitro* time kill analysis in narrow interval (minutes)

The initial inoculums composed of 0.1 ml bacterial suspensions (1.5 x $10^8$ to 5.5 x $10^8$cfu/ml) in the 0.8 ml medium containing 2.5%PG (w/v) and 0.1 ml fetal bovine serum (FBS) as a source of organic material. After incubation at 37°C for 1, 30, 60, 120, 240 and 480 minutes, respectively, 20 ml phosphate buffered saline (PBS) was added. One ml of the inoculated bacteria was diluted with 9 ml PBS. A number of viable colonies of ten fold serial dilution samples was determined by pour plate technique on TSA plate and incubated at 37°C for 48 hours. The reduction of colony forming units of bacterial cells to 5 or more logs was determined for its bactericidal efficacy (Rosso *et al*., 2002).

### 3.3 Scanning electron microscopy

Suspension of PG susceptible *S. agalactiae* in NSS was incubated with 2.5% PG (w/v) at 37°C for 8 hours. Tubes of non-viable bacteria were filtered and the bacterial cells were gold coated to examine an appearance of cell surface under scanning electron microscope (SEM) (JEOL JSM 5410LV, Japan). The viable bacteria in NSS without PG were used as a culture control. Before preparation of the culture sample for SEM, the survival of the cultivated bacteria were tested by inoculating the culture on TSA plate and incubated at 37°C for 48 hours.

## 4. Determination of the PG teat dip efficacy by experimental challenge

### 4.1 Preparation of challenge suspensions

Trials were performed with field bacterial isolates, *Staphylococcus aureus* and *Streptococcus agalactiae,* causing bovine mastitis in Thailand. The bacterial suspensions were freshly prepared on the day before challenge by inoculating 2 to 3 colonies of plated stock culture in the 100-ml flasks contained 50 ml TSB and incubated at 37°C for 24 hours. The cultures were diluted with TSB to produce a culture concentration of ~5x$10^7$ cfu/ml as determined by standard plate count in PCA.

### 4.2 Selection of experimental cows

Trials were conducted in research herds from the Faculty of Veterinary Science, Chulalongkorn University, Thailand. All Holstein Friesian cows in first lactation were housed a fix-stall barn and were milked in a stanchion barn. The quarters eligible for inclusion were selected in the trial, except for those infected with microorganisms of the same species as challenge microorganisms and quarters in which teats were deformed, abnormal, or injured prior to or during the trial. Sample size per group was calculated following the protocol previously proposed by Casagrande *et al*. (1978). The infected quarters having a 25 and 5% infection rate in the control group (p1) and the experimental group (p2), repectively, were anticipated and calculated by the following equation:

$$n = A*[1 + sqrt(1 + 4D/A)]\ 2 / (4*D*D)$$

n = sample size

D (difference anticipated between two groups)
= p1 (control infection rate) - p2 (experimental infection rate)

A = variable from calculation

$$A = [\ Z1*sqrt(2*p*(1-p) + Z2*sqrt(\ p1*(1-p1) + p2*(1-p2)\ )\ ]^2$$

Z1 = critical one-tailed value for Type I error rate (usually 5%): Locate the 0.95 probability and the corresponding Zi value, which by interpolation is 1.645.

Z2 = critical one-tailed value for Type II error rate (usually 10%): Locate the 0.90 probability and the corresponding Zi value, which by interpolation is 1.281.

p (average infection rate)
= [p1 (control infection rate) + p2 (experimental infection rate)]/2

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### 4.3 Experimental challenge method

The teat dip evaluation procedure was carried out by experimental exposure study according to the National Mastitis Council (Hogan *et al*., 1990). Each group of the 73 and 81 quarters eligible for new intramammary infections (IMI) was used for controlling infectious trial against *S. aureus* or *S. agalactiae*, respectively. Each teat of tested cow was immersed approximately 25 mm depth in a vessel contained 50 ml of freshly prepared bacterial suspension after removal of milking machine at the afternoon milking. Immediately, after challenged with each of the bacterial suspension, two contralateral teats were dipped in 50 ml of the PG teat dip and the remaining two teats were undipped controls. The experimental challenge was performed every day for 5 days. A quarter of new infected with the challenge bacteria was immediately received treatments.

### 4.4 Milk collection and sampling schedule

The bacteriological status of mammary quarters was determined by collection and culturing of duplicate milk samples from each quarter one week before bacterial challenge started. The milk samples from each quarter were collected and analyzed everyday for 7 days. All milk samples should be collected immediately before a regular milking by using standard procedure (Harmon *et al*., 1990). Briefly, three or four streams of foremilk are discarded from each quarter before sanitizing teat ends with cotton swabs soaked in an excess volume of 70% alcohol and then collecting samples, no more than two teats should be sanitized per swab. The far teats were sanitized first and the near teats were the last to avoid contaminating the near teats with the collector’s fingers. When collecting milk samples, the near teats were milking sampled first and the far teats were the last to avoid contaminating the near teats with the collector’s fingers.

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### 4.5 Criteria for diagnosis of infections

All milk samples were examined microbiologically according to standard procedures (Harmon *et al*., 1990). Four milk samples (10 µl) from each quarter of experimental cows were streaked across quadrant of TSA plates containing 5%

sheep blood and incubated at 37°C for 48 hours. The TSA plates were examined to identify the present microorganisms and checked for purity. Criteria for diagnosis of pathogens were made based on colony characterization in which the color, shape, size, texture, consistency and odor.

Hemolysis pattern are generally classified as alpha, beta or gamma according to the appearance of zones around isolated colonies growing on. The white (completed clear zone), greenish color and no change in the medium surrounding the colony are the hemolytic reactions of beta, alpha, and gamma, respectively.

Gram's reaction and potassium hydroxide (KOH) test were used to divide almost all bacteria into two large groups. For Gram's staining, each suspected bacterial colony was picked up on the glass slide and stained with crystal violet solution (crystal violet 2 gm., ammonium oxalate 0.8 gm. in 20% ethanol 100 ml.) for 1 minute. The slide was flooded with Gram's iodine solution (iodine crystals 1 gm., potassium iodide 2 gm. in distilled water 300 ml.) for 1 minute and washing off with decolorizer (acetone 50 ml and 95% ethyl alcohol 50 ml) for 2-3 seconds. The slides were flooded with safranin O counterstain (safranin 0.25 in 20% ethanol 100 ml.) for 2 minutes before gently rinsing with water. The cell morphology was examined under light microscope with the oil immersion lens. For KOH test, the tested colonies were picked up from the blood agar plate with a heat sterilized loop and mixed in a drop of KOH on a clean glass slide. The Gram-negative organisms were stained in pink from Gram's reaction and showed thickened, formed mucoid gels and threads of mixture in KOH test**,** whereas the Gram-positive bacteria were stained in blue and remained fluid in KOH test.

The gram-positive colonies were transferred from the original sample plate to blood agar containing 5% sheep blood. The pure culture was examined for coagulase test to identify the differentiation among streptococci, staphylococci and micrococci from the pure colony on blood agar plate. Bacteria exhibiting a catalase negative reaction were examined by coagulase test to distinguish the potentially *S. aureus* from the usually Coagulase-negative staphylococci (CNS)

while catalase negative bacteria were tested with both the CAMP reaction and esculin hydrolysis to classify the group of streptococci.

The catalase test was used to identify organisms which produce the catalase enzyme, particularly useful in differentiation of dual staphylococci and micrococci, which are catalase-positive; dual streptococci and enterococci, which are catalase-negative. One colony from a plate culture was placed on a slide and added one drop of hydrogen peroxide ($H_2O_2$). The bubble formation produced indicating a positive reaction.

The coagulase test was used to distinguish the non-pathogenic species Staphylococci from the potentially pathogenic species of *S. aureus* that can convert fibrinogen to fibrin by using its coagulase enzyme. A loopful of the organism was added to a tube of citrated rabbit plasma and thoroughly mixed to suspension with the loop and incubated the tube at $37^{o}$ C for 1 to 4 hours. The tube was examined at 30 minutes to 1 hour intervals for the first couple of hours to observe the presence of a clot by tipping the tube gently to observed clot alongside in tube. A test that showed any degree of clotting within 24 hours was considered coagulase positive.

The CAMP reaction and esculin hydrolysis were used to differentiate the potentially pathogenic species Streptococci. The CAMP reaction is an acronym for Christie, Atkins, Munch, Petersen, the discoverers of this phenomenon. Streaked *S. aureus* ATCC25923 by using an edge of a loop in a straight line down the center of a blood agar plate and also streaked strains of suspected streptococci at right angles to the standard *S. aureus* 2-3 centimeters apart. The sign of enhanced hemolysis in the shape of an “arrowhead” was observed where the *S. aureus* and streptococcal growth are in the nearest proximity after incubation at $37^{o}$ C for 24 hours. At the same time as CAMP test was plated on blood agar plate and the same colony was inoculated into the test tube containing Bile-Esculin medium. The presence of a dark brown color was determined the ability of an organism to hydrolyze the glycoside esculin to esculatin and glucose in the presence of bile (10 - 40%). The CAMP-positive, bile esculin-negative streptococcus can be reported as *S. agalactiae* (Group B streptococcus). A new IMI in a quarter was diagnosed

when the same bacterial species was isolated based on: 1) *S. aureus* or *S. agalactiae* was isolated from a clinical quarter, 2) two consecutive milk samples yielded ≥500 cfu/ml of the same pathogen, or 3) three consecutive milk samples contained 100 to 400 cfu/ ml of the same pathogen.

The gram-negative colonies were inoculated in McConkey agar at $37^o$ C for 24 hours to prepare the pure culture. The pure colony were analyzed by using biochemical tests followed NMC recommendations (Hogan *et al.,* 1990) including, Triple Sugar Iron (TSI) agar, Sulfur Indole Motility (SIM) test, Citrate utilization test, Oxidase test and urea hydrolysis test:

The triple sugar iron agar (TSI) test was a differential medium containing lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. The isolated colony was transferred with a sterile wire to streak on the agar slant and also stabbed the butt approximately 1/8 inch from the bottom of the TSI tube. The inoculated tubes were incubated at $37^o$ C for 24 hours and observed color change on the ability of hydrogen sulfide production and carbohydrate fermentations.

Sulfur indole motility (SIM) test was a test to differentiate among bacteria based on three tests including motility, sulfur reduction (cysteine desulfurase) and indole production (tryptophanase). Briefly, the isolated colony was inoculated in SIM tube and incubated at $37^o$ C for 48 hours. The sulfur-containing amino acids were degraded into pyruvate, ammonia and hydrogen sulfide by cysteine desulfurase-producing bacteria and the iron in the medium reacted with hydrogen sulfide to produce the characteristic black precipitate in the medium. The tested tube was filled with kovacs reagent (para-dimethylamino-benzaldehyde 5 gm. in 25ml of concentrated HCl and 75ml of Amyl alcohol) to the top of the tube. The bacterial motility was observed as growth away from the stab line. The tryptohanase-producing bacteria used amino acid tryptophan as carbon and energy source to produced indole formation in pink color.

Simmons citrate agar test was used to determine the ability of organisms in utilize citrate as a carbon source. Simmons citrate agar contained sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen and the pH indicator bromthymol blue. The isolated colony was streaked the surface of the agar slant with a sterile wire and incubated at $37^o$ C for 24 hours. A positive reaction was indicated by observing growth with an intense blue color in the agar slant.

The oxidase test was used to characterize distinct between the families of Enterobacteriaceae and Pseudomonadaceae, the cytochrome c oxidases-producing bacteria. The cytochrome oxidase is involved with the reduction of oxygen at the end of the electron transport chain. Briefly, the suspected colony was transferred onto filter paper saturated with a drop of oxidase reagent (1%Tetramethyl-p-phenylenediamine dihydrocholoride) which was also a redox indicator. A positive reaction produced from colony of *Pseudomonas spp.* was a dark blue whereas colony from the families of Enterobacteriaceae was colorless within 10-15 seconds.

Urea hydrolysis test was used for the detection of urease-producing bacteria. The isolated colony was inoculated in tube of 3 ml urea and incubated at $37^o$ C for 24 hours. A pink color formation indicated the bacteria that breakdown of urea to ammonia and $CO_2$.

**4.6 Treatment methods**

The infected cows were separated from their herd and immediately received treatments after a new IMI was confirmed. The infected quarter was milked frequently to reduce infected organisms until the milk was dried. The infected cow was injected with oxytocin 5 IU intravenously to stimulate an effective milk let down. The infected quarters were given an antibiotic Lactaclox® (Ampicillin 75 mg, Cloxacillin 200 mg) by intramammary infusion and Butasyl® (Phenylbutazone 18.6 mg/mL) for anti-inflammatory treatment by intramuscular injection.

### 5. Determination of the efficacy of PG teat dip by natural exposure

#### 5.1 Experimental cows

The trial was performed at a commercial dairy farm in Rajchaburi province, Thailand. Approximately 33 and 51 lactating Holstein Friesian cows were used in the field trial of experiment 1 and 2, respectively. The cows were housed in a free-stall barn as an area for loafing and feeding together with an access to pasture. Cows were milked in a double-two, side-opening, low line parlor. In each trial, the herd was divided into 3 groups by randomly balanced in parity and stage of lactation between treatment groups. In the experiment 1, the cows were randomly grouping to maintain a constant number of lactating cows in each group during the 1-week trial. Positive control group 1 (n = 11 cows) treated with iodophore teat dip, group 2 (n = 11 cows) treated with PG teat dip, and Negative control group 3 (n = 11 cows) was an undipped controls. In the experiment 2, the herd was divided into 3 groups, Positive control group 1 (16 cows) treated with iodophore teat dip, group 2 (n = 18 cows) treated with PG teat dip, and group 3 (n = 17 cows) treated with the combination of iodophore followed with PG teat dip. Ear-tag numbers, taped tails, and paint on the back were labeled to identify different groups. Colors were used to identify the three dips, and all dairy personnel and investigators were blinded of the identity or differences among the three dips.

#### 5.2 Sampling Schedule and Procedures

All milk samples were collected immediately before a regular milking according to standard procedure (Harmon *et al.,* 1990). Bacteriological status of mammary quarters was determined by collecting and culturing the duplicate milk samples to confirm infections at the beginning and end of the trial. The third sample was collected from specific quarters and cultured if the results from the first 2 samples were different. The result from the third sample was used to confirm the determination of infection status. Single milk sample from each quarter from all lactating cows of experiment 1 was collected and analyzed every day for 7 days whereas quarter milk samples from the cows of experiment 2 were

collected every week for 1 month. Cows were classified to have an infection whenever 2 consecutive samples contained at least 100 cfu/ml of the same organism. All quarters were eligible for new infections during the trial except those previously identified to be infected with the same species of tested organisms. For new cows entering a group or cows leaving a group, the bacteriological status was also determined by culturing the duplicate milk samples as described (Nickerson *et al*., 1986).

### 5.3 Laboratory Culture Procedures

The standard procedures were previously described in 4.5 and summarized in the Figure 1.

## 6. Determination of the effect of PG on bovine mammary leukocytes (*in vitro*)

### 6.1 Isolation of bovine mammary leukocytes

After disinfection of the mammary gland of tested cows, a volume of 20 ml of PBS without endoxin at pH 7.2, 37°C was infused intramammaryly under sterile condition. Mammary gland lavage fluid samples were recovered by immediate manual aspiration into sterile container and kept at 4 °C. Samples (0.1 ml) of the lavage fluids were inoculated onto blood agar and incubated aerobically at 37°C for 24 hours for detection of bacterial contamination. The cell pellet was collected by centrifugation at 400 x g, 4 °C for 10 minutes, and washed twice with 15 ml of cold PBS. The cells viability was assessed after exposure to trypan blue. A volume of 0.5 ml from each cell suspension was added with 0.1 ml of 0.4% Trypan blue stain in microtubes to assess cell viability. The mixtures were standed at 37°C for 5 minutes. The coverslip were placed over the hemocytometer counting chamber and using a Pasteur pipette to drop of the cell suspension at the edge of the “V” shape of the chamber. Allow the suspension drew into the chamber by capillary action and placed the chamber on the light microscope. The non-viable cells were stained and being excluded visually under a light microscope at 400X magnification (40X objective lens). Total mammary gland leukocytes per milliliter were calculated based on viable cells and also the viability more than 90% were used.

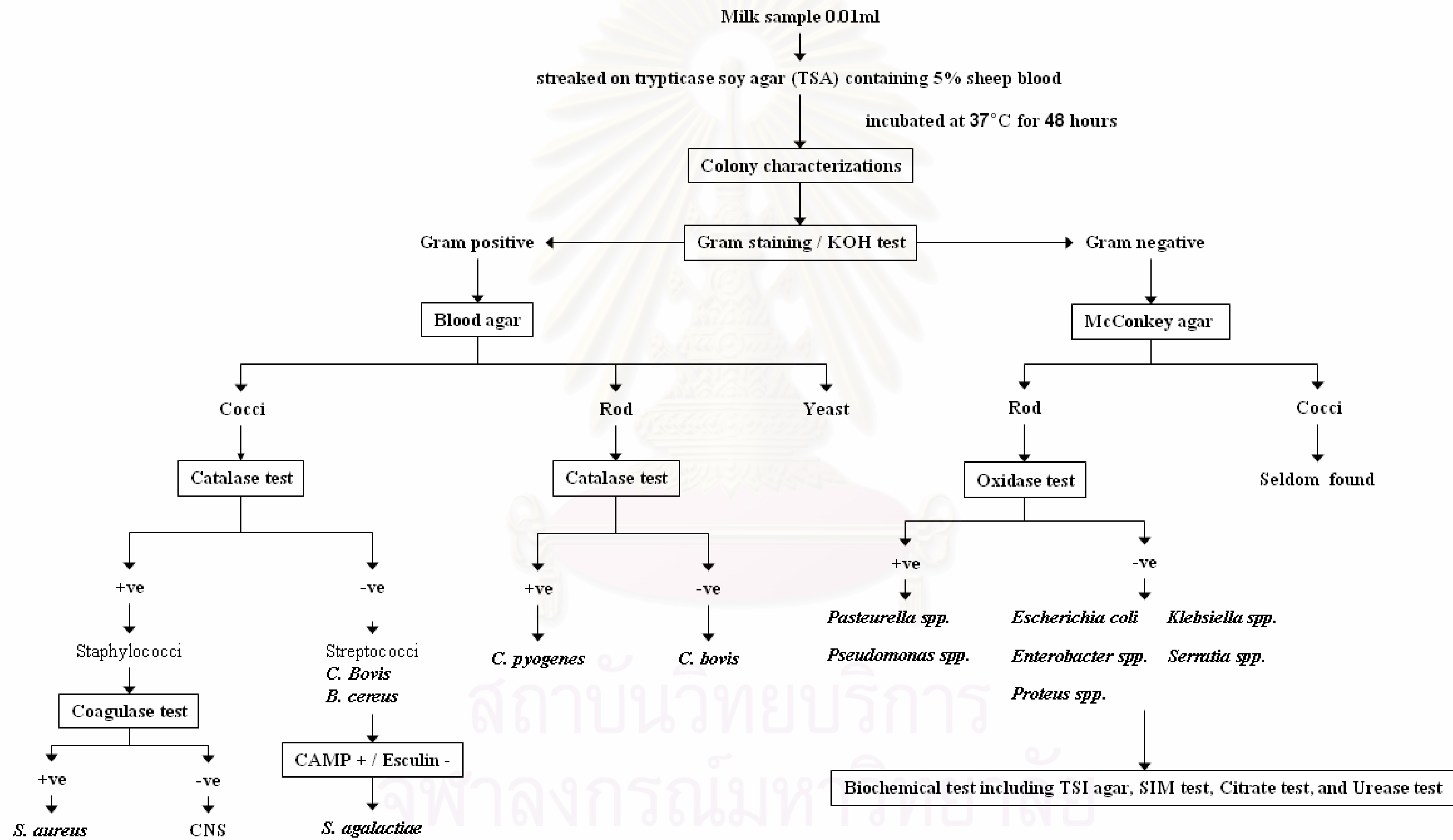



**Figure 8** Scheme for identification of mastitis causing bacteria

**6.2 Exposure method**

The cells were adjusted to $5 \times 10^5$ cells/ml in complete Dulbecco's Modified Eagle's Medium (DMEM). The volume of 150 µl of the cell suspensions was mixed together with 150 µl of PG solution 10, 20 and 50 mg/ml to make 0.5, 1 and 2.5% PG (w/v) of final concentration into 96-well sterile culture plate and incubated in 5% $CO_2$ incubator under humidified conditions at 37 °C for 30 minutes. The distilled water were used as a vehicle control. The reactions were stopped by centrifugation at 5,500 rpm at 4 °C for 10 minutes and supernatant fluid was discharged. The cell pellets were used to determine cytotoxic reaction in experiment 6.3.1 and *in vitro* phagocytosis assay in experiment 6.4.

