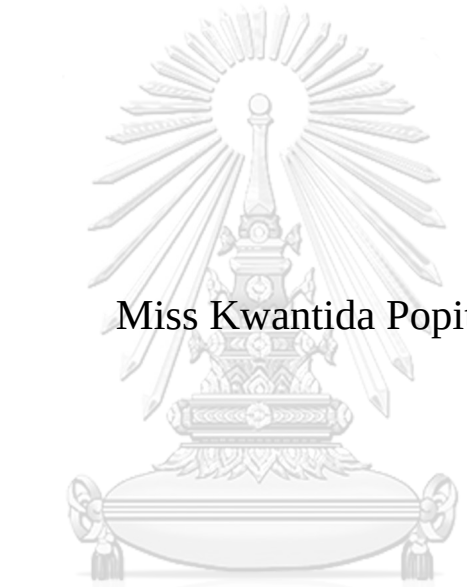
# POTENTIAL OF ANTIMICROBIAL PEPTIDES AGAINST BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS

Miss Kwantida Popitool





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# ศักยภาพของเพปไทด์ต้านจุลชีพต่อแบคทีเรียที่ก่อให้เกิดโรคเต้านมอักเสบในโคนม

น.ส.ขวัญธิดา โพพิทูล



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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| Field of Study | Biotechnology |
| Thesis Advisor | Assistant Professor SARINTIP SOOKSAI, Ph.D. |
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ขวัญธิดา โพพิทูล : ศักยภาพของเพปไทด์ต้านจุลชีพต่อแบคทีเรียที่ก่อให้เกิดโรคเต้านมอักเสบในโคนม. ( POTENTIAL OF ANTIMICROBIAL PEPTIDES AGAINST BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS) อ.ที่ปรึกษาหลัก : ผศ. ดร.ศรินทิพ สุกใส, อ.ที่ปรึกษาร่วม : รศ. ดร.กิตติศักดิ์ อัจฉริยะขจร

โรคเต้านมอักเสบเป็นโรคที่พบได้บ่อยในโคนม สาเหตุของการเกิดโรคเต้านมอักเสบในโคนมคือการติดเชื้อแบคทีเรียผ่านทางรูเปิดของเต้านม โดยทั่วไปโรคเต้านมอักเสบต้องใช้ยาปฏิชีวนะในกระบวนการรักษา อย่างไรก็ดี การใช้ยาปฏิชีวนะในปริมาณที่ไม่เหมาะสมส่งผลให้เกิดการดื้อยาปฏิชีวนะในแบคทีเรียและเกิดสารตกค้างในโคนมและผลิตภัณฑ์จากนม การรักษาทางเลือกในการต้านแบคทีเรียก่อโรคเพื่อลดการใช้ยาปฏิชีวนะคือการใช้เพปไทด์ต้านจุลชีพซึ่งเป็นส่วนประกอบของภูมิคุ้มกันที่มีมาแต่กำเนิดของสิ่งมีชีวิต เพปไทด์ต้านจุลชีพประจุบวกทั้งสามชนิด ได้แก่ เพปไทด์ Pm11, Pep64 และ L10 ได้รับการประเมินฤทธิ์ต้านจุลชีพต่อแบคทีเรียแกรมบวกและแบคทีเรียแกรมลบ พบว่า เพปไทด์ Pm11 ได้แสดงฤทธิ์ต้านจุลชีพที่มีศักยภาพดีที่สุดด้วยค่าความเข้มข้นของเพปไทด์ที่น้อยที่สุดที่ฆ่าแบคทีเรีย 99.9 เปอร์เซ็นต์ ของแบคทีเรียที่ใช้ทดสอบ (MBC) อยู่ในช่วง 2.5-10.0 ไมโครโมลาร์ และค่า $IC_{50}$ อยู่ในช่วง 0.32-2.07 ไมโครโมลาร์ จากการติดตามระยะเวลาในการออกฤทธิ์ของเพปไทด์ Pm11 ในการต้านแบคทีเรียที่ก่อให้เกิดโรคเต้านมอักเสบในโคนมต่อหน่วยเวลา เพปไทด์ Pm11 แสดงฤทธิ์การต้านจุลชีพที่มีศักยภาพในการฆ่า *Streptococcus agalactiae* (SCM1084), *Streptococcus uberis* (SCM1310) อย่างสมบูรณ์ภายใน 1 ชั่วโมง และแสดงการฆ่าที่สมบูรณ์ต่อ *Staphylococcus aureus* (CM967), *Escherichia coli* (SCM1249) ภายใน 4 ชั่วโมง อย่างไรก็ดี เพปไทด์ Pm11 ที่ความเข้มข้น 10.0 ไมโครโมลาร์ไม่สามารถฆ่าเชื้อ *Klebsiella* spp. (SCM1282) ได้อย่างสมบูรณ์ แม้ว่าการรอดชีวิตของเชื้อ *Klebsiella* spp. (SCM1282) ลดลงถึง 3 $\log_{10}$ CFU/ml ภายใน 2 ชั่วโมง แต่พบว่าเชื้อสามารถกลับมาเจริญเติบโตได้อีกภายใน 12 ชั่วโมง การสังเกตผลของเพปไทด์ Pm11 ต่อการทำลายเชื้อก่อโรคด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่า มีการเปลี่ยนแปลงทางสัญฐานวิทยาของเชื้อก่อโรคอย่างชัดเจน การทดสอบความเป็นพิษของเพปไทด์ Pm11 ต่อการแตกของเม็ดเลือดแดงแกะ พบว่า เพปไทด์ Pm11 ที่ความเข้มข้น 0-160 ไมโครโมลาร์ ส่งผลต่อการแตกของเม็ดเลือดแดงแกะอยู่ในระดับต่ำ แต่พบว่า ฤทธิ์การต้านจุลชีพของเพปไทด์ Pm11 ลดลงเมื่ออยู่ในสภาวะน้ำนมยูเอสที



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Kwantida Popitool : POTENTIAL OF ANTIMICROBIAL PEPTIDES AGAINST BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS. Advisor: Asst. Prof. SARINTIP SOOKSAI, Ph.D. Co-advisor: Assoc. Prof. KITTISAK AJARIYAKHAJORN, D.V.M., Ph.D.