Into 500 µl of cell suspensions in sterile conical tube, added 500 µl at various concentration of PG (10, 20 and 50 mg/ml) to make 0.5, 1 and 2.5% PG (w/v) of final concentration, respectively or distill water as vehicle control and incubated in 5% $CO_2$ incubator under humidified conditions at 37 °C for 30 minutes. The treated cells were harvested by centrifugation at 5,500 rpm at 4 °C for 10 minutes, cell pellets were use for cytotoxic reaction in experiment 6.3.2; whereas, the treated cells were fixed by adding 1 ml of 1% paraformadehyde for scanning electron microscope examination in experiment 6.5.

**6.3 *In vitro* cytotoxicity assay**

**6.3.1 XTT reduction assay**

A 3,3,-[(phenylamino)-carbonyl]-3,4-tetrazolium-bis-(4-methoxy-6-nitro)-benzensulfonic acid hydrate (XTT) was prepared at 1 mg/ml concentration in DMEM at 37°C without serum, and the phenazine methosulfate (PMS) was prepared at 5 mM (1.53 mg/ml) in PBS. Fresh XTT and PMS were mixing together to prepare 0.025 mM PMS-XTT solution by mixing 25 µl of the stock 5 mM PMS with 5 ml of XTT (1 mg/ml). The pellet cells in 6.2 which was previously washed twice with cold PBS were filled into each of 96 wells with 50 µl of PMS-XTT mixture solution (final concentration, 50 µg of XTT and 0.38 µg

of PMS per well) and inclubated in 5% $CO_2$ under humidified conditions at 37 °C for 4 hours. The XTT assay is based on the reaction of the cleavage of yellow tetrazolium salt XTT to from an orange formazan dye by mitochondrial dehydrogenase enzyme in active cell, this metabolic pathway become inactive after cell death. The colorimetric determination of XTT formazan product was measured spectrophotometrically at 450 nm. Cell viability was expressed as a percentage of the control culture. The percentage of cytotoxicity was calculated by the following equation (Lu *et al*., 1997):

$$\% \text{ cytotoxicity} = \left( \frac{OD_{450nm}\text{ (control)} - OD_{450nm}\text{ (sample)}}{OD_{450nm}\text{ (control)}} \right) \times 100$$

**6.3.2 Flow cytometric method with propidium iodide**

Flow cytometric analysis was performed as described previously (Warrington *et al*., 2003). Briefly, the treated cell pellets in 6.2 were washed twice with cold PBS. The treated cells were fixed by resuspending in 2 ml of cold 70% ethanol and stored at 4 ºC for 24 hours. The cells were collected at the indicated time and washed with PBS and then resuspended in 1 ml of PBS containing RNase A 100 µg/ml at 37 ºC for 30 minutes. Propidium iodide was added to a final concentration of 40 µg/ml, and the mixture was then incubated in dark at 37 ºC for 30 minutes (Glozak *et al*., 1996). Samples were analyzed for fragmented DNA content by a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). Excitation was done at 488 nm, with emission filter at 600 nm. CellQuestTM Pro software (Becton Dickinson) was used for sub G0/G1 phase for DNA distribution in apoptotic process. Ten thousand cells in each sample were analyzed and expressed as percentage of total cells.

**6.4 *In vitro* phagocytosis assay by NBT reduction method**

The treated cells in 96 well plates in 6.2 were washed twice with 250 µl of DMEM medium before incubating with 800 µg/ml of zymosan and 600 µg/ml of

NBT for 60 minutes. The 96-well plates were centrifuged at 5,500 rpm for 10 minutes followed by three-times washing with 200 μl methanol to eliminate the unreduced NBT dye. The cell pellets were air- dried and added 120 μl of 2 M KOH and 140 μl of DMSO and absorbance of the turquoise blue in reaction mixture was read at 570 nm by microplate reader. The percentage of NBT reduction or % phagocytosis was calculated by the following equation (Segal AW and Levi AJ., 1975):

$$\% \text{ phagocytosis} = \left( \frac{OD_{570nm}\text{ (sample)} - OD_{570nm}\text{ (control)}}{OD_{570nm}\text{ (control)}} \right) \times 100$$

**6.5 Scanning electron microscopy**

The treated cell pellets in conical tube in 6.2 were fixed by adding 1 ml of 1% paraformadehyde. The cell suspensions were filtered through Millipore filter before examining the cells morphological appearance SEM. Sample preparation for microscopy was performed and viewed under JEOL JSM 5410 LV scanning electron microscope by using standard operating conditions.

**7. Determination of immunomodulatory effect of PG on bovine udder**

**7.1 The experimental cows**

Three non-pregnant cows of the Holstein Friesian breed were used. The tested cows were milked for at least one lactation. At ten day prior to the final milking, all cows were dried by taking off concentrate feed and moved to a free-stall barn without an access to pasture. The cows were milked every other day and intramammaryly injected one syringe of Bovaclox® (Cloxacillin 500mg, Ampicillin 250mg) per quarter after final milking to dry tested milking cows. The dry cows were treated to dry and stop milking for at least 6 weeks before the experiment start**.** The mammary glands of the dried cows were examined to be free from abnormalities and samples from the tested udder quarter were also bacteriologically negative.

### 7.2 Preparation of PG test solution

Solution of 1%PG (w/v) was freshly prepared under sterile condition by dispersing PG powder in distilled water without endotoxin and stirred until homogenous. The contaminated bacteria were examined. The PG tested solution was then streaked onto a Trypticase Soy Agar plate containing 5% sheep blood for a purity examination. A standard plate count was carried out by diluted 1 ml of the PG test solution into 9 ml of PBS and ten fold serial dilutions was performed. A number of viable colonies were determined by using pour plate technique on PCA plates.

### 7.3 Udder preparation and intramammary infusion

The udder quarters were randomly selected from three non-lactating cows. The tested udders were cleaned the teat skins with wet single service towels individually. Before experimental started, all teats were dipped with the teat antiseptic for approximately 30 second contact time and wiped to dry with a single service towel. The teat ends were sanitized with cotton swabs soaked in 70% alcohol. This sanitization procedure eradicates the bacteria or contaminated materials in the teat orifice. Each treated group of 3 udders was infused through the teat canal with 12.5, 25 and 62.5 ml of 1% PG, respectively, and PBS was used in control group. A syringe with plastic infusion cannula about 1 centimeter long, replaced a needle was used for PG infusion, the cannula was inserted about half-way up into the streak canal. After emptying the PG syringes, the teat was pinched off and the PG dispersion was palpated up into the gland.

### 7.4 Clinical examination

A variety of systemic and local responses were used to examine the clinical to intramammary infusion of PG. The treated cows were observed and recorded its general appearance, such as normal, dehydration, depression, pyrexia, anorexia and coma. The quarters were considerate visually abnormal udder secretion, such as flake, clot or watery and also udder physical change included swelling, red, worm and painful of gland. Clinical examinations included fever (°C; determined by rectal temperature), heart rate (beats/min), pulse frequency (times/min),

respiration rate (breaths/min), and rumen contraction rate (contractions/min) were performed every day during the trial for 15 days.

### 7.5 Blood collection and analysis

The blood samples were withdrew from both PG treated and control (untreated) cows before infusion at day 0 and at day 1, 2, 5, 8 and 15 post-infusion. The volume of blood samples 2.5 ml of were withdrew from Jugular venipuncture with needle 18 gauge x 1.5 inch length and flowed freely into the blood collection vial containing 2.5 mg ethylenediaminetetraacetic acid (EDTA) powder, to make final concentration at 1 ml of blood sample per 1 mg EDTA as anticoagulant. Blood samples were carried out for counting of total white blood cells (WBC) which was performed in a Cell-DynR3500 (Abbott diagnostics, Abbott Laboratories, USA). The differential blood count was examined by staining a sample blood smear with Wright's stain. Briefly, the blood samples were gently inversed and dropped on a flat surface of glass slide. One end of the spreader slide was placed at approximately 30 degree angle onto the drop of blood on the slide and pushed in opposite direction to the blood drop with rapid motion to make a thin film of blood smear on slide. The blood smear slide was air dry quickly before overlaying with excess Wright's stain to completely cover the blood smear for 4 minutes. Added 2.0 ml of distilled water and blowed gently to mix until metallic sheen appeared and allowed to stand for 4 minutes. The slides were rinsed with distilled water for 30 seconds and air dried at room temperature before examination. The 200 cells per smear were classified of total lymphocyte, monocyte, eosinoplhil, basophil, polymorphonuclear and band neutrophil under a light microscope at 1000X magnification and calculated the proportions of each leukocyte types.

### 7.6 Udder secretion collection and analysis

Udder secretion samples were collected from the three PG treated cows at the day before infusion (day 0) and at day 1, 2, 5, 8 and 15 post-infusion. After teat end sanitization, a volume of 10 ml of mammary gland secretion was recovered by immediate manual aspiration into sterile container and kept at 4 °C. All udder

secretion samples were examined microbiologically according to standard procedures (Harmon *et al*., 1990). A volume of 1 ml of each samples from manual aspiration were used for Somatic Cell Count (SCC) in 8.6.1 and the remaining of 9 ml samples were centrifuged at 400 x g, 4 °C for 10 minutes to separate bovine mammary cells. The supernatant was collected and carried out to measure bovine lactoferrin by Enzyme-Linked Immunosorbent Assay (ELISA) in 8.6.2; whereas, the cell pellets were washed twice with 15 ml of cold PBS and centrifuged, the cell pellets were resuspended in 5 ml of cold PBS and use to assess cell viability by aliquot 0.5 ml of each cell suspension sample into microtubes and added 0.1 ml of 0.4% Trypan blue stain. The mixtures were standed at 37°C for 5 minutes before filling in a hemocytometer chamber for cell counting. The non-viable cells were stained whereas the viable cells were not stained with trypan blue as observed under a light microscope at 400X magnification. Total counts of mammary gland leukocytes per milliliter were calculated based on viable cells. The remaining of 4.5 ml cell suspension in PBS was adjusted to make $5x10^6$ cell/ml in cold PBS for analysis the immunophenotyping of leukocyte cell surface markers in 8.6.3 by flow cytometer.

#### 7.6.1 Somatic cell counts method

The udder secretion samples of 1 ml from 8.6 were added with 20 µl of fixative solution (Eosin G 0.1g in 3.5% Formaldehyde solution) and incubated at 50 ºC for 20 minutes. The volumes of 100 µl of fixed samples were mixed with 10 ml of detergent and warmed in water bath at 80 ºC for 10 minutes. Total somatic cell counts (SCC) in udder secretion samples were monitored by using an Electronic Coulter Counter ZM®.

#### 7.6.2 Measuring of bovine lactoferrin by ELISA technique

The 1 µl of capture Goat anti-Bovine Lactoferrin antibody was diluted with 100 µl of coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) in each well of microplate and incubated for 60 minutes. The capture antibody solutions were aspirated and washed three times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0. Added 200 µl of blocking solution (50 mM Tris, 0.14 M

NaCl, 1% BSA, pH 8.0) in the coated microplate and incubated for 30 minutes. After incubation, discarded the blocking solution and washed excess blocker three times with wash solution. The standards (Bovine lactoferrin calibrator) were diluted in the conjugate diluents buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0). The 100 µl of various concentrations from 7.81 to 500 ng/ml of the standards or sample were transfer to the assigned wells and incubated at ambient temperature for 60 minutes. The samples and standards solutions were discarded and wash each well 5 times with wash solution. The 100 µl of Goat anti-Bovine Lactoferrin-HRP conjugate were transferred to each well and washed out after 60 minutes incubation. The plate was washed 5 times with wash solution and added 100 µl of 3,3´,5,5´- tetramethylbenzidine (TMB) substrate solution. The enzymatic color reaction was allowed to develop at room temperature for 30 min; the substrate yielded a blue solution which was turned to yellow after 100 µl of stop solution (2 M $H_2SO_4$) was added into each well. The plate was then measured the absorbance at 450 nm using a microtiter plate reader.

**7.6.3 Surface marker analysis by flow cytometer**

The cell suspension (5 x $10^6$ cells/ml) after trypan staining for assuring the viability more than 90% from 8.6 were used for immunofluorescence labeling. A direct staining procedure was used to identify CD45+ leucocytes bearing the other markers of interest as described. Mammary cell suspensions were immediately labeled for flow cytometry with CD45 (Mouse anti-ovine CD45 with RPE, MCA2220PE) and one of the following antibodies: CD14 (Mouse anti-human CD14 with RPE-Cy5) or MHC class II (Mouse anti-ovine MHC class II with FITC, MCA2228F). The stained cells were analyzed in a FACStar Plus flow cytometer (Becton Dickinson Immunocytometry systems, Mountain View, CA) with standard optical equipment using an argon ion laser at 200mW tuned to 488 nm. Data were acquired with a FACstation. The software for data collection and analyses was Cellquest, version 1.2.2. (Becton Dickinson Immunocytometry System). Fifth thousand events were collected in list mode. The following parameters were collected forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1), PE fluorescence (FL2) and PerPc fluorescence (FL3). Leucocytes were identified by their expression of CD45. The CD14+

leucocytes were evaluated by PerPc fluorescence and SSC while MHC II+ leucocytes were evaluated by FITC fluorescence.

**8. Statistical analysis**

The results of PG teat dip efficacy in time-killed analysis in experiment 3. were mean values with duplicate replication. Differences between the percentages of quarters become infected in the determination of the efficacy of PG teat dip in experiment 4. and 5. were analyzed by using an approximated *t* statistic defined as; $t = [(xl/nl) - (x2/n2)l/[xl + x2)/(n\ ln2)]^{0.5}$, where x1 = number of new IMI in control quarters, x2 = number of new IMI in treated quarters, nl = (number of control quarters)(time unit), and n2 = (number of treated quarters)(time unit). The unit of time was quarter days at risk. A teat dip is considered efficacious if the percentage of reduction is at least 40%. The rate of new IMI in the treated group compared with that of in the control group was expressed as follows: 100[(x1/n1) - (x2/n2)]/(x1/n1), where x1, x2, n1, and n2 were defined (Hogan *et al.*, 1990). The determination parameter of the effect of PG on bovine mammary leukocytes in experiment 6 was examined in 10 samples. Conventional statistical methods were used to calculate means and standard error (SE), data were analyzed using one-way analysis of variance (ANOVA), significant differences was assumed if $P \leq 0.05$. To ascertain significant differences of %cytotoxicity and %phagocytosis between the concentrations of PG, turkey's test was applied between means. In the experiment 7.5, the total WBC was log-transformed before analysis. Total WBC and percentages of differential blood cell populations were means±SEM and the significance of time effects was analyzed using ANOVA for repeated measures. Total SCC, total numbers and lactoferrin concentrations in udder secretions were log-transformed before analysis. The expression of different surface antigens on leucocytes was expressed as proportions (%). The difference between treatment groups for each parameter was determined by analysis of variance. The significance of time effects was determined by analysis of variance for repeated measures. The significance of differences between groups was compared at each sampling occasion by using appropriate standard errors. Cell numbers and proportions were means±SEM. Probabilities less than 0.05 were considered significant.

# CHAPTER IV

## RESULTS

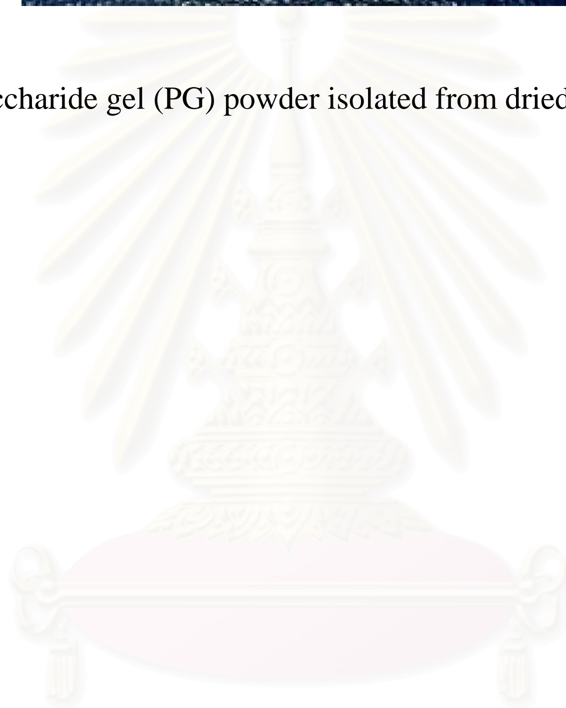
### 1. Extraction of polysaccharide gel (PG) from fruit-rinds of durian

Extraction of water-soluble PG yielded approximately 7% w/w of dried fruit rinds. The pale beige PG gel dried powder was shown in Figure 9. In this experiment, PG powder was a pooled sample from several laboratory scale extractions to make one uniform PG sample. Viscosity and pH of the 3% w/v of PG in water were 652 ±1.01 cps and 2.54±0.01, respectively. The aqueous PG gel at 3% PG (w/v) concentrations was shown in Figure 10.

### 2. Formulation of PG teat dip

The postmilking teat dip was successfully prepared; the formulation composed of 2.5 g PG, propylene glycol 10 ml, glycerin 10 ml and Ringer's solution to make 100 ml, the appearance of PG teat dip was shown in Figure 11. The property of PG teat dip product was shown in Table. 8. The product appearance after freshly prepared was homogenous, no air bubbles, easy flow and pale beige in color; and it appearance was not changed after stand at room temperature for 5 months. The viscosity was 852±2.79 cps after freshly prepared and increased to >10,000 cps after stand for 2 months, however, the viscosity decreased by agitation. The pH of postmilking teat dip was 2.53±0.03 after freshly prepared, which was changed to 2.71±0.02, 2.42±0.02, 2.55±0.04, 2.33±0.01 and 2.38±0.03, after stand at ambient temperature for 1, 2, 3, 4 and 5 months, respectively. The mean viscosity from triplicate measurements was increased whereas the mean pH values were rather stable after stand for 5 month (Table 8).

**Figure 9.** Polysaccharide gel (PG) powder isolated from dried fruit-hulls of durian.



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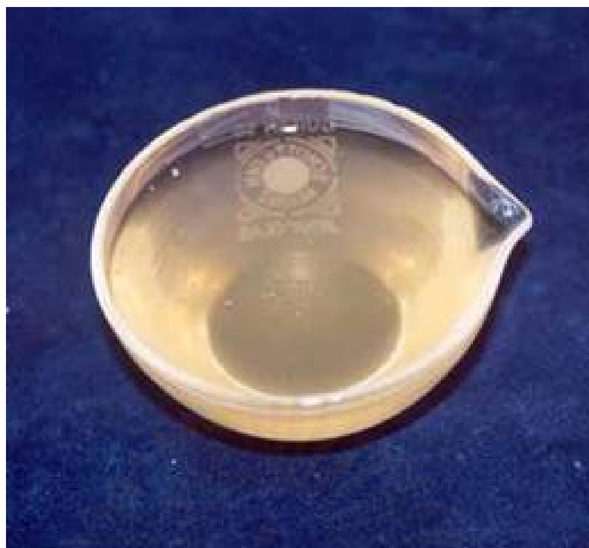

**Figure 10.** The PG gel dispersing in distilled water at 3% PG (w/v) concentrations

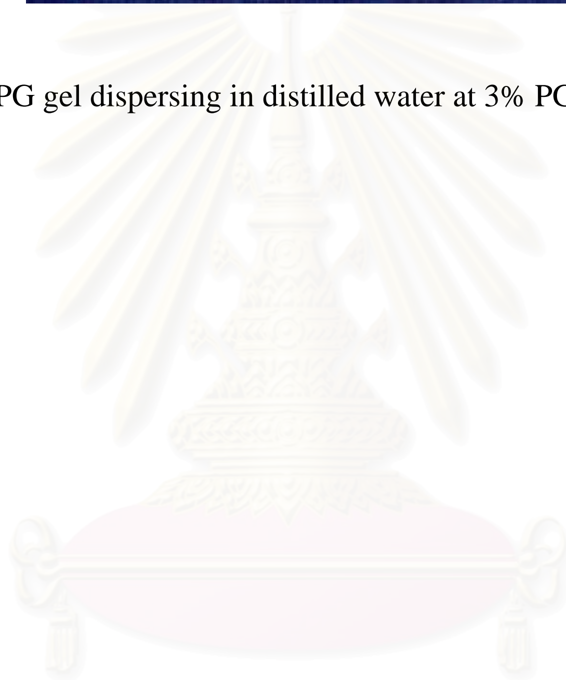

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**Figure 11.** The postmilking teat dip prepared from durian polysaccharide gel (PG)

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**Table 8.** Property of the posmilking teat dip prepared from durian polysaccharide gel (PG)

| **Antiseptic products of PG teat dip** | | | | | |
|---|---|---|---|---|---|
| **Freshly prepared** | **After stand at ambient temperature (months)** | | | | |
| | **1** | **2** | **3** | **4** | **5** |
| Appearance: homogenous | Appearance: homogenous | Appearance: homogenous | Appearance: homogenous | Appearance: homogenous | Appearance: homogenous |
| Flow: easy | Flow: easy | Flow: easy (after agitation) | Flow: easy (after agitation) | Flow: easy (after agitation) | Flow: easy (after agitation) |
| Air bubbles: none | Air bubbles: none | Air bubbles: none | Air bubbles: none | Air bubbles: none | Air bubbles: none |
| Color: pale beige | Color: pale beige | Color: pale beige | Color: pale beige | Color: pale beige | Color: pale beige |
| Viscosity: 852 cps | Viscosity: 2679 cps | Viscosity: >10,000 cps | Viscosity: >10,000 cps | Viscosity: >10,000 cps | Viscosity: >10,000 cps |
| pH: 2.53 | pH: 2.71 | pH: 2.42 | pH: 2.55 | pH: 2.33 | pH: 2.38 |

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**3. Determination of the efficacy of PG teat dip using time kill analysis *in vitro***

***In vitro* time kill analysis in broad interval (hours)**

A modified, *in vitro* time kill analysis was carried out to determine the time killing rates of post-milking teat antiseptic prepared from PG. Nine bacterial isolates were tested by applying the PG teat dip over tested bacteria deposited on sterile petri dish surface. The study was performed to evaluate the killing time of tested mastitis-causing bacteria. *Escherichia coli* ATCC 25922 as well as *Staphylococcus aureus* ATCC 25923 was used as standard controls for Gram-negative and positive bacteria, respectively. The time killing profiles of tested bacteria were shown in Figures 12, 13 and 14. The efficacy of PG teat dip against *Staphylococcus* spp. in Figures 12a and 12b demonstrated that bacterial counts decreased 1-2 logarithms from initial counts ($5x10^5$ CFU/ml) after 2 hours incubation in PG teat dip and decreased close to zero count in 8 hours. Time-kill analysis of *Streptococcus* spp. including *S. agalactiae* (Figure 13a), *S. uberis* and *S. porcinus* (Figure 13b) demonstrated that bacterial count declined to zero within 4, 2 and 8 hours, respectively. Time-kill analysis of *E. coli* (Figures 14a) showed slowly declined close to zero count in 8 hours; whereas, both *Klebsiella* sp. and *Pseudomonas* sp. (Figures 14b) showed that colony counts declined to zero at 8 hour incubation. The killing rate of PG teat dip was shown as percentages of bacterial cells reduction were summarized in Table 9. The result demonstrated that all tested bacteria were reduced soon as exposure to PG, bacterial cells reduced more than 80-90%, except for *S. agalactiae*, *S. porcinus* and *S. uberis* were 100% reduction, after 2 hours of incubation. The *E. coli* ATCC25922 and *S. aurues* ATCC25923 showed 89.45% and 80.81% cells reduction, respectively. The field isolates were reduced in their number more than 99% within 4 hours. The percentage reduction of most bacteria was completely 100% within 8 hours of incubation, except for *E. coli* ATCC25922, *S. aurues* ATCC25923 were 99.9%, and field isolate of *S. aurues* was 99.6%. Killing ability of PG was performed a time dependent manner.

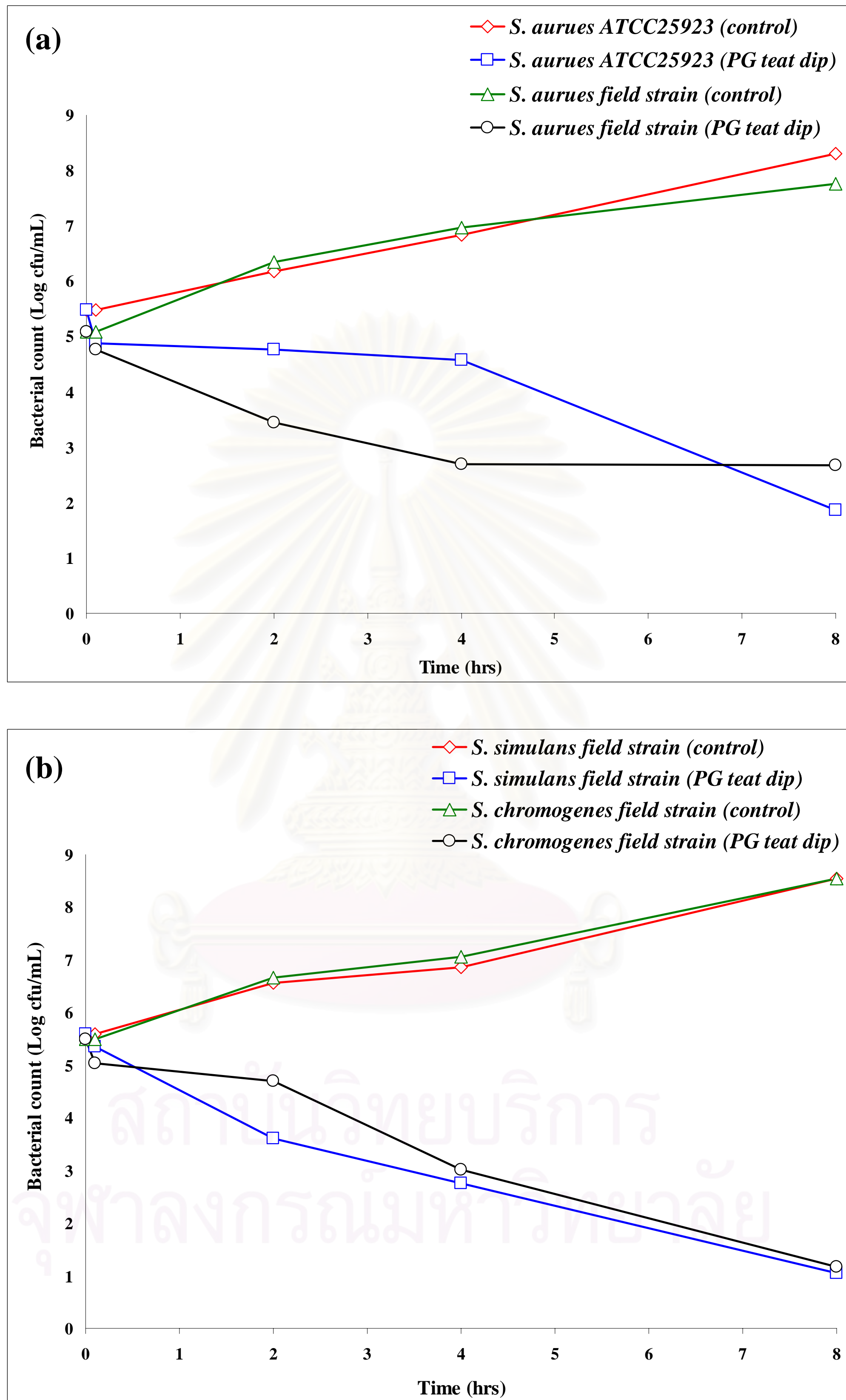


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**Figure 12:** Time-kill analysis of postmilking teat dip prepared from durian polysaccharide gel against *Staphylococcus spp.*; (a) Contagious matitis pathogens; *Staphylococcus aureus*, (b) Coagulase-negative Staphylococci (CNS); *Staphylococcus simulans* and *Staphylococcus chromogene*

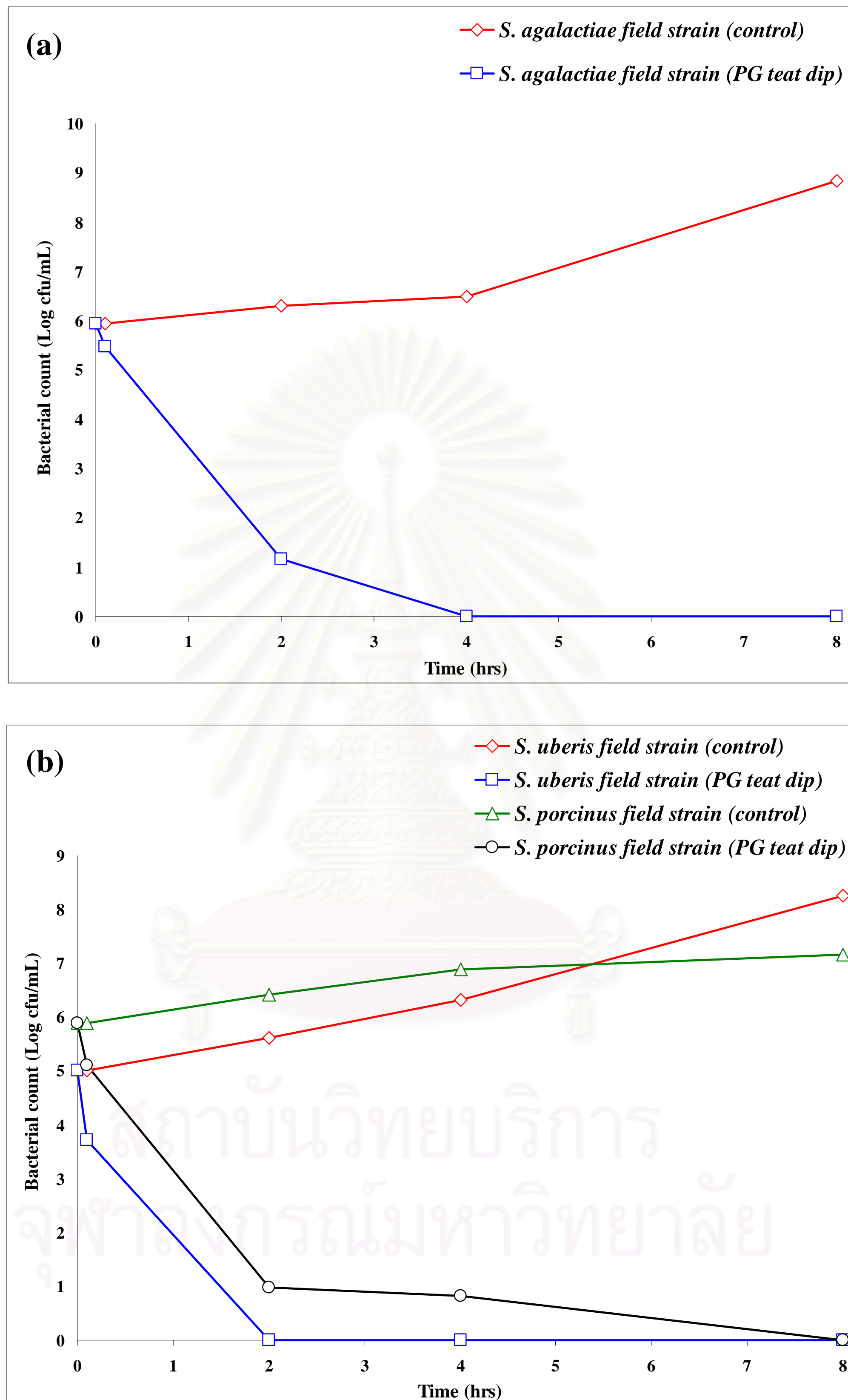


**Figure 13:** Time-kill analysis of Postmilking teat dip prepared from durian polysaccharide gel against *Streptococcus spp.*; (a) Contagious matitis pathogens; *Streptococcus agalactiae*, (b) Environmental mastitis pathogens; *Streptococcus uberis* and *Streptococcus porcinus*

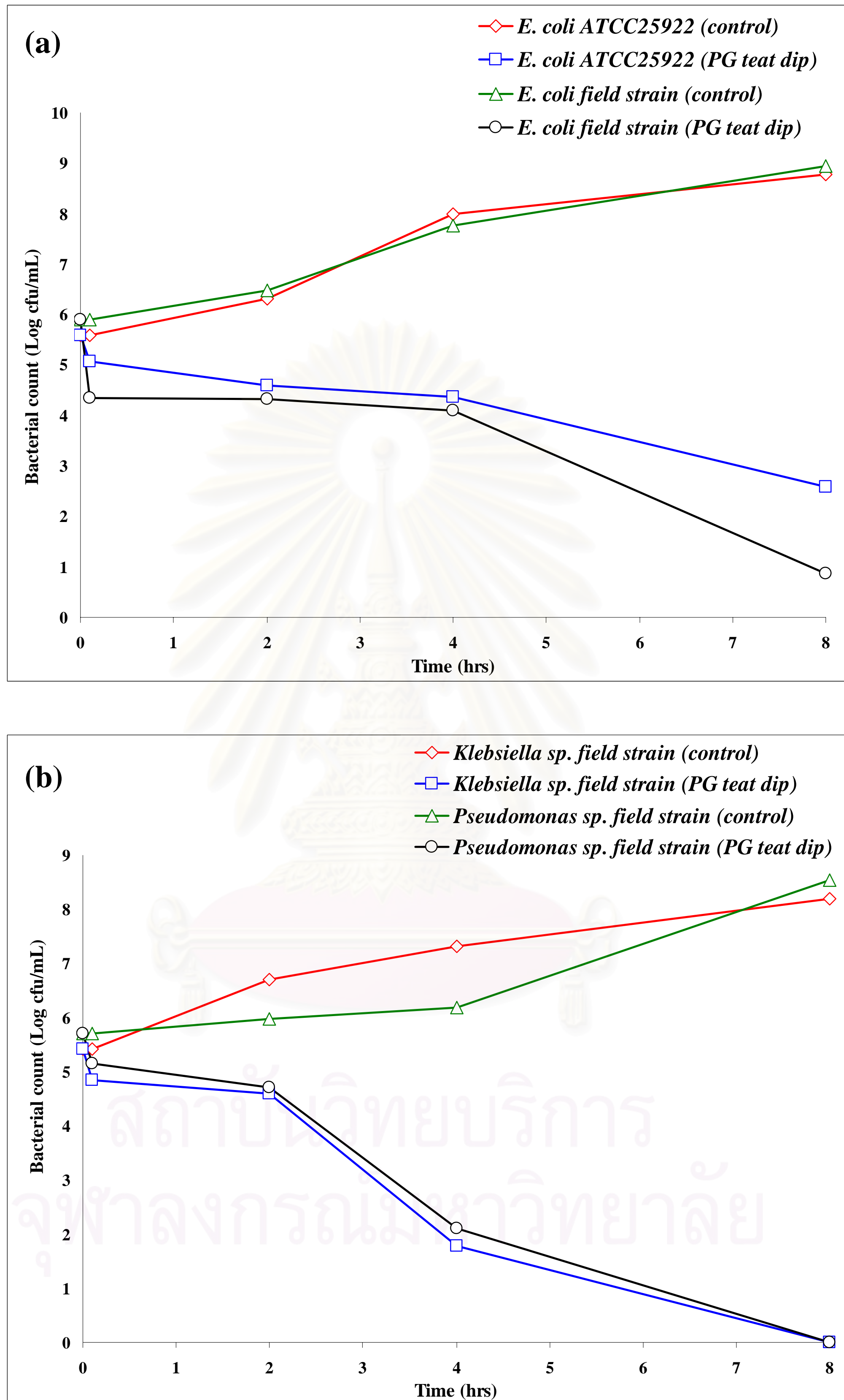


**Figure 14:** Time-kill analysis of Postmilking teat dip prepared from durian polysaccharide gel against mastitis-causing gram-negative bacteria; (a) *Escherichia coli*, (b) *Klebsiella* sp and *Pseudomonas* sp.

**Table 9**. The killing rate of PG teat dip against varieties of mastitis pathogens

| Mastitis pathogens | % Reduction of bacteria at times incubation (hrs) | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| *Staphylococcus aureus* ATCC 25923 | 74.41 | 80.81 | 84.54 | 99.98 |
| *Staphylococcus aurues* | 51.85 | 97.65 | 99.59 | 99.62 |
| *Staphylococcus chromogenes* | 65.56 | 84.13 | 99.97 | 100.00 |
| *Staphylococcus simulans* | 41.03 | 98.26 | 99.85 | 100.00 |
| *Streptococcus agalactiae* | 65.65 | 100.00 | 100.00 | 100.00 |
| *Streptococcus porcinus* | 88.12 | 100.00 | 100.00 | 100.00 |
| *Streptococcus uberis* | 94.90 | 100.00 | 100.00 | 100.00 |
| *Escherichia coli* ATCC 25922 | 69.03 | 89.45 | 83.88 | 99.90 |
| *Eschericha coli* | 97.27 | 97.33 | 98.45 | 100.00 |
| *Klebsiella* specie | 73.08 | 85.00 | 99.98 | 100.00 |
| *Pseudomonas* specie | 72.91 | 90.00 | 99.98 | 100.00 |

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### *In vitro* time kill analysis in narrow interval (minutes)

*In vitro* time kill analysis were carried out to assess antibacterial activity of post-milking teat antiseptic prepared from PG against five field isolates of mastitis-causing streptococci from dairy cows including *S. agalactiae, S. dysagalactiae, S. uberis, S. bovis* and *S. acidominimus*. The killing effect of PG teat dip against contagious mastitis-causing streptococci, *S. agalactiae* and *S. dysagalactiae*, was shown in Figure. 15a; whereas, environmental mastitis-causing streptococci including *S. uberis, S. bovis* and *S. acidominimus* was shown in Figure 15b. The result demonstrated that the bacterial counts of both *S. agalactiae* and *S. dysagalactiae* notably decreased within 1 minute and close to zero counts at 480 minutes exposured. The time killing profile of the three environmental mastitis-causing streptococci also illustrated the similar results. The percentages of bacterial cells reduction were summarized in Table 10. The result illustrated that the PG teat dip immediately killed all pathogens more than 90% within 1 minute and more than 99% within 60 minutes. The percentage reduction of *S. uberis* and *S. bovis* were completely 100%; whereas *S. agalactiae, S. dysagalactiae* and *S. acidominimus* were 99.99, 99.97 and 99.98, respectively, after 240 minutes exposured to PG teat dip. The other ingredients within preparation including propylene glycol 10% and glycerin 10% were confirmed no bactericidal effect to tested bacteria by using agar diffusion test (data not showed).