Bovine mastitis is a common disease in dairy cows. The majority causes of bovine mastitis disease is an intramammary bacterial infection via the teat orifice. In general, antibiotics are used for bovine mastitis treatment, its inappropriate usage leads to antibiotic-resistance problem and residue in meat and dairy products. One of the alternative ways against pathogen to reduce antibiotic usage is antimicrobial peptides which are innate immune of life. Three cationic peptides as Pm11, Pep64, and L10 peptides were evaluated for an antimicrobial activity to gram-positive and gram-negative bacteria. Pm11 has shown the best potent antimicrobial activity with minimum bactericidal concentration (MBC) range from 2.5-10.0 µM and $IC_{50}$ 0.32-2.07 µM. Time kill kinetics of Pm11 peptide against bovine mastitis pathogens were ascertained, Pm11 is exhibited strong activity to completely killing *Streptococcus agalactiae* (SCM1084), *Streptococcus uberis* (SCM1310) within 1 hour and completely killing *Staphylococcus aureus* (CM967), *Escherichia coli* (SCM1249) within 4 hour. However, Pm11 peptide at 10.0 µM could not be completely killing *Klebsiella* spp. (SCM1282), even though 3 $\log_{10}$ CFU/ml of *Klebsiella* spp. (SCM1282) was decreased within 2 h, but it was returning to grow up within 12 h. Visual observation under the scanning electron microscope of Pm11 peptide attacked to the pathogens shown the distinct morphological changes. The cytotoxicity assay confirmed that Pm11 peptide at the concentration 0 to 160 µM had low hemolytic activity to sheep red blood cells. Nonetheless, the antimicrobial activity of Pm11 peptide was reduced in UHT milk.



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# CHAPTER I

# INTRODUCTION

## Background and Rationale

Thailand has dairy industry that is well-developed. Dairy milk has a nutritional value, making the milk popular for consuming and processing to the other products (Haug, Hostmark, and Harstad 2007). To gain quality products, health care and disease control in a cow are necessary. The common disease of a dairy cow is bovine mastitis-caused by microbial infection. The disease can spread directly from the infected udder to other and arise from unsanitary housing and equipment (Bradley 2002). In general, antibiotics are necessarily used for bovine mastitis treatment. Antibiotics act in order to kill bacteria which cause of disease in dairy cows due to their strong and immediate for controlling the virulent microorganisms. The inappropriate use of antimicrobial drug for mastitis treatment may increase risk of antimicrobial residue in milk and enhances antimicrobial resistance in animal and environmental microorganisms (Kromker and Leimbach 2017) In the same way, farmer lack of income during the time using antibiotics and the consumer will not assure of consuming the milk product. Moreover, as bacteria grow resistant to conventional antibiotics, alternative treatments are being investigated, such as antibodies, probiotics, bacteriophages and antimicrobial peptides (AMPs).

AMPs are originally developed from host defense peptides which obtained from a component of the innate immune system. AMPs have been reported about activity against bacterial infectious disease and alternative treatment to treat the microbial infection in nowadays (Sheena Ramdeen and Boucher 2015 ). Over the past

decade, many researches using derived peptides from the organisms to treat a variety of diseases were conducted. Most AMPs are generally cationic and amphipathic and very short in length, containing 5 to 40 amino acid residues. Each peptide has an individual structure, which makes the different polarities property. The efficiency of peptides against pathogens has been reported since the particular structure and type of the amino acids which the element can result in damage on pathogens by the occurrence of electrostatic interaction between the peptides and pathogen surface (Sharma, Verma, and Sharma 2014; Matsuzaki et al. 1997) and then AMPs bind to cytoplasmic membrane and disrupt the inner membrane causing bacterial cell death. Using AMPs to treat infectious diseases were reduce the possibility of bacterial drug resistance. Besides, they display broad-spectrum and safe with non-toxic side effects or less (Sharma, Verma, and Sharma 2014).

Furthermore, antimicrobial peptides that have only 2-15 amino acids in length (Ramesh et al. 2016) which have ability to against the pathogens, called short antimicrobial peptides (SAMPs). The potential of SAMPs which can transmit through microbial membranes has been reported that the smaller size may simplify access to target sites on the pathogen surface (Mishra et al. 2013) and SAMPs act as natural ligands for many cell surface receptors is the cause of action against of wind range of pathogen strains (Sharma et al. 2018). Furthermore, the SAMPs are convenience to synthesize by chemical techniques and low cost (Ramesh et al. 2016).



Therefore, for clinical development, bioactive of several peptide molecules can first be optimized *in vitro*. Particularly, peptides in cathelicidins and alpha-helical cationic peptides family are subgroup of cationic SAMPs have been reported the potential to damage pathogen cells. In previous studies Pm11 is an alpha-helix peptide

derived from pleurocidin which found in skin mucous secretions of winter flounder (*Pleuronectes americanus*) has a strong potential against *in vitro Streptococcus* sp. causing oral disease (Zhang et al. 2016a). L10 peptide is peptide-derived from N-terminal modification of bovine lactoferrin which found in bovine neutrophils with activity to kill multi-drug resistance Gram negative bacteria (Mishra et al. 2013) . Pep64 peptide is lactoferricin based pentapeptide exhibits activity against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Sharma et al. 2018). As described earlier, demonstrate that the potential of selected peptides can develop to antimicrobial peptides drug for bovine mastitis treatment.

Consequently, the research to offering the alternative treatment for bovine mastitis by using antimicrobial peptides instead of antibiotic drugs should be consider. This study had investigated the potential of SAMPs against the laboratory strain Gram-negative and Gram-positive bacteria and the selected peptide was further testing the antimicrobial activity against bovine mastitis pathogens. Time kill of the peptide to against pathogens had observed both in media and in UHT milk. The cytotoxicity of the peptide had studied by investigated the hemolytic activity of peptide to sheep red blood cells. Finally, the morphology of microbial cells treated by peptide had observed by scanning electron microscope.



**Research objectives**

To investigated effect of selected antimicrobial peptides on bovine mastitis pathogens.

**Expected outcome**

To offer the potential of peptides for antimicrobial effect against bovine mastitis pathogens

# CHAPTER II

# THEORETICAL AND LITERATURE REVIEWS

## 2.1 Bovine mastitis

### 2.2.1 Causes and symptoms of bovine mastitis

Bovine mastitis is the most common disease in dairy cattle. Bovine mastitis is an inflammation of mammary gland tissue, resulting in redness, pain, warm and swollen udder, and the milk abnormal. There are several risk factors to serve mastitis including pathogens, cows and environmental factors. However, the major causes of bovine mastitis are pathogens infection as bacteria mycoplasma yeasts and mold. The pathogens can infect by entering the teat orifice as indicated in Figure 1. The mechanisms of the immune system induced the white blood cells to clear pathogens and cytokines or chemokines are rush results in inflammation.

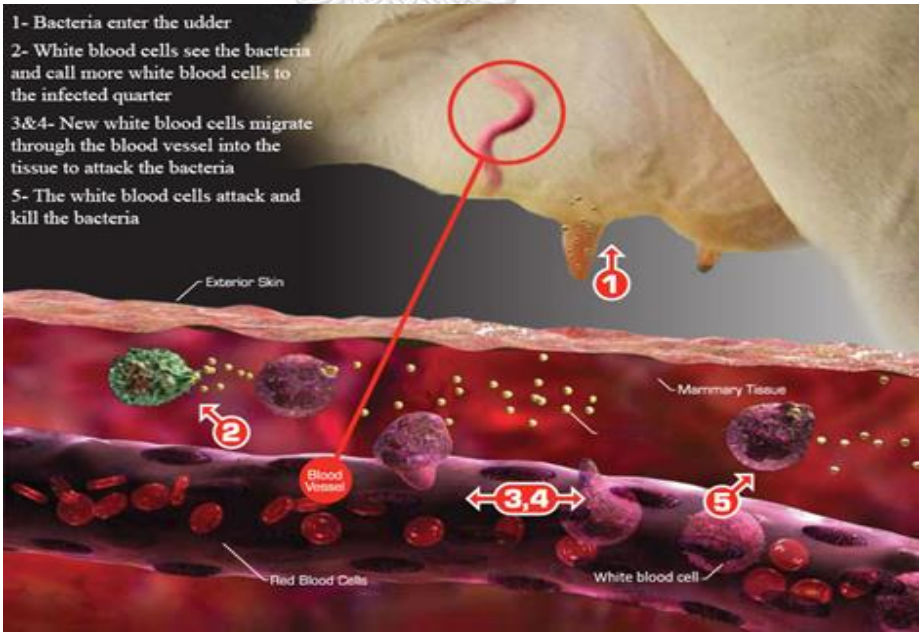



**Figure 1** Causes of bovine mastitis

(Kalmus 2019)

Bovine mastitis can be classified into three types based on the degree of inflammation: subclinical, clinical, and chronic mastitis. Sub-clinical is defined as inflammation of the mammary gland, which does not evince the visible abnormality of udder and milk, but milk yield decreases with increasing somatic cell count. Contrarily, clinical bovine mastitis shows visible inflammation such as redness, swollen udder, and fever, including abnormal milk that presence of flakes and clots. The last one is chronic mastitis define as an inflammation that lasts for several months, with clinical flare-ups occurring at irregular intervals (Cheng and Han 2020).

### 2.2.2 Detection and monitoring for bovine mastitis

#### 2.2.2.1 Cow side test

- **Palpation of the udder** is a routine diagnostic for mastitis detection in dairy cows. Careful udder palpation is especially helpful for the detection of fibrosis that resulted from chronic subclinical contagious infection. Palpation is also valuable after acute clinical mastitis resolution to detect glandular changes associated with infarction, abscessation, or chronic infection (Moroni et al. 2018).



- **California mastitis test** is a simple tool for estimating the amount of DNA and white blood in milk related to somatic cell count (SCC). The California mastitis test (CMT) reagent lyses the cell wall of leucocytes. Especially, polymorphonuclear leukocytes (PMNs) have large nuclei (DNA) results in gel formation. The degree of gel formation can be used to estimate the numbers of WBCs in the milk sample. The test is subjectively read as negative, trace, +1, +2, and +3. The scores equate well with somatic cell levels as listed in Table 1 (Moroni et al. 2018).