### Scanning electron microscopy

An appearance under scanning electron microscope of viable *S. agalactiae* in NSS without PG as culture control was shown in Figure 16a and 16b. The non-viable *S. agalactiae* after incubated with 2.5% PG at 37 °C for 8 hours clearly showed PG covered on the outer cell surface and aggregation of bacterial cell as showed in Figure 16c and 16d.

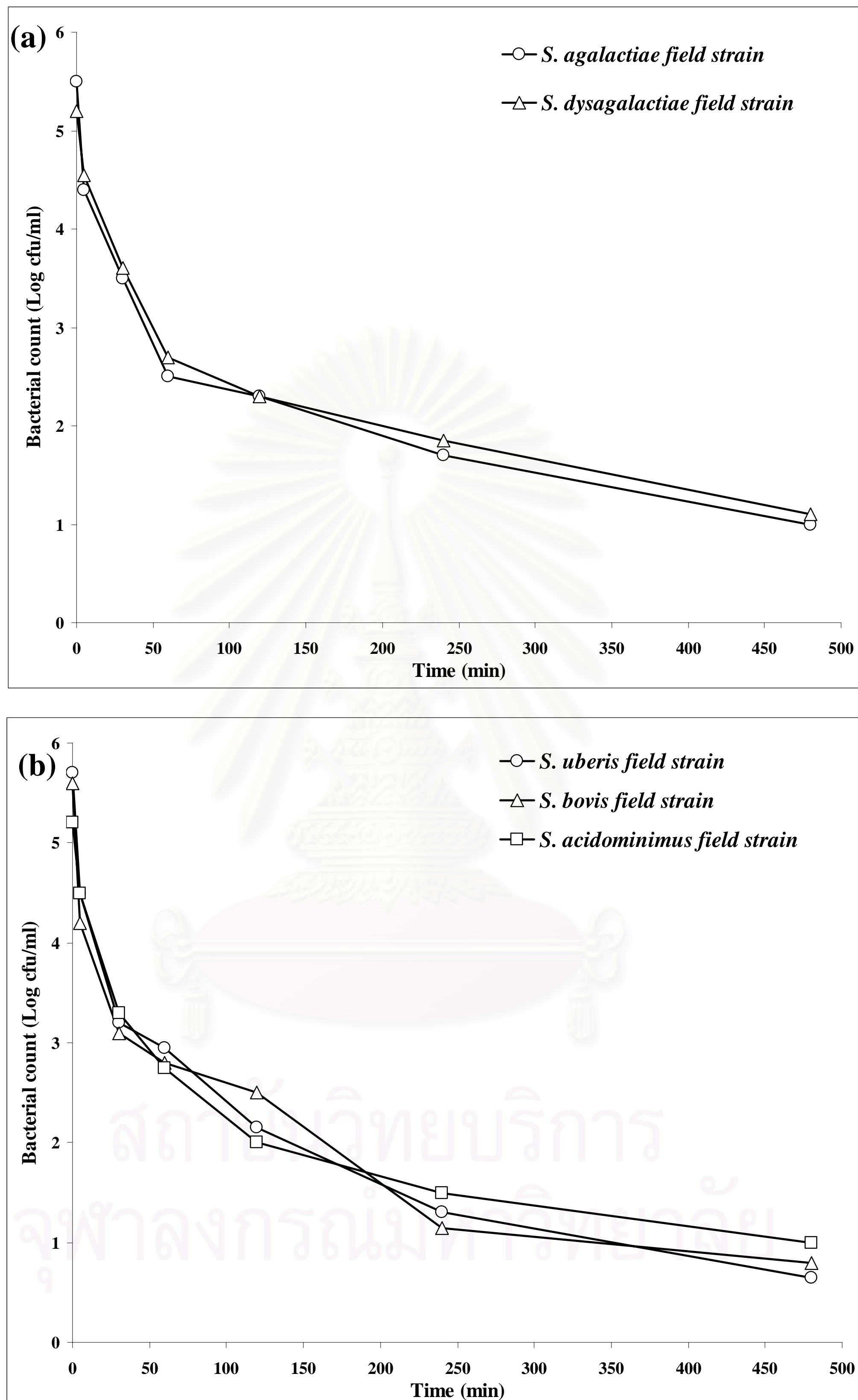


**Figure 15:** Time-kill analysis of Postmilking teat dip prepared from durian polysaccharide gel against *Streptococcus spp.*; (a) Contagious matitis pathogens; *Streptococcus agalactiae* and *Streptococcus disagalactiae* (b) Environmental mastitis pathogens; *Streptococcus uberis*, *Streptococcus bovis* and *Streptococcus acidominimus*

**Table 10**. The killing rate of PG teat dip against *Streptococci isolates* from mastitic cows

| **Mastitis pathogens** | **% Reduction of bacteria at times incubation (min)** | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **30** | **60** | **120** | **240** | **480** |
| *Streptococcus agalactiae* | 92.00 | 98.76 | 99.88 | 99.95 | 99.99 | 100.00 |
| *Streptococcus disagalactiae* | 92.35 | 96.64 | 99.76 | 99.87 | 99.97 | 99.99 |
| *Streptococcus uberis* | 90.67 | 99.71 | 99.85 | 99.97 | 100.00 | 100.00 |
| *Streptococcus bovis* | 96.67 | 99.74 | 99.89 | 99.94 | 100.00 | 100.00 |
| *Streptococcus acidominimus* | 93.75 | 98.88 | 99.72 | 99.94 | 99.98 | 99.99 |

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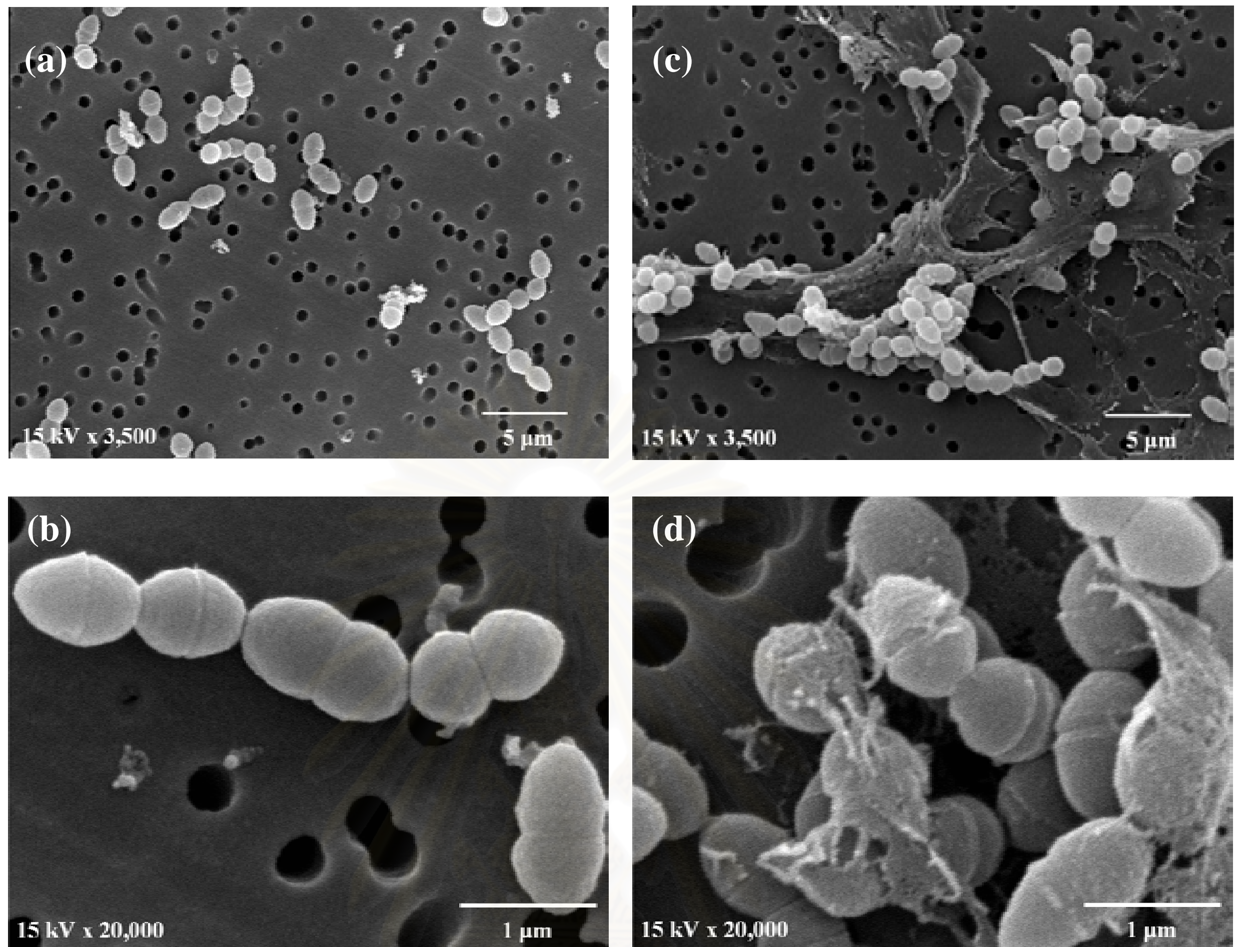


**Figure 16**. Scanning electron micrographs of *S. agalactiae* after incubation at 37°C for 8 hour; (a), Viable cells (x 3,500) in NSS without PG; (b) Viable cells (x 20,000) in NSS without PG; (c) Non-viable cell (x 3,500) with 2.5%PG; (d) Non-viable cells (x 20,000) with 2.5% PG.

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**4. Determination of the PG teat dip efficacy by experimental challenge**

The inhibitory efficacy of PG teat dip on new intramammary infections (IMI) was studied *in vivo* by experimental challenge procedure as described by the National Mastitis Council with the contagious mastitis pathogens (*S. aureus* and *S. agalactiae*). The experimental challenge with the tested bacteria was performed every day for 5 days. Each group of the 73 and 81 quarters eligible for IMI was used for controlling infectious trial against *S. aureus* or *S. agalactiae*, respectively. Infection data were collected during the trial period of evaluation an efficacy of the PG teat dip prepared for protection the udders against mastitis bacteria and the results were summarized in Table 11. The infection rates for *S. aureus* in undipped control and dipped groups were 6.85% and 0%, respectively; whereas, *S. agalactiae* were 6.17% and 0% in control and dipped groups, respectively. The PG teat dip treated group showed marked reduction in the number of new IMI caused by of both *S. aureus* and *S. agalactiae* at 100% ($P < 0.05$). The new IMI per 100 quarter days at risk for infection with *S. aureus* and *S. agalactiae* of the PG-treated cows were 1.02 and 0.8, respectively. Teat skin irritation was not observed during treated with PG teat dip through the study period.

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**Table 11**. Summary of the efficacy of PG teat dip against *Staphylococcus aureus* and *Streptococcus agalactiae* by experimental challenge

| **Challenge organisms** | **Experiment groups** | **Eligible quarters for new IMI** | **New IMI** | **Quarter day at risk for new IMI** | **New IMI per 100 quarter days at risk** | **Reduction rate (%)** |
|---|---|---|---|---|---|---|
| *S. aureus* | PG group | 73 | 0 | 505 | 0 | 100* |
| | Control group | 73 | 5 | 492 | 1.02 | |
| *S. agalactiae* | PG group | 81 | 0 | 640 | 0 | 100* |
| | Control group | 81 | 5 | 626 | 0.8 | |

* Significant difference from it control ($P < 0.05$)

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**5. Determination of the efficacy of PG teat dip by natural exposure**

The determination of the efficacy of postmilking teat dip prepared from PG from durian rinds for preventing mastitis infection was carried out in a commercial dairy farm in Rajchaburi province, Thailand in June 2007 according to natural exposure (protocol of NMC).

First experiment, the 33 lactating Holstein Friesian cows were randomly divided in three different experimental groups as showed in Table 12. The infection data were collected during the 7-days trial period and the results were summarized in Table 13. The un-dipped (negative control) group showed the highest incidence density of total infection followed by positive control group and PG teat dip group, the incidence density values were 4.17, 1.95 and 1.47, respectively. Incidence density of total infection in PG teat dip group was significantly lower than that of negative control group ($P$ = 0.037); on the contrary, bacterial free samples collecting in PG teat dip group was significantly higher than that of negative control group ($P$ = 0.037). Incidence density of total contagious pathogens was 0.76 in negative control group inasmuch as infection of *S. aureus* and *C. bovis* found only in negative control group with the same identical incidence density value of 0.38. The highest incidence density of environmental pathogens was found in negative control undipped group (incidence density of 3.41) followed by positive control group (incidence density of 1.95) and PG teat dip group (incidence density of 1.47). The incidence densities of CNS and environment streptococci in negative control group were 2.27 and 0.38 and also yeast-positive sample was 0.76 of incidence density. The incidence densities of CNS and environment streptococci in positive control group were 1.56 and 0.39; whereas, incidence densities of CNS and environment streptococci in PG teat dip group were the same identical incidence density value of 0.73. The rate difference between PG teat dip comparing with negative or positive control groups was summarized in Table 13. Negative value of the rate difference indicated that incidence density of PG teat dip group is higher than the other groups. The incidence density of the mastitis pathogens were lower in the PG teat dip group (1.47) than that of negative (4.17) and positive control group (1.95), except for incidence density of infection with streptococci other than *S. agalactiae*.

**Table 12.** Summary of experimental groups eligible for new IMI and quarter-days at risk in comparison with positive and negative control groups by natural exposure in experiment 1

| Experimental group | Number of experimental cows | Quarters eligible for new IMI | Quarter-days at risk |
|---|---|---|---|
| Iodophore teat dip (Positive control) | 11 | 37 | 259 |
| PG teat dip | 11 | 40 | 280 |
| Un-dipped (negative control) | 11 | 39 | 273 |

**Table 13**. Efficacy of PG teat dip in comparison with the iodophor (positive control) and undiped (negative control) groups by natural exposure (Experiment 1)

| Type of mastitis pathogens | New IMI per 100 quarter day at risk (Incidence desity) | | | PG teat dip bearing with positive control group | | PG teat dip bearing with negative control group | |
|---|---|---|---|---|---|---|---|
| | Iodophor group | PG group | Un-dipped group | Rate difference | *P* | Rate difference | *P* |
| **Total infection** | 1.95 | 1.47 | 4.17 | 0.48 | 0.449 | 2.70* | 0.037 |
| **Total contagious pathogens** | 0.00 | 0.00 | 0.76 | - | - | 0.76 | 0.241 |
| *Staphylococcus aureus* | 0.00 | 0.00 | 0.38 | - | - | 0.38 | 0.747 |
| *Streptococcus agalactiae* | 0.00 | 0.00 | 0.00 | - | - | - | - |
| *Corynebacterium bovis* | 0.00 | 0.00 | 0.38 | - | - | 0.38 | 0.747 |
| **Total environmental pathogens** | 1.95 | 1.47 | 3.41 | 0.48 | 0.449 | 1.94 | 0.103 |
| Coagulase-negative staphylococci | 1.56 | 0.73 | 2.27 | 0.83 | 0.301 | 1.54 | 0.123 |
| *Streptococcus spp.* | 0.39 | 0.73 | 0.38 | - 0.34 | 0.530 | -0.35 | 0.510 |
| *Escherichia coli* | 0.00 | 0.00 | 0.00 | - | - | - | - |
| *Pseudomonas spp.* | 0.00 | 0.00 | 0.00 | - | - | - | - |
| Yeast | 0.00 | 0.00 | 0.76 | - | - | 0.76 | 0.241 |
| **No bacteria growth sample** | 98.05 | 98.53 | 95.83 | 0.48 | 0.499 | 2.70* | 0.037 |

* = significant differences ($P \leq 0.05$).

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Second experiment, the three experimental groups in 51 lactating Holstein Friesian cows were used for the preventive trial showed in Table 14. The infection data were collected during the 4-week trial period and the results are summarized in Table 15. The two dips combination group (iodophor teat dip followed with PG teat dip) showed surprisingly highest incidence density of total infection at 4.63 followed by in iodophor teat dip (positive control) group at 1.00 and PG teat dip group showed the lowest value at 0.85. Bacterial-free samples collecting from PG teat dip group, positive control and combination group were 97.86, 97.49 and 95.37%, respectively. The incidence density of total contagious pathogens was not found in positive control and PG teat dip groups except in combination group was at 0.46 due to *C. bovis* infection. The incidence density of total environmental pathogens found in this experiment was 1.00, 0.85 and 2.78 in positive control, PG teat dip and the two dips combination groups, respectively. The incidence density of infection with CNS in positive control, PG teat dip and the two dips combination groups was 1.51, 1.28 and 1.39; whereas, incidence density of infection with streptococci other than *S. agalactiae* was 0.55, 0.85 and 0.46, respectively. The new IMI by *Escherichia coli* occurred in the two dips combination groups (incidence density of 2.31); whereas, *Pseudomonas spp.* infection was found only in positive control group (incidence density of 0.05). The rate difference between PG teat dip group compared with positive control or two dips combination groups were summarized in Table 15. The incidence density of the mastitis pathogens in the PG teat dip group was lower than that of positive and two dips combination groups, in particularly, incidence density of *E. coli* infection (P = 0.02). But incidence density of infection with streptococci other than *S. agalactiae* in the PG teat dips was the highest.

**Table 14.** Summary of experimental groups eligible for new IMI and quarter-weeks at risk in comparison with positive control and two teat dip combination groups by natural exposure in experiment 2

| Type of treatment | Number of experimental cows | Quarters eligible for new IMI | Quarter-weeks at risk |
|---|---|---|---|
| Iodophor teat dip (Positive control) | 16 | 53 | 200 |
| PG teat dip | 18 | 62 | 236 |
| Iodophore teat dip followed by PG teat dip | 17 | 54 | 200 |

**Table 15**. Efficacy of PG teat dip in comparison with the iodophor (positive control) and two teat dips combination groups by natural exposure (Experiment 2)

| **Type of mastitis pathogens** | **New IMI per 100 quarter week at risk (Incidence desity)** | | | **PG teat dip bearing with positive control group** | | **PG teat dip bearing with two teat dips combination group** | |
|---|---|---|---|---|---|---|---|
| | **Iodophor group** | **PG group** | **Combination group** | **Rate difference** | ***P*** | **Rate difference** | ***P*** |
| **Total infection** | 2.51 | 2.13 | 4.63 | 0.38 | 0.526 | 2.5 | 0.081 |
| **Total contagious pathogens** | 0.00 | 0.00 | 0.46 | - | - | 0.46 | 0.466 |
| *Staphylococcus aureus* | 0.00 | 0.00 | 0.00 | - | - | - | - |
| *Streptococcus agalactiae* | 0.00 | 0.00 | 0.00 | - | - | - | - |
| *Corynebacterium bovis* | 0.00 | 0.00 | 0.46 | - | - | 0.46 | 0.466 |
| **Total environmental pathogens** | 1.00 | 0.85 | 2.78 | 0.15 | 0.455 | 1.93 | 0.108 |
| Coagulase-negative staphylococci | 1.51 | 1.28 | 1.39 | 0.23 | 0.583 | 0.11 | 0.592 |
| *Streptococcus spp.* | 0.50 | 0.85 | 0.46 | -0.35 | 0.559 | -0.39 | 0.552 |
| *Escherichia coli* | 0.00 | 0.00 | 2.31 | - | - | 2.31* | 0.020 |
| *Pseudomonas spp.* | 0.50 | 0.00 | 0.00 | 0.5 | 0.461 | - | - |
| Yeast | 0.00 | 0.00 | 0.00 | - | - | - | - |
| **No bacteria growth sample** | 97.49 | 97.86 | 95.37 | -0.37 | 0.526 | 2.49 | 0.081 |

* = significant differences ($P \leq 0.05$).

## 6. Determination of the effect of PG on bovine mammary leukocytes (*in vitro*)

### Isolation of bovine mammary leukocytes

The bovine leukocytes were isolated from mammary glands by stripping out secretion under sterile condition. Mammary gland lavage fluids were confirmed for bacterial-free purity by inoculation the samples onto TSA plate containing 5% sheep blood and the cell pellets were assessed for cells viability by trypan blue staining. The result showed that all lavage fluid samples were bacteriologically negative and more than ninety percentages of viable cells was obtained.

### Exposure method

The cell suspensions ($5 \times 10^5$ cells/ml in complete DMEM) were mixed together with PG solution to make 0.5, 1 and 2.5% PG (w/v) of final concentration or distill water as vehicle control and incubated at 37 °C for 30 minutes. After reactions were stopped, the cell pellets were used for cytotoxic and phagocytosis assays.

### *In vitro* cytotoxicity assay

#### XTT reduction test

To examine the PG-induced cytotoxicity, cell viability for bovine mammary leukocytes was determined using XTT reduction assay. Cell viability was represented by the detection of enzyme mitochondrial dehydrogenase activity. The cytotoxic effect of PG on bovine mammary leukocytes evaluated by XTT reduction assay was summarized in Figure 17. The results showed that PG induced cells cytotoxicity in a concentration-dependent manner. The PG induced percentage of cells cytotoxicity to 27.60±2.81% after exposed to 2.5 %PG which was significantly higher than that of the 1 and 0.5% PG and the vehicle control (Figure 17). However, cell cytotoxicity with PG at 0.5 and 1% was not significantly different from that of the vehicle control. PG at the low concentration ≤ 1% was not toxic to the bovine mammary leukocytes by XTT reduction test.

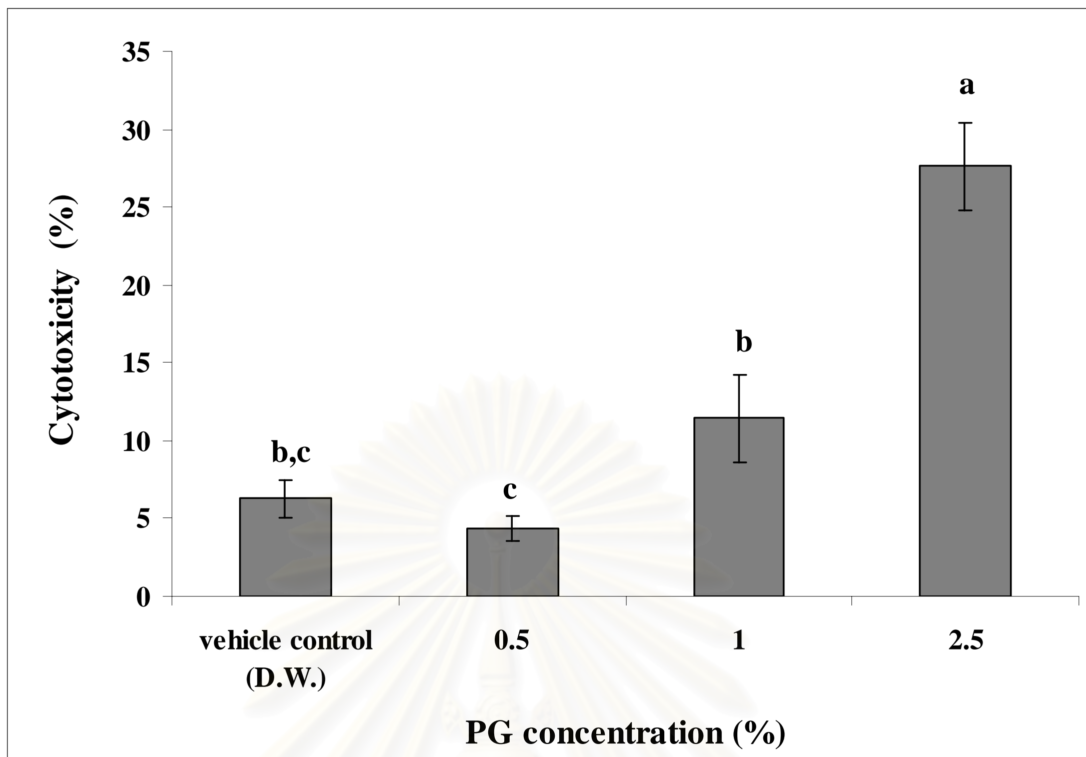


**Figure 17** Percentage cytotoxicity (mean ± S.E.) of bovine mammary leukocytes in ten independent experiments with duplicate replication determined by using XTT reduction assay. a, b and c = significant differences ($P \leq 0.05$).

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**Flow cytometric method with PI**

The samples were analyzed for fragmented DNA content as an indicator of cell death after reacted with PI by flow cytometer was shown in Figure 18. The cytotoxic effect of PG on bovine mammary leukocytes was summarized in Figure 19. The percentage of cytotoxicity on bovine mammary leukocytes after exposured to 0 (control), 0.5, 1 and 2.5%PG (w/v) were 4.87±0.56, 15.22±1.27, 25.35±1.34 and 70.08±3.97, respectively. PG reduced the viable cells in a concentration-dependent manner, the 2.5%PG concentration exhibited significantly highter cytotoxicity than that of the 1 and 0.5 %PG and control (Figure 19). PG at the low concentration at 0.5 and 1%PG showed low cytotoxic effect to bovine mammary leukocytes by this assay.

***In vitro* phagocytosis assay by NBT reduction method**

The *in vitro* phagocytic assay by NBT reduction method was carried out to determine the phagocytic effect of PG in bovine mammary leukocytes. The effect of PG on phagocytic activity of bovine mammary leukocytes was evaluated and summarized in Figure 20. The results indicated that bovine mammary leukocytes were more activated after exposured to 1% (w/v) PG for 30 min, the percentages of phagocytosis was 38.66±4.65, than that of the value compared -4.83±1.41% in untreated control. However, 0.5 and 2.5%PG exhibited phagocytic activity on bovine mammary leukocytes, the percentage of phagocytosis were 4.97±3.87 and -10.87±2.95%. respectively, which were not significantly different from the control (Figure 20).

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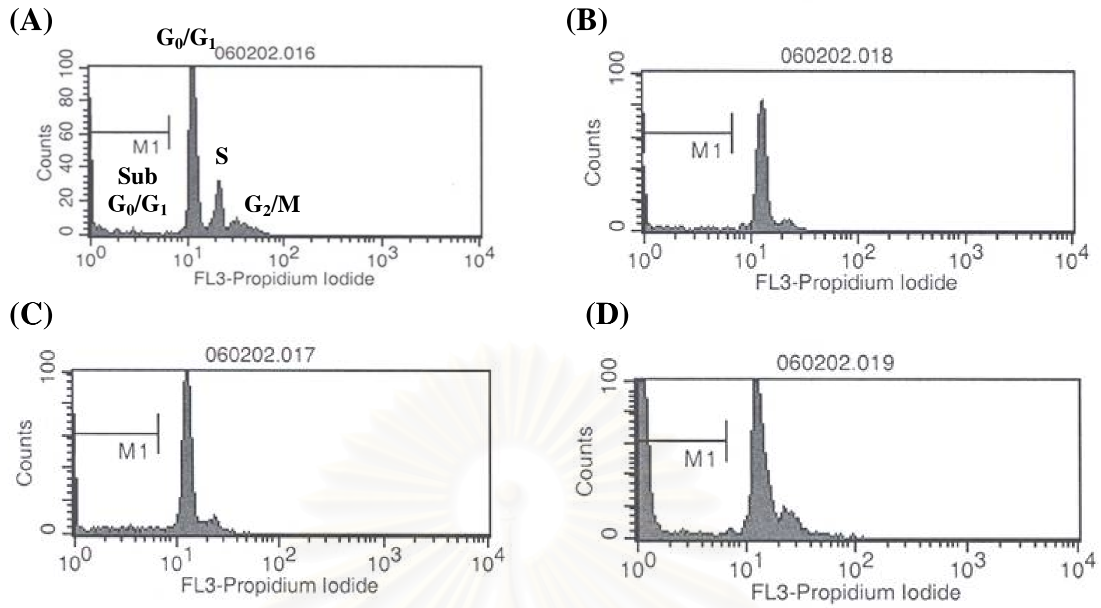


**Figure 18** Apoptotic cell death accessed according to the percentage of cells with sub $G_0/G_1$ peak as representative histogram of PG-treated bovine mammary leukocyes compared with control A) D.W. (control); B) 0.5%PG; C) 1%PG and D) 2.5%PG

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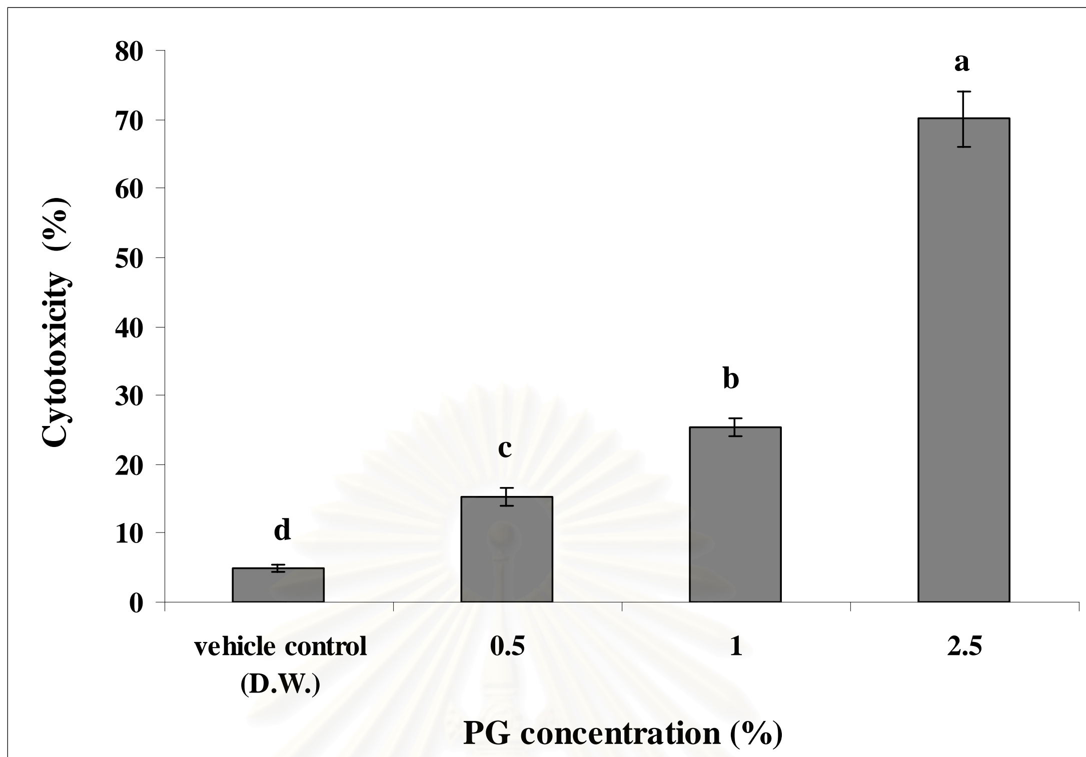


**Figure 19.** Percentage cytotoxicity (mean ± S.E.) of bovine mammary leukocytes in ten independent experiments with duplicate replication determined by flow cytometric with propidium iodide. a, b, c and d = significantly different ($P \leq 0.05$)

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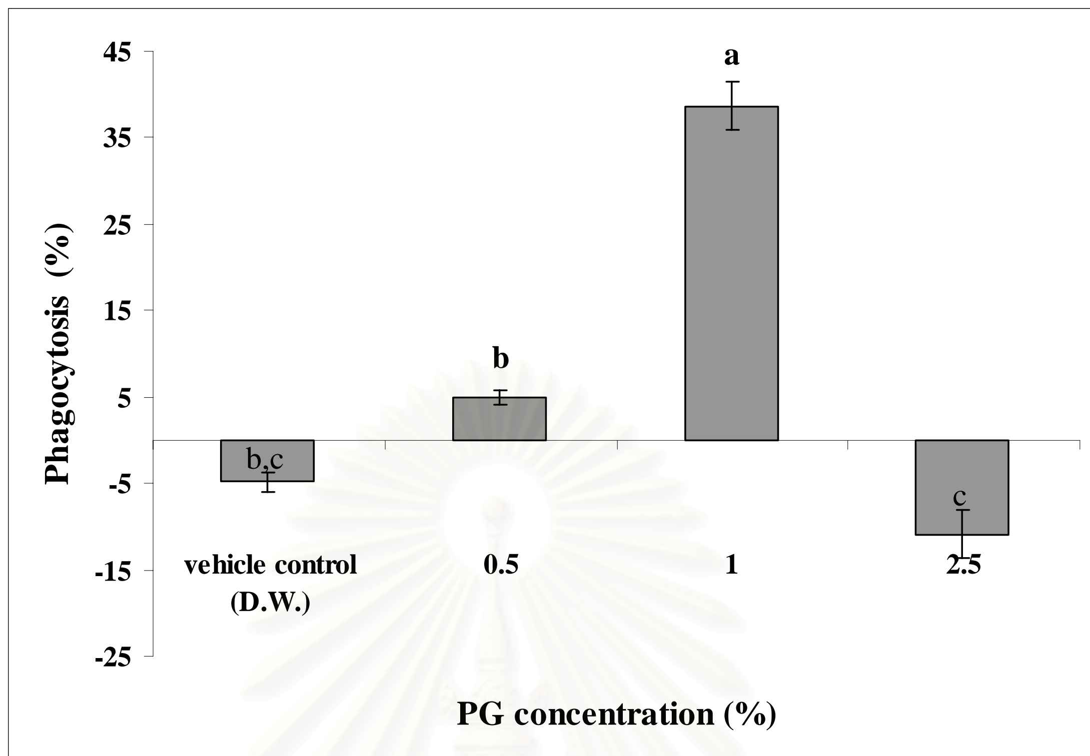


**Figure 20** Percentage of phagocytic activity (mean ± S.E.) of bovine mammary leukocytes in ten independent experiments with duplicate replication determined by NBT reduction assay. a, b and c = significantly differences ($P \leq 0.05$).

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**Scanning electron microscopy**

The examination of the morphological appearance of macrophages after exposed to the various concentrations of PG was carried out by scanning electron microscopy. The scanning electron micrographs were showed in Figure 21. The picture showed the reactive morphology of bovine mammary macrophages, marked pseudopodal formation was observed in sample treated with 1%PG (Figure 21c) compared to the non-reactive cells in control (Figure 21a). The trifling pseudopodia observed in macrophages exposed to 0.5%PG (Figure 21b) but the pseudopodal formation of macophages were inhibited with 2.5%PG treatment (Figure 21d). These results were corresponding with the phagocytosis examination in NBT reduction method.

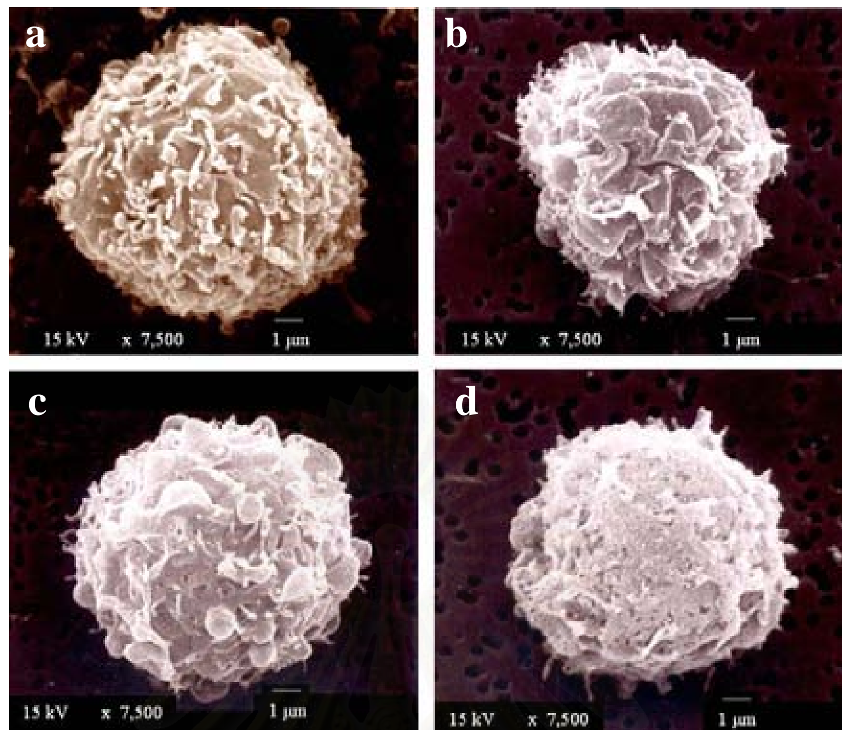


**Figure 21.** Scanning electron micrograph of bovine mammary macrophages treated with PG (x 7,500); (a), Distill water; (b), 0.5% PG; (c), 1% PG and (d), 2.5% PG

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**7. Determination of immunomodulatory effect of PG on bovine udder**

**The experimental cows**

The non-lactating cows in dry period were used to evaluate the effect of PG on bovine mammary gland immunity. The mammary gland secretion samples of dried cows were collected and examined for being free of infection before the experiment start**.** The uninfected cows that produced bacterial-negative secretion samples were used in this experiment.

**Preparation of PG test solution**

Solution of 1%PG (w/v) was freshly prepared without endotoxin. The contaminated bacteria were examined, No growth of bacteria on both TSA plate containing 5% sheep blood and PCA plate were observed which was indicated the sterility of tested solution.

**Udder preparation and intramammary infusion**

The udder quarters were randomly selected from three non-lactating cows. Each treated group of 3 udders was infused through the teat canal with 12.5, 25 and 62.5 ml of 1% PG, respectively, and PBS was used in control group by using syringe with plastic infusion cannula. The treated cows were neither suffering during insertion of teat cannula nor abnormal acute symptoms of mammary gland.

**Clinical examinations**

The clinical examinations were performed every day during the trial for 15 days. The clinical parameters including rectal temperature, heart rate, pulse frequency, respiratory rate and rumen contraction rate were not significantly different between treated group and untreated control group. Both systemic symptoms and udder change were not observed during the study period. The slightly sticky sample and increasing in udder secretion volume were observed at 24 hours and mostly disappeared at 48 hours after infusion of PG test solution.

**Blood collection and analysis**

The EDTA-blood samples were determined total white blood cells (WBC) which was performed in automatic instrument (Cell-DynR3500) and the differential blood count was examined by staining a sample blood smear with Wright's stain. The total WBC was shown in Figure 22. The result demonstrated that the total WBC count was significantly different at day 8 post-infusion in comparison with three normal cows in control. The proportions of blood leukocyte populations were shown in Table 16. The proportions of polymorphonuclear neutrophil (PMN), eosinoplhil, monocyte and lymphocyte were not significantly different between PG treated group and untreated control group; however, the basophil and band neutrophil were not found. The differential absolute counts of PG treated group were calculated form proportions of each type of leukocytes and shown in Figure 23. The number of neutrophil from initial counts (9,164.67±6,356.00) was dropped after intramammary infusion of PG from day 5 post-infusion to 2,258.67±471.33 and 1,727.67±524.75 at day 8, whereas eosinoplhil, monocyte and lymphocyte were not significantly changed.