**Table 1** Relationship CMT scores to approximate somatic cell counts

| CMT Score | Approximate Somatic Cell Count |
|---|---|
| Negative | 0-200,000 |
| Trace | 150,000-500,000 |
| +1 | 400,000-1 million |
| +2 | 800,000-5 million |
| +3 | >5 million |

(Moroni et al. 2018)

- **pH indicator paper** is a kit for detecting the more alkaline pH of the milk. Normally, the milk from cows without mastitis has a pH of approximately between 6.5 to 6.7. While the milk from cows with mastitis is often close to pH 7.4, resulting from the plasma in milk (Moroni et al. 2018).

**2.2.2.2 Somatic cell count (SCC)** is an indicator of the detection of both resistance and susceptibility of cows to mastitis. They can be used to monitor the level or occurrence of subclinical mastitis in herds or individual cows. Usually, the somatic cell in milk contains 75% of leucocytes and 25% of epithelial cells. Moreover, the concentration of erythrocytes can be found approximately 0 to $1*10^6$ cell/ml. The increasing of somatic cells is caused by plenty of factors as the stage of lactation, cow's age, season and stress of cows. However, the most crucial factor affecting to somatic cell increasing in milk is pathogens infection. The major increase in somatic cells is due to the inrush of neutrophils into milk to oppose and respond to the pathogens infection (Sharma, Singh, and Bhadwal 2011). Mastitis increases the relative proportion of neutrophils in mammary secretions up to 95%. Moreover, the

species and severity of pathogens can also affect the degree of inflammation (Moroni et al. 2018).

**2.2.2.3 Electric conductivity (EC)** the technology is used to monitor the electrical conductivity of milk. The increasing $Na^+$, $Cl^-$ and decreasing levels of lactose and $K^+$ are detected for mastitic milk. The conductivity value is displayed by the EC tool and compared with an established threshold value to determine the possibility that an individual cow has mastitis (Moroni et al. 2018).

**2.2.2.4 Immune assay** is the method developed based on ELISA for detecting *S. aureus*. One of the important pathogens is to serve bovine mastitis. For the principle, the magnetic bead-based ELISA using beads coated with anti-*S. aureus* monoclonal antibody for detecting Staphylococci pathogen (Deb et al. 2013).

**2.2.2.5 PCR base diagnosis**

- **Multiplex PCR-based detection** is used for the diagnosis of multiple pathogens in mastitic milk. This method can identify multiple pathogens in a single reaction at the same time (Deb et al. 2013).



- **Real-time PCR-based detection** is an alternative method for in vitro culture for detecting pathogens in milk. Both contagious and environmental pathogens in milk are detected with this method

- **microRNA detection** is a novel diagnostic tool for bovine mastitis detection (Srikok et al. 2020). MicroRNAs are endogenous, small, and noncoding RNAs that are generated by various cell types. Scientist confirms the presence of various microRNAs in the cow genome and well-characterized these microRNAs in cows. The in vitro challenge studies involving *E. coli* lipopolysaccharides and *S. aureus* enterotoxin B showed alterations in the expression of varied microRNAs as

MIR184, MIR24-3p, MIR148, MIR486 and LET7A-5p were unique microRNAs associated with *E. coli* intramammary infection.

### 2.2.3 Economic consequences of bovine mastitis

#### 2.2.3.1 Factors of the economic effect of bovine mastitis

The economic effect of bovine mastitis, both subclinical and clinical mastitis, is due to milk production losses, milk quality changes, culling, treatment, and risk of other diseases. The associated costs can be divided as the following factors (Halasa et al. 2007).

- **Milk production losses** are found in cows with bovine mastitis both subclinical and clinical mastitis. Production losses due to subclinical mastitis are considered to be a direct relationship between somatic cell count. However, milk production does not improve after the complete recovery of subclinical mastitis. Thus, the assumed relationship might underestimate production losses due to subclinical mastitis.

- **Milk product quality** from a cow with mastitis has influenced the quality of milk. Some of these changes cause less efficient milk processing and might result in products with less valuable properties. Moreover, antibiotics in mastitis treatment increase the risk of antibiotic residues that are not safe. All the milk quality problems lead to the discard of milk produced by the cows, which means that feeding costs for amount of milk having to be taken into account in the calculations.

- **Culling** of a cow with mastitis has a higher risk of being culled. The cost of premature replacement of animals is due to mastitis, which is probably one of the largest economic loss areas.

- **Treatment of cows** with bovine mastitis, antibiotic drugs are necessary to treat infected animals, which are a direct cause of economic damage. Besides, the economic damage is associated with veterinary services. Moreover, spending time for diagnosis of a clinical mastitis case. Veterinary services may be mandatory for each clinical mastitis case.

- **Mastitis management** includes the use of materials and commodities that cost money. These materials can either be renewable (for instance, disinfectants and) or non-renewable (for example, a new milking parlor).

#### 2.2.3.2 Economic impact of bovine mastitis

The economic impact of mastitis at the herd level and the weight (percent) of this impact's components in a Holstein dairy herd under tropical conditions. Table 2 the mastitis indices used to be records at the farm and the Holstein Cattle Association of Minas Gerais State database (real indices). In 2011-2012, economic losses around 61,623 U$/year. The factor that affects economic loss is the reduction of milk production, including clinical and subclinical mastitis. In the same way, in 2012-2013, the factors that affect economic loss are reducing milk production and increasing economic losses that up to 91,552.69 U$/year in Table 2 (Guimarães et al. 2017).

In Thailand, the economic losses of bovine mastitis having in many factors and average expenses for bovine mastitis cows that around two hundred thousand baths per year (Pannium n.d.).