**Udder secretion collection and analysis**

Udder secretion samples were collected from the PG treated cows on the day before infusion (day 0) and on day 1, 2, 5, 8 and 15 post-infusion. A volume of 10 ml of mammary gland secretion was recovered by immediate manual aspiration into sterile container and kept at 4 °C. All udder secretion samples were examined microbiologically, quantity of bovine lactoferrin, Somatic Cell Count (SCC), and analysis for immunophenotyping of leukocyte cell surface markers by flow cytometer

The result of bacterial examinations, demonstrated that contaminated bacteria were not detected through out the studies.

Total SCC in udder secretion samples were monitored by using an Electronic Coulter Counter ZM®. The log SCC values (Mean±SE) of each experimental group was plotted vs. time (days) as shown in Figure 24.

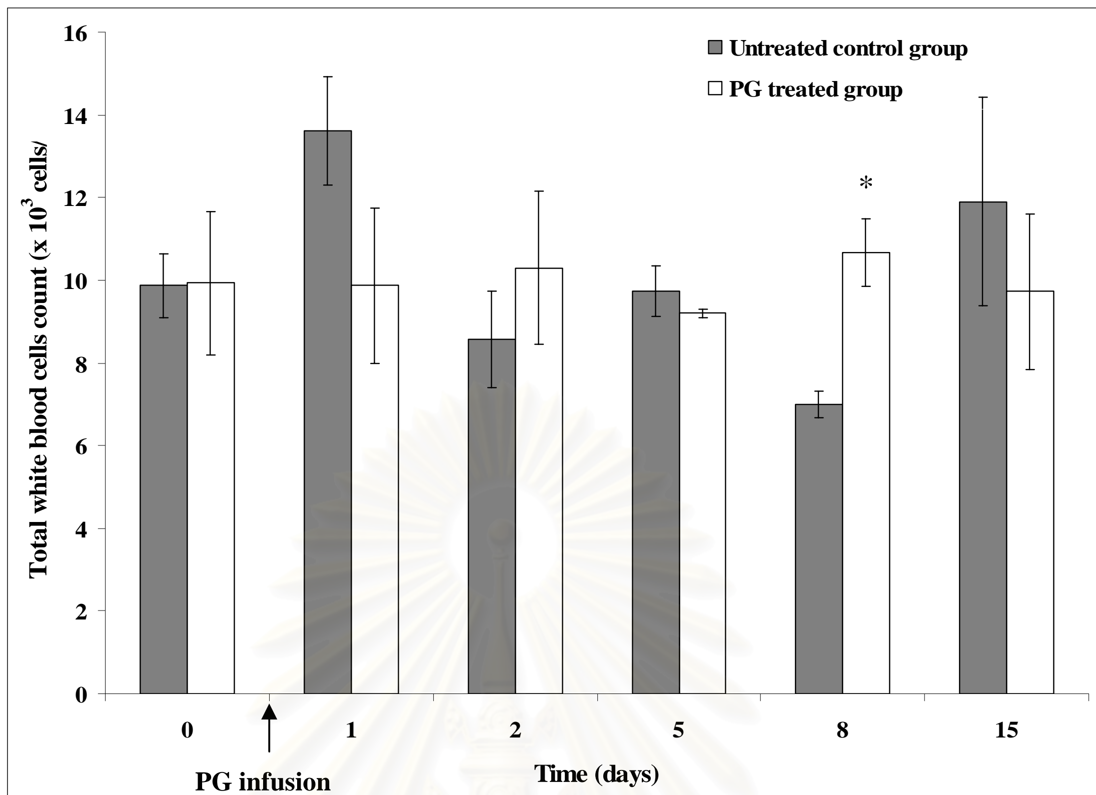


**Figure 22:** Total white blood cell count (mean ± SE) of blood in non-lactating cows at day 0 before intramammary infusion of PG and 1-15 days post-infusion. * = significantly different from its control ($P \leq 0.05$).

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**Table 16:** Proportion of blood leukocytes in untreated control cows and PG-treated cows at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| Blood leukocytes | | Proportion of blood leukocytes (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Day before infusion | Day after infusion | | | | |
| | | 0 | 1 | 2 | 5 | 8 | 15 |
| Neutrophil | Control | 33.33±4.18 | 48.00±7.00 | 46.00±7.77 | 29.67±3.48 | 29.00±1.53 | 39.00±5.55 |
| | Treatment | 25.67±5.46 | 27.67±7.22 | 27.33±8.57 | 24.67±5.33 | 17.00±5.69 | 31.00±8.02 |
| Eosinophil | Control | 7.33±2.96 | 6.67±3.84 | 7.67±1.76 | 6.67±2.40 | 6.67±1.86 | 7.00±3.79 |
| | Treatment | 9.67±2.33 | 7.00±3.51 | 7.33±1.20 | 8.67±3.33 | 4.67±2.73 | 6.00±3.16 |
| Lymphocyte | Control | 53.00±5.51 | 39.33±4.18 | 41.67±6.17 | 68.00±5.51 | 58.00±4.51 | 48.00±4.51 |
| | Treatment | 60.00±5.57 | 60.67±10.73 | 60.33±7.54 | 60.67±8.67 | 74.00±8.54 | 57.33±10.41 |
| Monocyte | Control | 6.33±0.33 | 6.00±0.58 | 4.33±1.33 | 5.67±0.88 | 6.00±1.15 | 6.00±0.58 |
| | Treatment | 4.33±1.20 | 4.67±0.88 | 4.00±0.58 | 6.00±0.01 | 4.33±1.20 | 5.67±0.33 |

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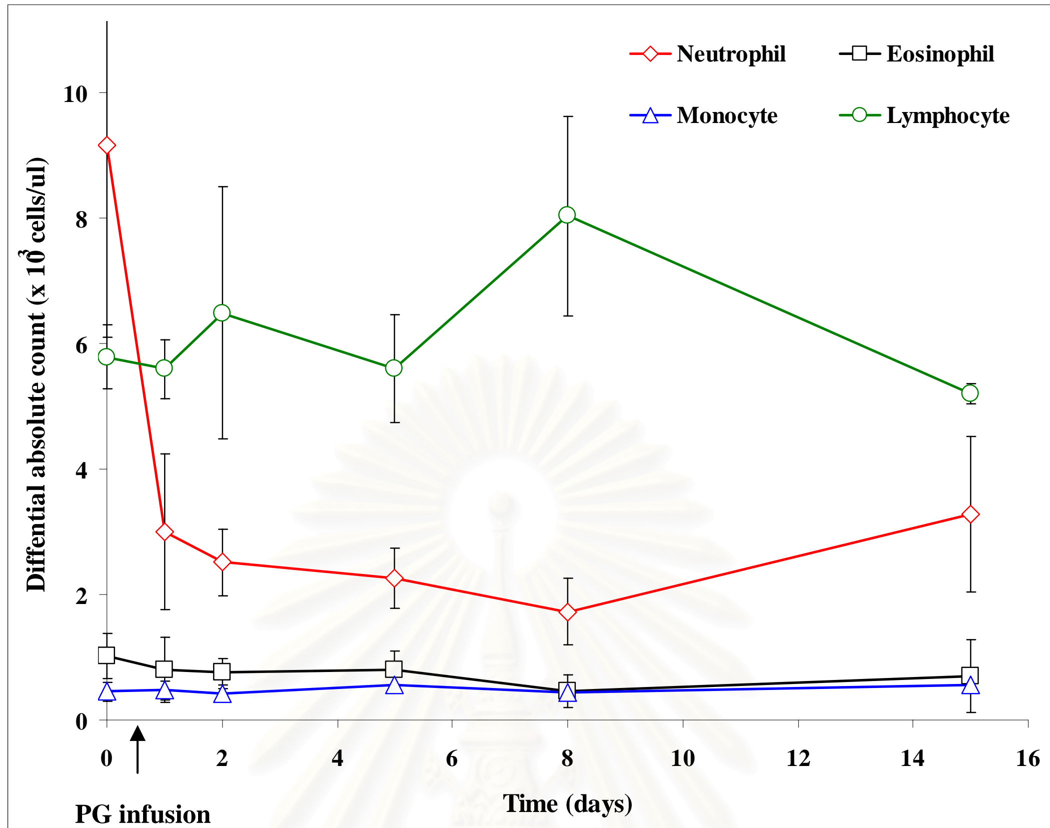


**Figure 23:** Deferential absolute count (mean ± SE) of blood in non-lactating cows at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

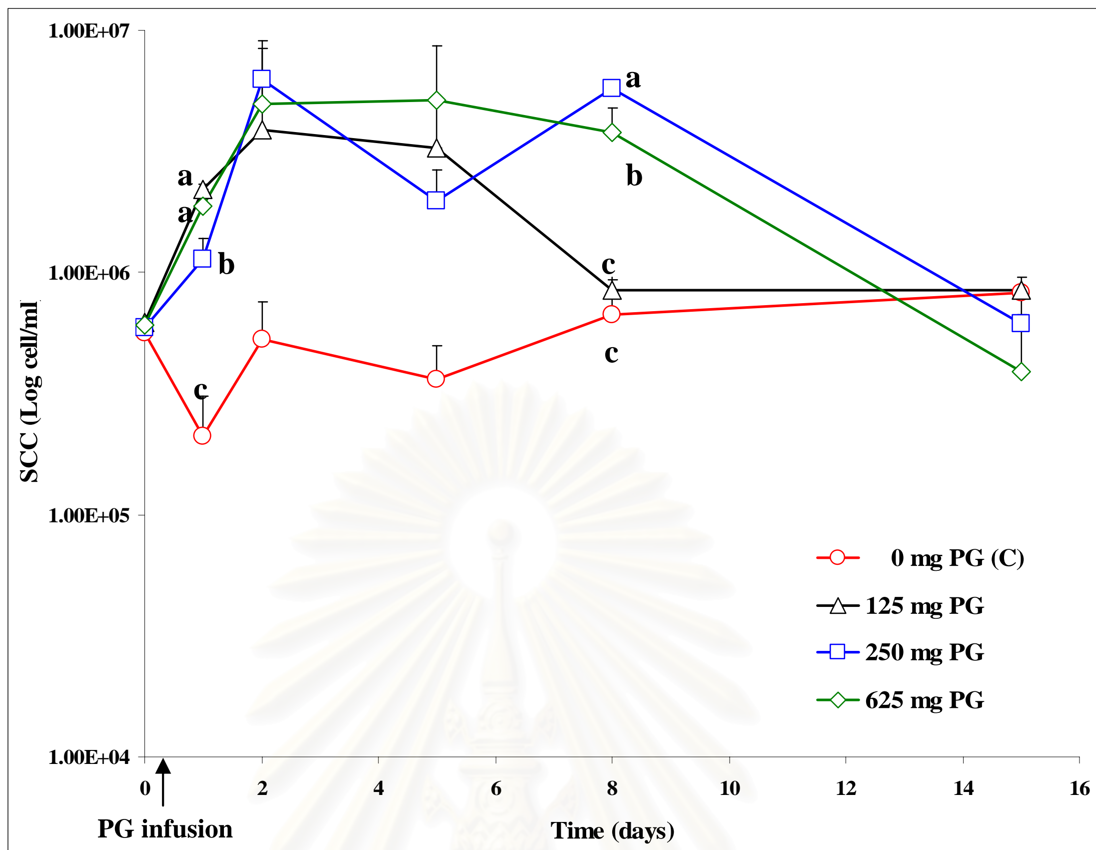


**Figure 24:** Somatic cell count (mean+SE) of udder secretion samples in non-lactating cows at day 0 before intramammary infusion of PG and 1-15 days post-infusion. a, b and c = significantly different ($P \leq 0.05$).

The result demonstrated that the mean value of SCC were not different in 0, 125, 250 and 625 mg PG groups at pre-infusion (day 0) in which log cells/ml were 5.75±0.14, 5.79±0.13, 5.77±0.05 and 5.78±0.1, respectively. After 1 day of intrammamary infusion, the log cells/ml of SCC at 125, 250 and 625 mg PG-treated groups were significantly increased to 6.34±0.02, 6.05±0.06 and 6.28±0.06, respectively, which were higher than the value of 5.32±0.13 log cells/ml in control group, the high level of SCC existed to day 5 post-infusion. The log cells/ml of SCC at dose 250 mg and 625 mg PG-treated groups were 6.76±0.21 and 6.58±0.03, respectively, which were significantly higher than that of the control group (5.83±0.09) on day 8, however, SCC level log cells/ml of 5.93±0.21 in 125 mg PG-treated groups was drop to the value as control group. The SCC in PG treated group were decline from day 8 to the same level of control group at day 15. The SCC level in treatment groups was a dose-independent manner.

The surface markers were analyzed by using direct immunofluorescence labeling with flow cytometer. Fifth thousand leukocytes from each experimental quarter samples were used to identify their expression of CD45 molecule bearing with CD14 or MHC class II molecules as showed in Figure 25. The CD14 molecule is not only macrophage marker but also plays an important role as a receptor for lipopolysaccharides (LPS). The expression of MHC class II molecules indicates a stimulation of the antigen-presenting activity of the mammary cells.

The percentages of CD14-positive leukocytes were shown in Table 17. The result demonstrated that percentages of CD14-positive leukocytes between control group and PG-treated groups were closely related. The average of CD14-positive leukocytes percentages in all treated and control groups were decreased from its initial counts during day 1 to 2 post-infusion but recovered on day 5. At day 8 post-infusion, both 125 mg and 250 mg PG-treated group showed the rising of CD14-positive leukocytes but not significantly higher than that of control group. The proportion of CD14-positive leukocytes was declined to level of control group on day 15. The absolute value of each samples calculated by percentages of CD14-positive leukocytes compared with SCC in PG treated group was higher than that of the control from day 1 to 8, showed in Figure 26.

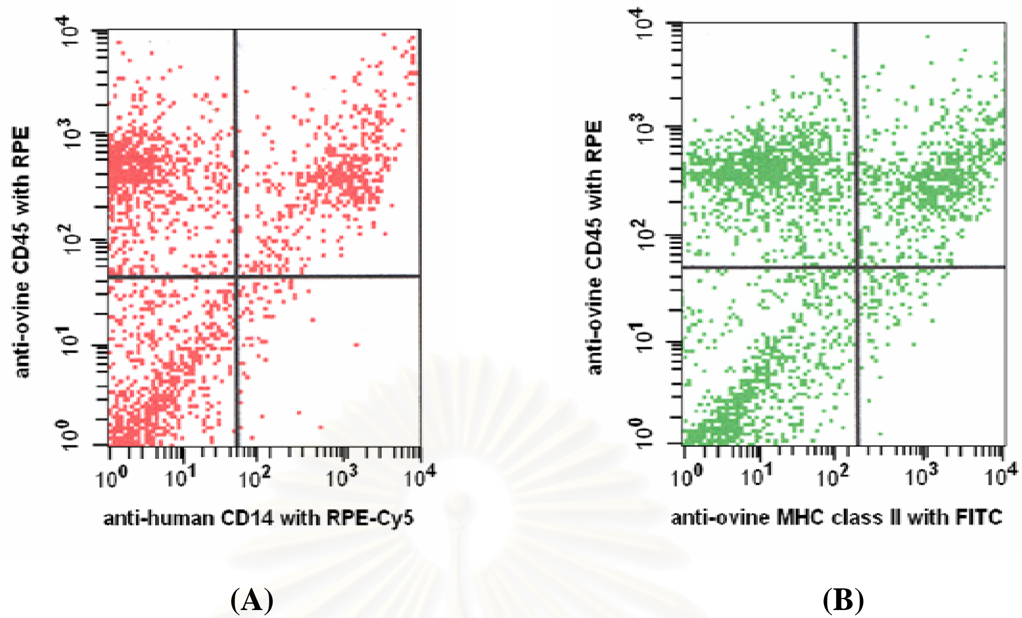


**Figure 25**. The 2-dimensions dot pot of different populations of bovine mammary leucocytes; (A) CD45 RPE-conjugated antibody bearing with CD14 RPE Cy5-conjugated antibody; (B) CD45 RPE-conjugated antibody bearing with MHC class II FITC-conjugated antibody.

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**Table 17.** Percentage of CD14-positive leukocyte (mean±SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| PG infusion (days) | Proportion of CD14-positive leukocyte (%) | | | |
|---|---|---|---|---|
| | Dosage of 1%PG solution (mg) | | | |
| | **0** | **125** | **250** | **625** |
| Pre-infusion 0 | 27.74±5.47 | 39.49±13.89 | 32.08±13.73 | 33.97±9.99 |
| Post-infusion 1 | 7.82±1.05 | 15.61±5.84 | 7.95±1.72 | 8.89±4.71 |
| 2 | 9.27±2.85 | 9.04±0.79 | 13.05±4.68 | 5.75±0.73 |
| 5 | 18.66±16.30 | 21.28±11.66 | 23.40±10.28 | 24.83±9.96 |
| 8 | 16.45±9.13 | 35.76±9.67 | 25.01±9.96 | 15.41±3.39 |
| 15 | 15.84±7.14 | 19.22±5.95 | 28.59±9.47 | 14.84±5.29 |

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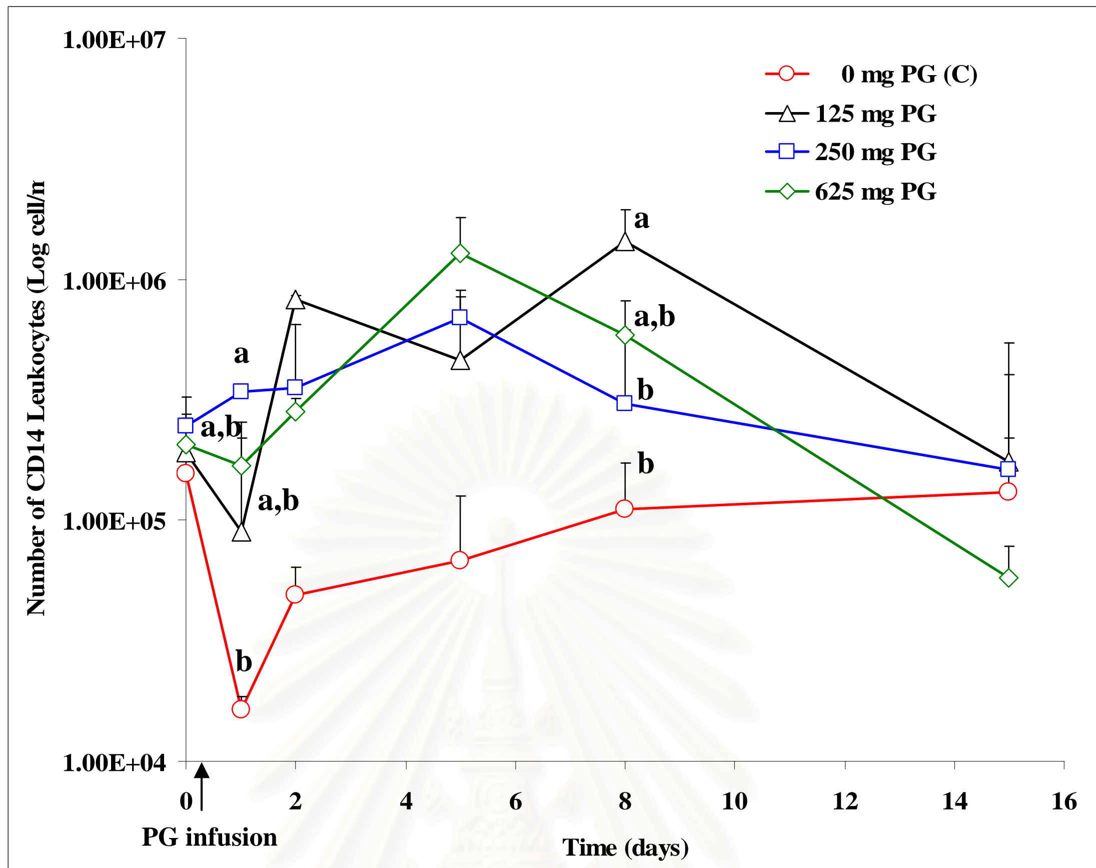


**Figure 26.** Number of CD14-positive leukocyte (mean+SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion. a and b = significantly different ($P \leq 0.05$).

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The number of CD14-positive leukocytes in 125 mg PG-treated group was significantly higher than that of control group after PG infusion for 8 days, before declined to the level in control group.

The percentages of MHC class II-positive leucocytes were shown in Table 18. The percentages of MHC class II-positive leucocytes between control and PG-treated groups were not significantly different throughout the studies. The absolute value of each sample was calculated by percentages of MHC class II-positive leucocytes in comparison with SCC was shown in Figure 27. The number of MHC class II -positive leucocytes in 125 mg PG-treated groups was significantly higher than that of the control group after PG infusion to day 5 and declined to normal control at day 15 as demonstrated in Figure 27.

The bovine lactoferrin concentrations (bLf) in udder secretion samples were determined by goat anti-bovine lactoferrin antibody coated ELISA plates. The concentrations of bLf were calculated by using linear regression of various standard concentrations (Bovine lactoferrin calibrator). The ELISA plates were measured the absorbance at 450 nm of products from horseradish peroxidase (HRP) catalyzed TMB-substrates by using a microtiter plate reader. The result demonstrated in Figure 28 that bLf level at day 0 before intramammary infusion of PG and 1-5 days were in close proximity. Till day 8 of 125 mg PG post-infusion, bLf level was higher than that of the other treated and control group. The concentration bLf seem retrogression to normal level at day 15 post-infusion.

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**Table 18:** Percentage of MHC class II-positive leukocyte (mean±SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| **PG infusion (days)** | **Proportion of MHC class II -positive leukocyte (%)** | | | |
|---|---|---|---|---|
| | **Dosage of 1%PG solution (mg)** | | | |
| | **0** | **125** | **250** | **625** |
| Pre-infusion 0 | 48.33±0.29 | 48.88±5.66 | 50.01±5.60 | 47.17±1.75 |
| Post-infusion 1 | 25.75±18.74 | 27.20±11.17 | 7.95±1.72 | 28.27±13.88 |
| 2 | 47.29±23.19 | 45.62±21.54 | 41.52±12.56 | 25.87±19.86 |
| 5 | 35.45±19.36 | 40.78±17.46 | 43.30±17.91 | 45.40±15.51 |
| 8 | 21.10±13.84 | 36.92±15.51 | 51.30±15.51 | 19.86±3.37 |
| 15 | 29.09±12.40 | 38.36±11.12 | 35.15±6.97 | 27.13±4.42 |

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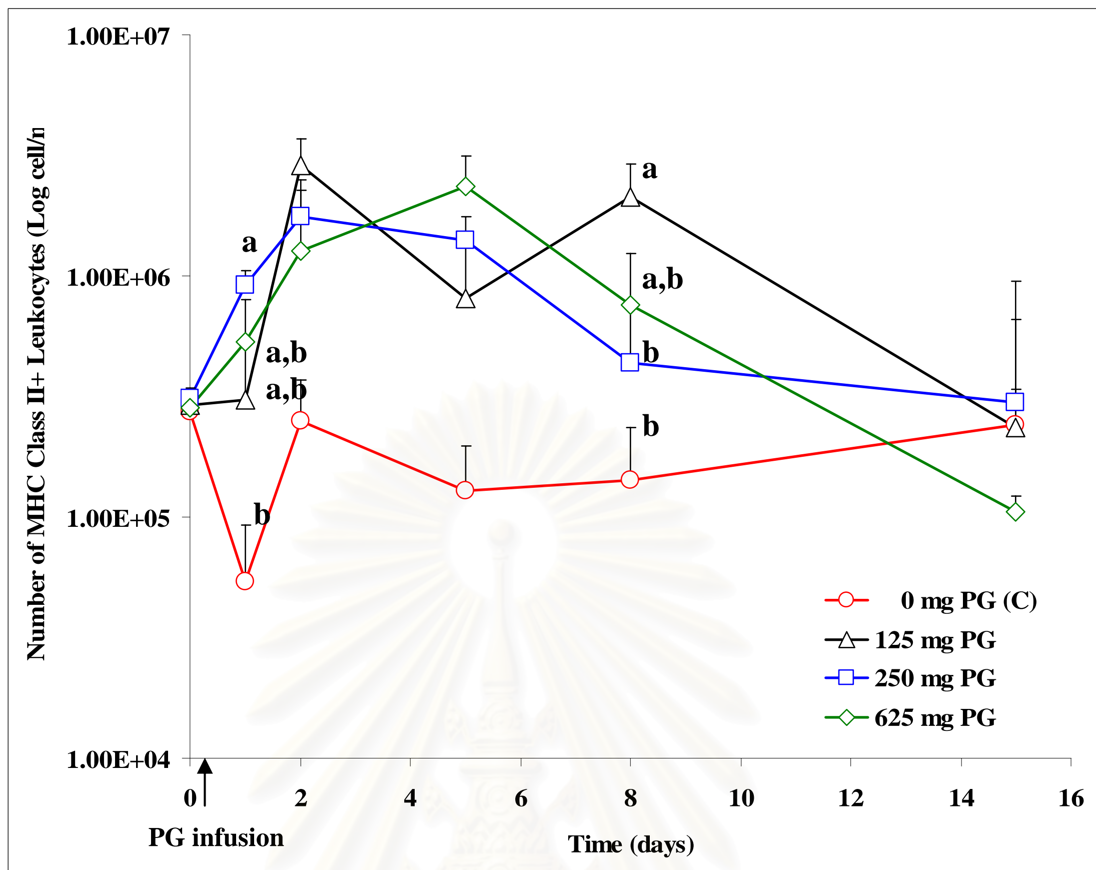


**Figure 27**. Number of MHC class II-positive leukocyte (mean+SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion. a and b = significantly different ($P \leq 0.05$).

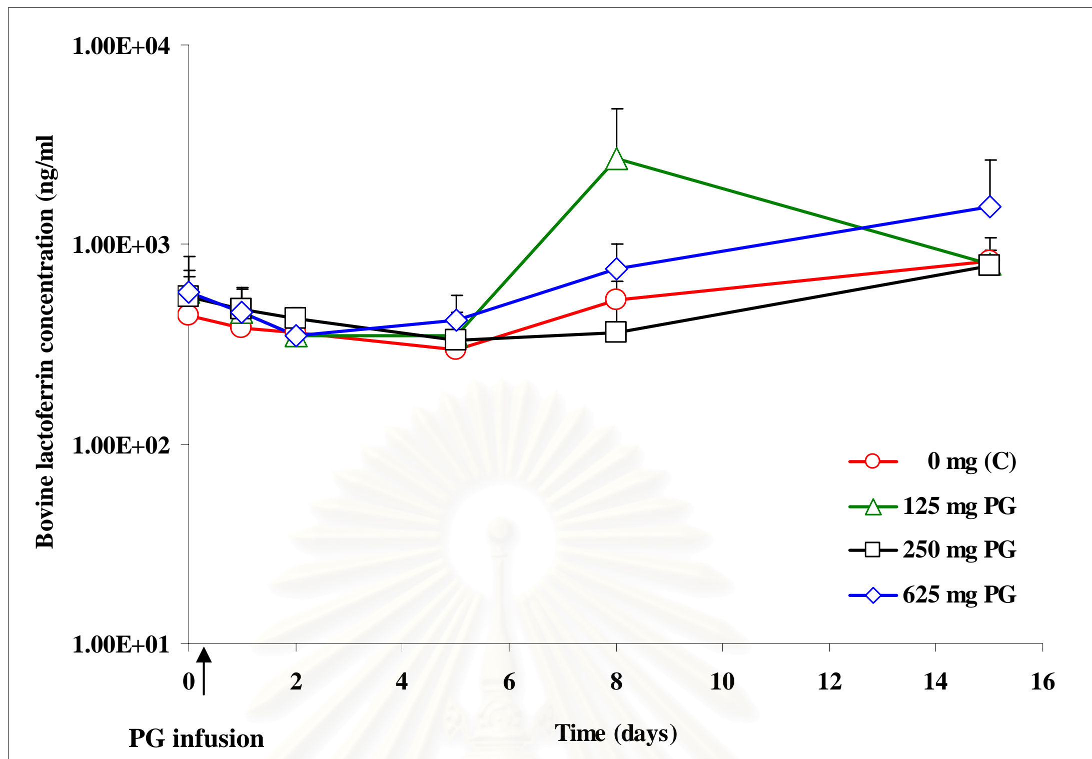


**Figure 28:** Concentrations (mean+SE) of bovine lactoferrin in udder secretion samples taken at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

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# CHAPTER V

# DISSCUSION AND CONCLUSION

## DISCUSSIONS

### Extraction of polysaccharide gel (PG) from fruit-rinds of durian

The polysaccharide gel (PG) was first isolated by Pongsamart and Panmuang 1998, from durian rinds. The PG was previously characterized as a pectic polysaccharide, polygalacturonic acid is the principal component together with neutral sugars side chain (Hokputsa *et al*., 2004). The major component including glucose 20.9%, galactose 4.9%, rhamnose 4.8%, fructose 3.7%, arabinose 1.2%, xylose 0.4% and galacturonic acids 67.9% have found in the polysaccharide gel (Greddit, 2002; Hokputsa *et al*., 2004). Percentage yield of semipurified extract, as pale beige powder, was 7% by weight of dried fruit-rinds. The result of semipurified PG obtained was about the same as previously study (Greddit, 2002; Nakchat, 2002; Hokputsa *et al*., 2004).

### Formulation of PG teat dip

The PG also has gelling property that suitable for gel formulation as a teat dip sanitizer. The PG has antibacterial activities against both of Gram-positive and Gram-negative bacteria and most of mastitis bacterial isolates from dairy cows (Pongsamart *et al*., 2005, Lipipun *et al*., 2006). Formulation of the postmilking teat antiseptic containing 2.5% PG (w/v) as an active ingredient was selected due to its effective concentration for inhibiting bacteria growth as well as gelling effect. In topical pharmaceutical formulations, glycerin and propylene glycol are used primarily for its humectants and emollient properties. The concentration normally used in topical products is about 30% and 15% for glycerin and propylene glycol, respectively (Rowe, 2003). In the formulation of PG teat dip, the concentration of 10% glycerin and 10% propylene glycol were used. The PG teat dip formulation showed rather stable at room temperature. After stand at room temperature for 5 month, a homogenously clear gel preparation was not change. Viscosity of gel was increased;

however, agitating decreased its viscosity. The prior study showed a similar result, PG gel preparation has found stable after storage at ambient temperature and freeze-thaw cycles stability test (Paphattarapong, 2005). The postmilking teat dip of PG presumed to be a promising the alternative attractive remedies for routine use in livestock.

**Determination of the efficacy of PG teat dip using time kill analysis *in vitro***

An *in vitro* time kill analysis was carried out to determine the efficacy of PG teat dip against field isolated of mastitis-causing bacteria by exposured the represented bacteria with the PG teat dip prepared in various times as indicated. The methods including exposure the bacterial suspension with FBS as a source of organic material and the method modified by exposure the bacteria on glass surface. On condition that the milking interval of dairy cattle is approximately 8-12 hours, Inhibitory effect, the PG teat dip against bacteria during 8 hours period was investigated. The standard bacterial suspension test was used for assuring uniform in both time killed analysis tests. The modified method on sterile glass surface were used for studying the efficacy of PG teat dip against the tested bacteria deposited on sterile Petri dish, as on bovine mammary teat skin. The studies were performed to evaluate the killing time of the tested mastitis-causing bacteria. Killing effect of PG was performed in a time dependent manner. The reduction rates were determined by the reduction of counting cfu/ml of bacteria against times. Most of tested bacteria were declined to nearly 100% or 100% within 8 hours of exposure to PG (Table 9). However, IMI need to be cognizant that the PG teat dip is unable to complete killing some tested bacteria. The survival bacteria may cause IMI. The research associated mastitis vaccine showed that a little bit, use of 1 ml of skimmed milk containing approximately 300 cfu of streptococci as intramammary bacterial challenge (Finch *et al.*, 1994). The reduction rate of Gram-positive bacteria extremely became lower in short time period, particularly species of streptococci group; on the contrary the killing effect of PG teat dip on gram-negative bacteria slowly slope downward as previous investigation by Nantawanit, 2001 and Phaunfoong, 2005.

Although the mechanism of antibacterial activity of PG from durian fruit-rinds has not been studied, however, there are some reports in great detail, insight into antibacterial mechanism. Most of the studies on the mechanism of antibacterial polysaccharide focused on their effects on cellular membranes, altering its function and in some instances pectin structure of cell membrane, the polysaccharide causes causing swelling and increasing all membrane permeability. According to the previous studied on bactericidal effect of chitosan, describe charged polysaccharide in chitosan binds and forms polyelectrolyte complex with bacterial cell wall. (Chung *et al.*, 2004). The similar mechanism of polyelectrolyte complex may also be explained for the mechanism of bacteriocidal effect of PG, the polyanionic charge of polygalacturonan in PG (Hokputsa *et al.*, 2004) possibly interacted with bacterial cell wall and destroyed its function. The PG attachment effect to bacterial cell surface was demonstrated by scanning electron micrographs of *S. agalactiae* (Figure 16) and *E. coli* ATCC 25922 after incubation with PG for 24 hours, the structure of cells pilli is destroy as demonstrated by (Nantawanit, 2001). The antibacterial nature of the PG studies against Gram-positive and Gram-negative bacteria is also found apparently related to galacturonic acid, a major components in PG as well as acidity in medium (Phaunfoong, 2005).

**Determination of the PG teat dip efficacy *in vivo***

The inhibitory efficacy of PG teat dip on new IMI was studied *in vivo* by experimental challenge and natural exposure procedure. The protocol are the standardize procedures for the evaluation of postmilking teat dips of the National Mastitis Council to assure uniform and accurate comparison of efficacy data that inconsistent among countries. In addition, the postmilking teat dipping application is now accepted, correctly uses, will reduce incidence of contagious mastitis pathogens. Experimental challenge study: This protocol is highly recommended for conducting both *S. aureus* and *S. agalactiae* for the experimental challenge. The PG teat dip treated group showed marked reduction in the number of new IMI caused by both of *S. aureus* and *S. agalactiae* by 100% ($P < 0.05$). The incidence density values per 100 quarter days at risk of the cows treated with PG teat dip were 1.02 and 0.8 against infection with *S. aureus* and *S. agalactiae*, respectively. In fact, more than 140 different microorganisms involved in bovine mastitis, and they live in the cows and

her environment. The previous study in 2004, Chiangmai province, Thailand of reported that the bacterial pathogens associated with IMI were CNS 27%, environmental streptococci 49.5% and yeast 29.4%. This study indicated that the majority of bovine mastitis caused by environmental and opportunistic bacteria, which is the most pathogen of mastitis and IMI around the world (Bartlell *et al*., 1992; Lafi *et al*., 1994). Natural exposure study: The efficacy of PG teat dip in this study was examined in a commercial dairy farm under natural exposure. The experiment divided into two studies in experiment 1 and 2, the infection data were collected during the 7-days and 4-weeks trial period, respectively. The results from dual experiment are rather relation. PG teat dip seems the protective factor, on the ground that it showed (1) highest the incidence density of no bacterial samples, (2) complete protecting of contagious mastitis pathogens, (3) prevent infection *Pseudomonas spp.*, the germicidal resistance bacteria, (4) reduce incident of environmental mastitis pathogens, particularly *E. coli* and (5) most of rate difference are positive when compared to both negative and positive control group, except incidence density of infection with streptococci other than *S. agalactiae*; however, most parameter is not significantly statistical different.

The prospective of postmilking teat dip includes high potency, specific against pathogens, prolong action, no toxicity, no irritation and induce wound healing. For the reason of high potency, specific against pathogens and prolong action, the PG teat dip was studied in both *in vitro* and *in vivo*. The *in vitro* study, the PG teat dip illustrated a high potency for growth inhibition and had specific killing all tested field bacterial isolates. The results of fast killing ability and prolong acting of PG was demonstrated in a time dependent manner. Moreover, methicillin-resistance *S. aureus* isolated from clinical bovine mastitis and bovine mastitis isolate of *Pseudomonas spp.* are susceptible to PG (Phaunfoong, T., 2005); althongh, these bacteria are resistant to most chemical bactericidal agent. Routinely a use of PG teat dip is expected that not only protected mammary gland infection in dairy cows but also prevented consumers from drug residues in milk consumed. In addition, PG has not induced toxicity in laboratory animals according to the previous studies (Pongsamart *et al*., 2001; Pongsamart *et al*., 2002). A large number of studies have been reported the antiseptic contaminated milk, especially milk iodine (Kammerlchmer *et al*., 1955; Fischer *et al*., 1993; Preiss *et al*., 1997) Although iodine residues in milk can be reduced by

lowering the concentration of available iodine in teat disinfectants, there is definite level of effective concentration in post-milking teat disinfection if the product is too dilute to be effective. *In vivo* studies, skin irritation on teat end was not observed during the study period, moreover the teat end skin was soft and skin lesion seem to be healed after dipped with PG teat dip for 4 weeks. Since, bioactivity of PG in both bactericidal and wound healing effects has been reposted. Previous studies demonstrated that the dressing films prepared from the durian polysaccharide gel enhance wound healing in pig and dog skin (*in vivo*) (Nakachat, *et. al*., 2001 and Siripoksupkul, *et. al*., 2004). The skin irritation always observes in which by routine uses of chemical antiseptics such as iodophor (Windholz, 1976), chlorhexidine (Schultze *et al*., 1970) and sodium Hypochlorite (Pankey and Philpot 1975). The irritative effect causes ecological changes in the skin including changes in temperature, composition of skin fluids and cell turnover. Skin lesion of teat ends destroy the first barrier of defense mechanism of mammary gland, the lesion areas are easily infected and may affect the bacterial flora of the skin, leading to colonization by specific types of organism (Maki and Hecht, 1982). Antiseptic teat dips prepared from PG of durian rinds, antibacterial from natural source, revealed a promising efficacy in protecting the contagious bacteria causing mastitis and also healing with softening the skin of teat end. The PG teat antiseptic seems to be a suitable product for routinely use as a postmilking teat dip in dairy farm.

**Determination of the effect of PG on bovine mammary leukocytes (*in vitro*)**

The XTT reduction assay and Flow cytometric procedure with PI was used for determining cytotoxic effect of PG on bovine mammary leukocytes. In the reaction mixture, XTT is cleaved by mitochondrial dehydrogenases of metabolically active cells and the amount of XTT formazan generated is directly proportional to the viable cell number (Seudiero *et al*., 1988). The staining with PI, a well known dye for the detection of the late apoptotic cells (Lecoeur, H., 2002), was used to clarify DNA content of bovine mammary leukocytes which exposed to PG. DNA content of treated cells was then determined by flow cytometric analysis. After mammary bovine leukocytes exposed to various concentration of PG at 37 °C for 30 minutes, the cytotoxic reaction was determined. The results in close proximity were demonstrated

by using two standard methods. The concentration at 0.5 and 1 %PG showed low effect to bovine mammary leukocytes, whereas, the percentage of cytotoxicity at 2.5%PG is upper inhibitory concentration 50% (IC50). The cytotoxicity result of PG on the leukocytes was in dose-dependent manner. The mechanisms of PG-mediated cell death are difficult to classify only by these two different modes, apoptosis and necrosis, under the present study. From results of the percentage of cell viability detected by XTT assay was lower than the percentage of cell death detected by PI staining method indicated that PG effected cell death by destruction via mitochondrial function was low; whereas, the Bovine mammary leukocytes damaged by exposure to PG probably mainly through an apoptosis pathway (Figure 18 and Figure 19). The leukocytes exposured to the high concentration of (2.5% w/v PG) induced in the DNA fragmentation. The present study presumed that a relatively minor mitochondrial dysfunction would be a signal for apoptosis, and the extensive collapses of mitochondrial membrane that potentially impaired respiration was likely resulted in energy dissipation leading to necrosis (Tsujimoto, 1997). On the other hand, its appeared definitively ascertained that the intranucleosomal cleavage of chromatin and the fragmentation of nuclear proteins were presented during apoptosis.