**Table 2** Economic impact in Holstein dairy herd under tropical conditions

| Economic impact components | Economic impact of mastitis U$/year (%) | |
|---|---|---|
| | Feb 2011-Jan2012 | Feb 2012-Jan2013 |
| Reduction of milk production (Clinical mastitis) | 21,886.06 (35.5%) | 31,761.97 (34.7%) |
| Reduction of milk production (Subclinical mastitis) | 26,157.33 (42.2%) | 18,537.44 (20.2%) |
| Milk disposal | 8,610.91 (14.0%) | 20,117.49 (22.0%) |
| Medicines | 3,435.16 (5.6%) | 8,999.60 (9.8%) |
| Labor | 26.17 (0.04%) | 76.20 (0.08%) |
| Cow culling | 1,507.50 (2.4%) | 12,060.00 (13.2%) |
| **Total** | **61,623.13** | **91,552.69** |

(Guimarães et al. 2017)

### 2.2 Bovine mastitis pathogens

The main cause of bovine mastitis in dairy cows is bacterial intra-mammary infection (IMI). Several bacteria are identified as causative pathogens of bovine mastitis. The bacterial pathogens can be divided into two types based on bacterial origin, including contagious bacteria, define as mastitis, can be transmitted cow-to-cow, particularly during milking. Contagious pathogens inhabit on udder and skin and then colonizing and growing into the mammary gland. Contrarily, environmental bacteria refer to the pathogens in not hygienic environments surrounding dairy cows such as the herd's unsanitary house, bed and food. Environmental bacteria are explained as opportunistic pathogens. The pathogens can enter into the teat during milking owing to the liner slippage or when a natural immunity of the cow is weak, causing clinical mastitis (Cheng and Han 2020).

### 2.2.1 Contagious bacteria

***Staphylococcus aureus*** is the most pervasive gram-positive bacteria associated with subclinical and clinical mastitis. *S. aureus* is a round-shape bacterium, facultative anaerobe, non-spore-forming, and catalase-positive. *S. aureus* does not induce an immune response in the cow as strong as *E. coli* or endotoxin. Thus, the infection of *S. aureus* is always milder, leading to chronic mastitis that lasts for a few months. The pathogen can produces toxins degradative enzyme which damage the mammary tissue, eventually decreasing milk production. Some strains of *S. aureus* are known as methicillin-resistant *S. aureus* (MRSA) that have the *mecA* gene result in pathogens resistant to β-lactam antibiotics. Moreover, the ability of *S. aureus* can build a cluster of the cell enclosed in a self-produced matrix that also is known as biofilm to protect it from environmental stress, adhere to mammary epithelial cells and acquire nutrients makes the pathogen an even harder target for the treatment of such infection (Cheng and Han 2020).

***Streptococcus agalactiae*** is a gram-positive coccus, facultative anaerobe, non-spore-forming, and catalase-negative. *S. agalactiae* is also known as Group B *Streptococcus* (GBS). It was first differentiated from other streptococci by Rebecca Lancefield after being isolated from milk and cows with bovine mastitis (Lancefield 1933). *S. agalactiae* was classified into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII) based on the capsular polysaccharide (Raabe and Shane 2019). Besides, the bacterium was found in the bovine gastrointestinal tract. *S. agalactiae* is the subclinical mastitis case with increased SCC and low milk production. This pathogen can survive unreservedly in the mammary glands of cows by forming biofilm results

in the pathogen adhere and it remains in the mammary gland, concomitantly enhancing resistance to host factors and nutrient deprivation (Ebrahimi et al. 2013).

***Mycoplasma* spp.** is a group of bacteria that lack a cell wall and less common than infection of other contagious, *S. aureus* and *S. agalactiae*. However, this pathogen is highly severe and derogate to secretory tissue, resulting in induced lymphatic node fibrosis and abscesses. Moreover, it is self-limiting. It produces biofilm and invades a host cell as well as it does not respond to antibiotic treatment. There is only control of *Mycoplasma* infection is usually regular monitoring and rapid segregation or culling of an infected cow (Cheng and Han 2020).

**2.2.2 Environment bacteria**

***Escherichia coli*** is gram-negative bacilli bacteria that is the most frequently found gram-negative pathogen. It invades the udder through the teat, proliferate and initiate an inflammatory response in a dairy cow. It can be found in the dairy cow's environment, as building and bedding of the herd, especially condition with humidity (Smith and Hogan 1993) . Mastitis from *E. coli* infection is usually clinical and ephemeral. Symptoms are ranging from mild with local signs as a red and swollen udder to severe of systemic signs as fever. Besides, clinical mastitis caused by *E. coli* can cause irreversible tissue damage of the mammary gland, outright milk production loss, sometimes even leading to the death of the dairy cow. *E. coli* rapidly induces an inflammatory response in the cow. The virulence factor to trigger the inflammatory response is the endotoxin, which is found on the outer membrane of *E. coli*, called as lipopolysaccharide (LPS). The binding of LPS to a toll-like receptor in association with other molecules, such as LPS-binding protein and cluster of differentiation 14 induces a series of signaling pathways (Chow et al. 1999). TLR4 engagement

activates myeloid differentiation and recruit members of the interleukin-1 receptor to lead to inflammation.

Nevertheless, *E. coli* infection is classified as an opportunistic pathogen with various virulence factors as toxins, invasins, adhesins, capsule production, ability to resist serum complement and iron scavenging. These factors are necessary to defeat the host's selection pressure and colonize as well as survive in the udder and cause inflammatory responses (Kaper, Nataro, and Mobley 2004). Besides, *E. coli* can remain in the mammary gland tissue, results in recurrent mastitis infections that are difficult to treat, possibly due to the ability to produce biofilm at different levels.