Effect of PG on phagocytosis of cellular innate immunity was investigated by *in vitro* NBT reduction method. NBT test which reflexes the production of superoxide anion by the activated phagocytes (Mills and Quie, 1981). The results indicated that bovine mammary leukocytes were the most induced of phagocytosis after exposured treated with 1% (w/v) PG for 30 min compared to the 0.5% PG treated and untreated control, but at 2.5%PG inhibited phagocytic activity of bovine mammary leukocytes (Figure 20). The NBT reduction assay was perform in many pectic polysaccharides from plants for activities related to induction of phagocytosis such as pectic polysaccharides from the pericarbs of mangosteen (*Garcinia mangostana* L.), root of ginseng (*Panax ginseng* ), leaves of Siberian tea (*Bergenia crassifolia* L.) and the leaves of maidenhair (*Ginkgo biloba* L.) (Chanarat *et al*., 1997; Shin *et al*., 2002; Popov *et al*., 2005; Hancianu *et al*., 2007). Immunostimulating polysaccharides were already observed in 1982 in the water extract of roots from *Angelica acutiloba* (Kumazawa *et al*., 1982). The relationship between the structure and the immunogenic effect is discussed in great detail by Paulsen and Barsett, in 2005. Degradation of the galactan backbone significantly reduced the immunogenicity; on

the other hand, removal of the external side chain of the molecule result in increasing activity more than native structure, particularly in complement system. However, branch-removed backbone decrease cytokine production from the Peyer's patch cells. It is concluded that the polygalacturonan backbone essential for the activity but side chains attached to the backbone is obscure.

The morphology of bovine mammary macrophages under scanning electron microscope was used to convince the reactive profile of PG. The results were corresponding to the phagocytosis examination. The PG induced marked pseudopodal formation of macrophages was observed in cells treated with 1%PG, but pseudopodal formation of the cells were inhibited with 2.5%PG treatment. The morphology of bovine mammary macrophages treated with 2.5%PG exhibited cellular shrinkage which was the characteristic morphological appearance of apoptotic cell (Catchpoole and Stewart 1993). These results indicated that PG at 1% (w/v) concentration was not only the highest phagocytosis induction but also the lowest cytotoxicity in comparison with 0.5 and 2.5 % PG. The concentration at 1% PG was used for immunomodulatory study *in vivo* by intramammary infusion of PG in non-lactating cows.

**Determination of immunomodulatory effect of PG on bovine mammary gland**

A single intramammary infusion of PG in non-lactating cows (dry period) was performed. Each treated group of 3 udders was infused through the teat canal with 12.5, 25 and 62.5 ml of 1% PG, respectively, and PBS was used in control group. In this experiment bacteriologically negative samples was strictly including: udder secretion of experimental cows before infusion of PG; PG test solution; and finally, udder secretion after infusion of PG, are used to assure that immunological change was the result of PG. The results suggested that directly induced to local immunity, since the systemic reactions were not found throughout study such as clinical symptoms, vital signs and hematogram.

PG stimulated transient immune responses in the bovine mammary gland after intramammary infusion of PG during the dry period. PG induction of the accumulation of SCC, number of CD14 and MHC class-positive leucocytes in udder secretions, however, these effects was transient responded in a dose independent

manner. The increase of the number of CD14 positive leukocytes by PG suggested the ability of bacterial recognition, especially of Gram-negative bacteria enhancing (Figure 25) (Wright *et al*, 1990). Since CD14 is not only monocytes and macrophages marker but also a receptor for lipopolysaccharides (LPS) of Gram-negative bacteria. LPS binds to (LPS-binding protein) LBP, an acute-phase protein that facilitates the transfer of LPS to membrane-associated CD14 on macrophages. This binding initiates signal transduction through the Toll-like receptors 4 (TLR-4) resulting in the release of pro-inflammatory cytokines by macrophages, such as tumour necrosis factor-alpha (TNF-α) and interleukins 1 (IL-1), IL-6, IL-8 (Dentener *et al*, 1990). PG induced accumulation of CD14 could affect cascade reaction in the immune system.

The increasing in MHC class II positive leukocytes susggested that PG inducted stimulation of the antigen-presenting cells activity in the bovine mammary glands. However, the MHC class II positive leucocytes were due to an increasing of antigen-presenting cells (APC) other than macrophages, the elevation of the number of CD14-positive cells was found coincide with MHC class II-positive cells in the PG-treated groups (Figure 25 and 26). The previous studies demonstrated that only a few lymphocytes in udder secretions expressed MHC class II (Taylor *et al*., 1994) and MHC class II-positive leukocytes was not due to an increase in B-cells after intramammary infusion of ß1,3-glucan in sheep and dairy cows (Persson and Colditz, 1999; Inchaisri *et al*., 2000). Intramammary infusion of saline resulted in any cellular reactions such as proportion of CD14 and MHC class II-positive leukocytes as well as in PG treated group. A similar response observed in the lactating gland might be due to mild irritation caused by saline (Saad and stensson, 1990). However, infusion of saline induced lower degree of accumulation of leucocytes then that of infusion of PG.

The induction of bLf in udder secretion seems to play a key role in the defence mechanisms in the mammary gland of dairy cows. Several studies have reported that Lf activates macrophages and induces IL-8, TNF-α and nitric oxide (NO) and phagocytosis-enhancing effect (Kaminska *et al*., 1995; Sorimachi *et al*., 1997; Dentener *et al*., 2003). Furthermore, the bLf has bacteriostatic and bactericidal properties attributed to its ability to chelate iron or to bind to the bacterial surface (Ziere *et al*., 1996). Studies have shown that Lf damages the outer membrane of

Gram-negative bacilli, causing the release of endotoxin (Ward, and Conneely, 2004). However, in the present study, bLf concentration in quarter secretion samples somehow found higher in the 125 mg PG treated group than that of and other two PG treated and control groups at day 8 post-infusion (Figure 27).

Stimulation of immune reactions in the udder during the dry period by using non-specific immunomodulators may be a suitable approach as the bovine mammary gland is particularly susceptible to new infections. However, most cases of clinical mastitis occur after parturition, probably due to immunosuppression during this period (Van Kampen and Mallard, 1997). The cellular and humoral immune status of the udder change substantially during involution. The higher resistance to infections during the steady dry period may be due to variation physiological differences such as higher concentrations of leucocytes and humoral factors such as lactoferrin (Concha, 1986; Burvenich *et al*., 1995; Persson and Colditz, 1998). The results in the present study concluded that intramammary infusion of PG into the bovine udder enhanced some aspects of the local immune responses and then reduced the risk of udder infections during the dry period. However, the effect was only 1-2 weeks post-infustion, the result suggested that respected infusion of PG closer to calving would be necessary to induce mammary defense against infections during the dry period.

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**CONCLUSION**

The postmilking teat dip preparation contained 2.5% PG (w/v) in 1% Ringer's solution with humectants including 10% propylene glycol and 10% glycerin was successfully prepared. The PG teat dip illustrated good killing effect to all tested isolates *in vitro* and high protective effect for mastitis prevention as demonstrated by using *in vivo* studies. The results indicated that the antiseptic teat dip of PG from durian rinds has potential to prevent bovine IMI in lactating cows. The immunostimulating potential of PG on bovine mammary gland was also assessed in non-lactating cows during dry period. Optimum concentration at 1% w/v PG showed the enhancement of the highest phagocytic activation. The single intramammary infusion of 1% w/v PG into the bovine mammary gland potentially enhanced some aspects of the local immune responses. The *in vitro* cytotoxicity assay was used for confirming no effect to bovine mammary leukocytes. Therefore, the PG is expected to be a promising biodegradable agent for mastitis control during the dry period.

As mention in study objectives; firstly, to determine the potential of durian polysaccharide gel as a natural antibacterial agent for developing a postmilking teat antiseptic, the postmilking teat dip was successfully prepared; the formulation composed of 2.5 g PG, propylene glycol 10 ml, glycerin 10 ml and Ringer's solution to make 100 ml. The product has not been changed it appearance after kept at room temperature for 5 month observation. *In vitro* time-kill analysis was examined against nine field bacterial isolates causing mastitis. The PG teat dip illustrated killing effect to all tested isolates within 1 minute. The reduction rates of most bacteria were absolutely declined to 100% or nearly 100% within 8 hours of incubation with PG. The PG teat dip inhibited incidence density for new intramammary infection (IMI) by 100% ($P < 0.05$) of both *S. aureus* and *S. agalactiae* demonstrated by using the experimental challenge protocol. The natural exposure study, there was not significant difference between PG teat dip group and iodophor (positive control) group. However, incident rate of PG teat dip group was higher than undipped group and combination (iodophor followed with PG teat dip) group.

Secondly, to determine the immunomodulatory potential of durian polysaccharide gel on bovine mammary gland immunity**.** The XTT reduction assay and flow cytometric procedure with PI were used for confirming no effect to bovine mammary leukocytes. The cytotoxicity of PG on the leukocytes was dose-dependent manner. Concentration at 2.5%PG exhibited significantly higher cytotoxicity than that of at 0.5 and 1 %PG and control. The immuno-stimulating potential of PG on bovine mammary gland was assessed by *in vitro* phagocytosis assay using NBT reduction. Bovine mammary leukocytes are activated after exposured to 0.5 and 1% (w/v) PG for 30 min compared to in untreated control, however, at 2.5%PG exhibited phagocytic inhibition on bovine mammary leukocytes. A single intramammary infusion of three different doses at 12.5, 25 and 62.5 ml of 1% PG caused a transient elevation of the somatic cell count, number of CD14 and MHC class-positive leucocytes in udder secretions. The concentration of bLf in quarter secretion in 125 mg PG group were somewhat higher than the other treated and control group at day 8 post-infusion. The total white blood cells count was significantly increased at day 8 post-infusion; however, differential counts of peripheral blood samples were not significantly different throughout the study in comparison with normal cows.

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# APPENDICES

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# APPENDIX A

# PREPARATION OF REAGENTS

**Blocking solution**

To make 1 liter of Wash Solution (50 mM Tris, 0.14 M NaCl, 1% BSA), the ingredients are 6.67g Tris, 5.8g NaCl and 1 g BSA were dissolved in DDW with continuously stirring. The solution was mixed well and adjusted the pH to 8 with 5 N NaOH. The solution was adjusted volume to 1000 ml.

**Conjugate diluents**

To make 1 liter of Wash Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0. 05% Tween 20), the ingredients are 6.67g Tris, 5.8g NaCl, 5 ml Tween 20 and 1 g BSA were dissolved in DDW with continuously stirring. The solution was mixed well and adjusted the pH to 8 with 5 N NaOH. The solution was adjusted volume to 1000 ml.

**DMEM medium**

DMEM powder was dissolved with deionized distilled water and the 3.7 g/l sodium bicarbonate was added. The solution was mixed well and adjusted pH to 7.2 with 2N HCl. Then, the solution was adjusted volume to 1,000 ml. This solution was sterilized by filtration (0.2 μm millipore filter membrane). Before use, this solution was supplemented with 10% FBS.

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**Nitro blue tetrazolium**

NBT was dissolved in distilled water at 2 mg/ml. A stock solution at 2 mg/ml is stable for 1-2 weeks in the dark at –20 °C. Prior to each experiment, the NBT was diluted with an equal volume of buffer containing 270 mM NaCl, 10.4 mM $Na_2HPO_4$, and 3.16 mM $KH_2PO_4$ (NBT working solution).

**Phosphate buffered saline (PBS)**

To make 1 liter of PBS, the ingredients including 8 g NaCl, 0.2 g KCl, 0.2 g $KH_2PO_4$ and 1.44 g $Na_2HPO_4$ were dissolved in deionized water. The solution was mixed well and adjusted the pH to 7.4 with 5 N NaOH. The solution was adjusted volume to 1,000 ml.

**PMS solution**

PMS was made up as 100 mM solution in PBS and stored at 4 °C for periods up to 1 month. Prior to each experiment, the PMS was diluted with PBS.

**Ringer's solution**

To make up in 1 liter distilled water, the ingredients including 6.5g NaCl, 1.4g KCl, 0.12g CaCl, 0.1g $NaHCO_4$ and 0.01g $Na_2HPO_4$ were dissolved in deionized water. The solution was mixed well and adjusted the pH to 7.4 with 5 N NaOH. The solution was adjusted volume to 1,000 ml.

**Wash solution**

To make 1 liter of Wash Solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20), the ingredients are 6.67g Tris, 5.8g NaCl and 5 ml Tween 20 were dissolved in DDW with continuously stirring. The solution was mixed well and adjusted the pH to 8 with 5 N NaOH The solution was adjusted volume to 1000 ml.

**XTT solution**

XTT solution were freshly made each day by dissolving XTT in hot DMEM (60 °C) to make the final XTT concentration of 1 mg/ml. Prior to each experiment, the XTT was diluted with PBS.

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**Zymosan A particles**

Zymosan A particles suspended in 0.15 M sodium chloride at the concentration of 4 mg/ml were placed in a boiling water bath for 30 min, then centrifuged for 30 min at 4000 rpm. The supernatant was discarded and the residue was resuspended in DMEM at 4 mg/ml and stored at –20 °C in small aliquots for at least a month.

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# APPENDIX B

## TABLE AND EXPERIMENTAL RESULTS

**Table 1**. The effect of polysaccharide gel form durian rinds on cytotoxicity of bovine mammary leukocytes after 30 minutes exposured as determined by using XTT reduction assay.

| Treatment of bovine mammary leukocytes | Percentage of cytotoxicity (mean±S.E.) | Number of studies |
|---|---|---|
| Vehicle control | $6.28^{b,c} \pm 1.18$ | 10 |
| 0.5 %PG | $4.37^{b} \pm 0.82$ | 10 |
| 1.0 %PG | $11.42^{b} \pm 2.77$ | 10 |
| 2.5 %PG | $27.60^{a} \pm 2.81$ | 10 |

a, b = significant differences ($P \leq 0.05$).

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**Table 2**. The effect of polysaccharide gel form durian rinds on cytotoxicity of bovine mammary leukocytes after 30 minutes exposured as determined by using flow cytometric with propidium iodide.

| Treatment of bovine mammary leukocytes | Percentage of cytotoxicity (mean±S.E.) | Number of studies |
|---|---|---|
| Vehicle control | $4.87^{d} \pm 0.56$ | 10 |
| 0.5 %PG | $15.22^{c} \pm 1.27$ | 10 |
| 1.0 %PG | $25.35^{b} \pm 1.34$ | 10 |
| 2.5 %PG | $70.08^{a} \pm 3.97$ | 10 |

a, b, c and d = significantly different ($P \leq 0.05$).

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**Table 3**. The effect of polysaccharide gel form durian rinds on phagocytic activity of bovine mammary leukocytes by using NBT reduction assay.

| Treatment of bovine mammary leukocytes | Percentage of phagocytic activity (mean±S.E.) | Number of studies |
|---|---|---|
| Vehicle control | $-4.83^{b,c} \pm 1.41$ | 10 |
| 0.5 %PG | $4.97^{b} \pm 3.87$ | 10 |
| 1.0 %PG | $38.66^{a} \pm 4.65$ | 10 |
| 2.5 %PG | $-10.87^{c} \pm 2.95$ | 10 |

a, b and c = significantly different ($P \leq 0.05$).

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**Table 4**. Somatic cell count (mean±SE) of udder secretion samples in non-lactating cows at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| PG infusion (days) | Dosage of 1%PG solution (mg) | | | |
|---|---|---|---|---|
| | **0** | **125** | **250** | **625** |
| 0 | 5.75±0.14 | 5.79±0.13 | 5.77±0.05 | 5.78±0.17 |
| 1 | 5.32±0.13[c] | 6.34±0.02[a] | 6.05±0.06[b] | 6.28±0.06[a] |
| 2 | 5.72±0.11 | 6.59±0.28 | 6.80±0.10 | 6.69±0.27 |
| 5 | 5.56±0.09 | 6.51±0.16 | 6.30±0.09 | 6.71±0.21 |
| 8 | 5.83±0.09[c] | 5.93±0.21[c] | 6.76±0.21[a] | 6.58±0.03[b] |
| 15 | 5.92±0.21 | 5.93±0.07 | 5.79±0.16 | 5.59±0.19 |

a, b and c = significantly different ($P \leq 0.05$).

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**Table 5.** Number of CD14-positive leukocyte (mean±SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| PG infusion (days) | Dosage of 1%PG solution (mg) | | | |
|---|---|---|---|---|
| | **0** | **125** | **250** | **625** |
| 0 | 5.19±4.49 | 5.28±4.94 | 5.39±4.91 | 5.31±4.78 |
| 1 | 4.21±3.34[b] | 5.11±4.95[a,b] | 5.53±4.29[a] | 5.22±4.95[a,b] |
| 2 | 4.69±4.18 | 5.91±4.49 | 5.55±5.47 | 5.45±4.56 |
| 5 | 4.83±4.77 | 5.66±5.58 | 5.84±5.31 | 6.11±5.71 |
| 8 | 5.04±4.79[b] | 6.16±5.71[a] | 5.71±5.48[a,b] | 5.77±4.46[a,b] |
| 15 | 5.61±5.12 | 5.35±5.24 | 5.21±4.76 | 4.76±4.31 |

a and b = significantly different ($P \leq 0.05$)

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**Table 6**. Number of MHC class II-positive leukocyte (mean+SE) in udder secretion samples at day 0 before intramammary infusion of PG and 1-15 days post-infusion.

| PG infusion (days) | Dosage of 1%PG solution (mg) | | | |
|---|---|---|---|---|
| | 0 | 125 | 250 | 625 |
| 0 | 5.44±3.21 | 5.46±4.55 | 5.49±5.15 | 5.46±4.03 |
| 1 | 4.73±4.59[b] | 5.49±5.39[a,b] | 5.96±5.87[a] | 5.73±5.42[a,b] |
| 2 | 5.40±5.09 | 6.46±5.92 | 6.24±5.55 | 6.11±5.99 |
| 5 | 5.11±4.85 | 5.91±5.75 | 6.15±5.90 | 6.37±5.90 |
| 8 | 5.15±4.97 [b] | 6.33±5.90[a] | 5.64±5.90[b] | 5.88±4.46[a,b] |
| 15 | 5.38±5.85 | 5.63±5.37 | 5.47±4.63 | 5.02±4.23 |

a and b = significantly different ($P \leq 0.05$).

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**Table 7.** Concentrations of bovine lactoferrin in logarithmic from (mean±SE) in quarter milk samples

| PG infusion (days) | Dosage of 1%PG solution (mg) | | | |
|---|---|---|---|---|
| | **0** | **125** | **250** | **625** |
| 0 | 2.64±0.05 | 2.74±0.10 | 2.68±0.18 | 2.66±0.02 |
| 1 | 2.57±0.07 | 2.65±0.06 | 2.64±0.12 | 2.63±0.12 |
| 2 | 2.53±0.11 | 2.53±0.05 | 2.62±0.05 | 2.51±0.11 |
| 5 | 2.26±0.31 | 2.32±0.33 | 2.30±0.32 | 2.37±0.36 |
| 8 | 2.69±0.13 | 3.08±0.39 | 2.45±0.24 | 2.81±0.18 |
| 15 | 2.86±0.18 | 2.90±0.01 | 2.88±0.09 | 2.94±0.33 |

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# VITA

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