***Streptococcus uberis*** is an environmental pathogen that causes recurrent mastitis, associated with clinical and subclinical infections. It is reported that the α-casein and β-casein components in milk induce biofilm production, and it helps *S. uberis* persist under environmental stress and resist antibiotic treatment. It has been detected in different parts of animals, including lips, skin, teat orifice, infected udders, teat canals, feces, and wounds (Krömker et al. 2014).



***Klebsiella* spp.** is gram-negative bacilli, an important opportunistic pathogen. In the dairy industry, *K. pneumoniae* is a known cause of primary environment-derived *Klebsiella* mastitis. Clinical mastitis is classified as the condition where an animal displays the physical symptoms of mastitis, milk production, and quality. Moreover, it has reported a significant increase in bacteriological cure after using antimicrobials for treating non-severe cases of *Klebsiella*-associated clinical mastitis. Mastitis negatively affects milk production and generally cows which do not normally recover production levels post-recovery (Podder et al. 2014).

***Enterococcus faecalis*** is the predominant *Enterococcus* spp., followed by *Ent. faecium*. They are environmental gram-positive bacterial pathogens that present in the organic bedding material of the herd. Both *E. faecalis* and *E. faecium* are resistant to several antibiotics due to biofilm formation (Cheng and Han 2020).

## 2.3 Treatment of bovine mastitis

**2.3.1 Five point-plan** is introduced by National Institute for Research in Dairying (NIRD) in the 1960s, which effectively controls contagious mastitis pathogens (Breen 2019). The five points are identified and treat clinical cases, post milking teat disinfection, Dry *cow* therapy (DCT), cull chronic cases and routine maintenance of milking machine (Bradley 2002). Regrettably, the five-point plan is not very effective against environmental pathogens. Therefore, this method should be coupled with other appropriate strategies to control mastitis infections (Down et al. 2016).

**2.3.2 Antibiotic is** also known as antibacterial, medications that kill the bacteria or slow down the growth of bacteria.



**2.3.2.1 Mechanisms of Antibiotic action** was divided into many functions that consist of inhibition of cell wall synthesis, disruption of cell membrane function, inhibition of protein synthesis, inhibition of nucleic acid synthesis and block or inhibit metabolism, as shown in Figure 2.

- **Inhibition of cell wall synthesis** by attacking and damaging the cell wall and inducing a pore on the cell wall caused by unbalance of an influx of water leads to cell thinks and bursts. These antibiotics as the beta-lactam group, cephalexin.

- **Disruption of cell membrane function** by the binding cell membrane and induce membrane permeability. Then, leads to leakage of the cell. Daptomycin

can insertion of calcium-independent and induce depolarization of cell membrane and destroys ion-concentration gradient.

- **Inhibition of protein synthesis** by attacking bacterial ribosomes that function to produce protein and then inhibit protein formation. The antibiotics as streptomycin, tetracycline which inhibit 30s ribosome, Macrolide, which inhibits 50s ribosome.

- **Inhibition of nucleic acid synthesis** by attacking DNA-related enzymes of bacteria. These antibiotics as ciprofloxacin, ofloxacin, quinolones, and fluoroquinolones.

- **Block pathway and inhibit metabolism** as sulfonamides; these drugs inhibit folic acid synthesis of bacteria. Since the synthesis is inhibited, bacterial growth is inhibited.

**2.3.2.2 Antibiotic treatment in mastitis cow** commonly uses beta-lactam group antibiotics that is the function to inhibit cell wall biosynthesis such as penicillin, ampicillin, tetracycline, gentamycin, etc.; moreover, they are used from appropriate antibiotics follow from species of pathogens. For drug administration, antibiotics are given by intra-mammary infusion, intramuscular or intravenous injections (Gomes and Henriques 2016).

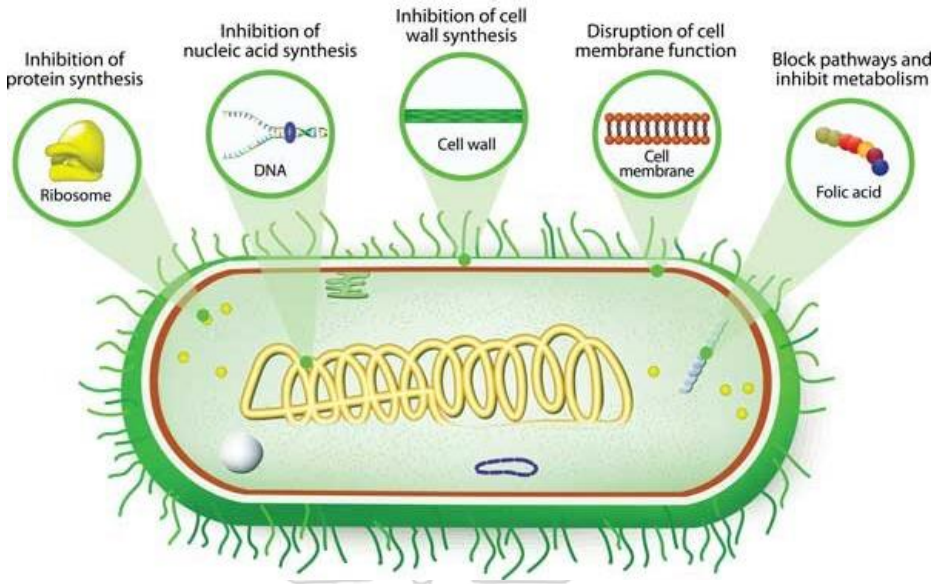



**Figure 2** Mechanisms of antibiotic action

(Calvo and Martínez-Martínez 2009)

**2.3.3 Vaccination** is a preventive mastitis treatment in herds. Mainly, vaccines are designed for *S. aureus*, *S. agalactiae* and coliform bacteria. The category of vaccines can be divided into three types based on field used as marketed vaccines, autogenous (herd-specific) vaccines, and experimental vaccines. The most commonly targeted udder pathogens are *S. aureus*, *S. agalactiae*, and *E. coli*. Vaccines which are against *S. aureus* and *S. agalactiae* contained either the whole organism (cellular lysates, inactive, and attenuated vaccines) or subunits (toxins, surface proteins, and polysaccharides) while for *E. coli*, the mutant core antigen J5 was used most commonly. Vaccines are also classified as mono or polyvalent according to the number of targeted pathogens that they contained (Ismail 2017). There are currently produced and using vaccines for mastitis followed by Table 3.

**Table 3** Summary of mastitis vaccine by type, trade name, manufacturer and administration

| Vaccine type | Trade name | Manufacturer | Administration |
|---|---|---|---|
| Coliform | ENVIRACOR™ J-5 | Zoetis | 3 shots: At 7 and 8 months of gestation and within 2 weeks of calving; 5cc SC or IM/shot |
| | J-VAC® | Merial | 2 shots: At dry-off and a boost 1 to 3 weeks prepartum; 2cc SC or IM/shot |
| | ENDOVAC-Dairy® | Immvac Inc. | 2 shots: During dry period and boost 2 or 3 weeks later; 2cc (IM)/shot |
| *S. aureus* | Lysigin® | Boehringer Ingelheim Vetmedica, Inc. | 3 shots: 5cc IM; boost 14 days later, and at 5-6 months |
| *Mycoplasma* | Mycomune® | AgriLabs | 3 shots: First 2 are 2 weeks apart followed by a last shot 2 to 3 weeks prepartum; 2cc SC/shot |

(Nickerson 2019)



## 2.4 Risk and side effects of antibiotic treatment

Antibiotics are the main and important treatment for bovine mastitis due to their strong and rapid killing and inhibiting virulent pathogens. Thus, proper dose and type of antibiotic with pathogens species should be a concern. However, the problem about antibiotic-resistant is a major global health concern due to the fact that inappropriate antibiotic usage in a cow with mastitis lead to adaptation of pathogens inducing genetic mutation which has antibiotic-resistant gene results in pathogens resistant to antibiotics; a major problem for bovine mastitis treatment. Moreover, the risk of using antibiotics is antibiotic residues in the cow that can spread to other farms or widespread pass farm workers and food processors and contaminated in milk products leading to antibiotic resistance, as shown in Figure 3.

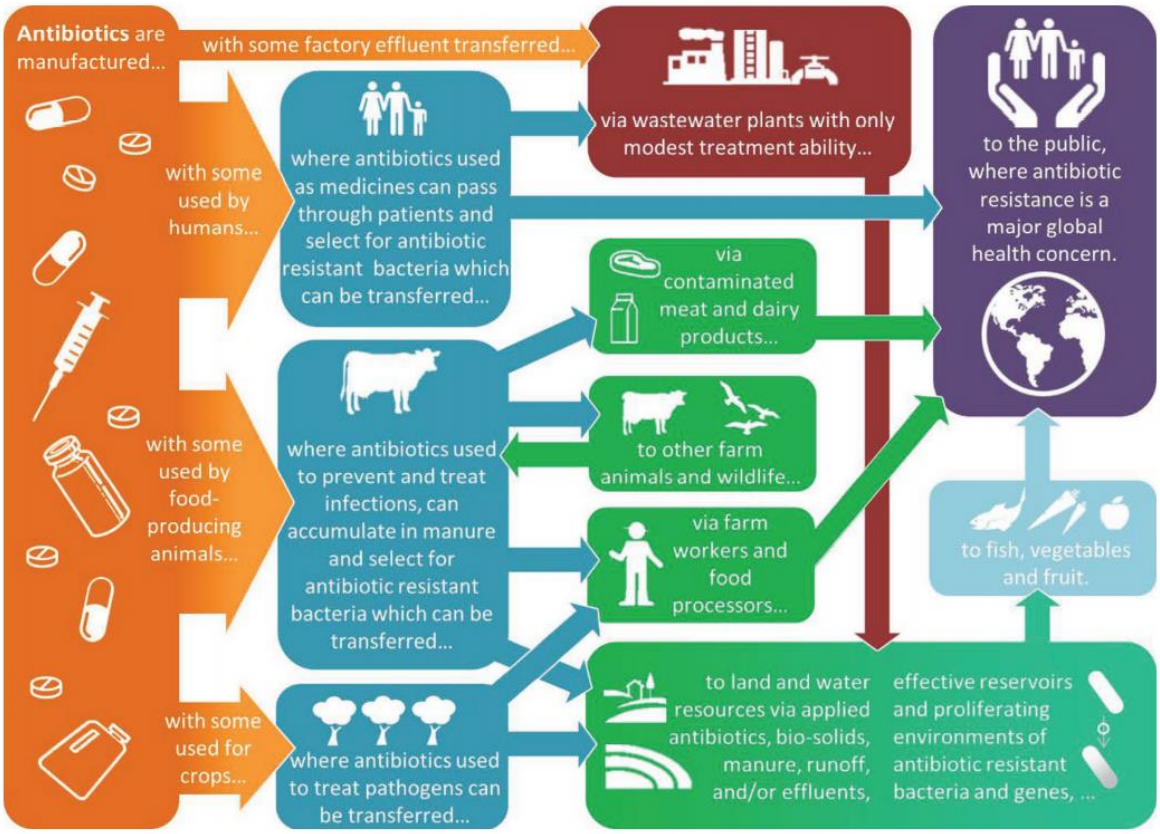



**Figure 3** Anthropogenic sources of antibiotic resistance and potential transmission routes to human populations

(Oliver et al. 2020).



### 2.5 Potential of Alternatives treatment

As mentioned above, even though antibiotics remain the main treatment strategy, their effectiveness is limited, not to mention the development of antibiotic-resistant strains of the pathogen has become a vital challenge in antibiotic treatment (Fair and Tor 2014; Suriyasathaporn et al. 2012). Besides, the increasing concern of antibiotic resistance in public health issues push the milk industries to reduce antimicrobial drugs. Therefore, seeking an alternative to antibiotic therapy, especially

natural alternative strategies, can be divided into several categories. The first category is plant-derived compounds, various plant-extracts that have been reported to antimicrobial properties. *Terminalia chebula* is a tree in the Combretaceae family which the ethyl acetate extract of the plant exhibit antimicrobial activity against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Bacillus megaterium* (Jäkel et al. 2012). Moreover, baicalin is a flavonoid extracted from *Scutellaria baicalensis* with pharmacological actions, baicalin's bacteriostatic mechanisms which is able to inhibit *E. coli* strains isolated from mastitic milk (Le, Fang, and Sekaran 2017). The second category is a phage therapy by using the bacteriophages (phages) or virus that propagate at the expense of bacteria. The phages can occupy the host cell systems, results in the synthesis of phage components and assembly to new phages within the bacteria host cell leads to bacterial cell lysis and the release of phage progeny that can begin a second phage cycle. Bacteriophages can be used for the alternative treatment of infections by various bacteria as *S. aureus*, *P. aeruginosa* and *Salmonella*. SalmoShield$^{TM}$ is the bacteriophages cocktail that is approved to treat burn wounds infected by *P. aeruginosa* (Souza et al. 2020). Some limits of this category are the occurrence of resistant development and releases of bacterial endotoxins. The third category is predatory bacteria such as Bdellovibrio (BALOs) has been considered a promising alternative to antibiotics treatment (Harini, Ajila, and Hegde 2013). These organisms are d-proteobacteria that multiply only upon entering Gram-negative pathogens such as *E. coli*, *Pseudomonas*, *Salmonella*. BALOs degrade prey cells by lysing them with various hydrolytic enzymes (such as DNAses and proteases). The next category is antibodies that oppose the invasion of pathogens. The immune system produces antibodies that recognize specific components of the pathogen and

neutralize them. Antibodies could be used to treat bacterial infections either by directly targeting the bacterial surface or indirectly by neutralizing the bacterial toxins and the virulence factors responsible for infection (Baumgarth, Tung, and Herzenberg 2005). The final category is antimicrobial peptides which is one of the best candidate of naturally alternative treatment with strong antimicrobial properties, include peptides from plant, vertebrate animal, mammalian, amphibian, insect, aquatic and microorganisms have been studies and developed for alternative antimicrobial treatment. As compared to antibiotics, antimicrobial peptides have the advantage of not inducing resistance even after prolonged exposure. Another advantage of antimicrobial peptides is their low toxicity and broad-spectrum activity against bacteria viruses and molds (Huan et al. 2020).

## 2.6 Antimicrobial peptides

As mentioned above, antimicrobial peptides (AMPs) are the alternative treatment to treat microbial infection in nowadays (Sheena Ramdeen and Boucher 2015 ). Over the past decade, many pieces of research using derived peptides from organisms to treat a variety of diseases are conducted. AMPs are oligopeptides with a varying number of amino acids, also known as host defense peptides. The AMPs substances are obtained from a component of the innate immune system in the host (Scott et al. 2002). The majority of AMPs belong to 12-50 amino acids. Besides, the AMPs have high efficiency against virulent microorganisms.



### 2.6.1 Classification of antimicrobial peptides

The classify of major categories of antimicrobial peptides based on source are peptides from plant, vertebrate animal, mammalian, amphibian, insect, aquatic and

microorganisms. Moreover, antimicrobial peptides based on the activity that inhere in antibacterial activity, antibacterial activity accounts for a large proportion of AMPs and has a board spectrum activity to common pathogenic bacteria, such as *Acinetobacter baumannii*, *Streptococcus* sp. and MRSA in clinical medicine and *S. aureus*. Many natural and synthetic AMPs like defensins, cecropins and nisin have exhibited strong inhibition activity to Gram-positive and Gram-negative bacteria. Antiviral Peptides (AVPs) show a strong killing effect on viruses mainly by (1) inhibiting virus attachment and virus-cell membrane fusion, (2) destroying the virus envelope, or (3) inhibiting virus replication. Antifungal Peptides (AFPs) are a subclass of AMPs that address fungal infections with enhanced drug resistance. Many AFPs have shown great anti-fungal activities against common pathogenic fungi, such as *Candida albicans*, *Aspergillus* and filamentous fungi (Huan et al. 2020).

### 2.6.2 Structure and Characteristic of antimicrobial peptide

The characteristic of the antimicrobial peptide is hydrophobic and hydrophilic part in a molecule that is known as an amphipathic molecule. Moreover, the peptide has a unique and diverse group of molecules based on their amino acid composition and structure. Therefore, antimicrobial peptides are classified into five subgroups (Brogden 2005).



**2.6.2.1 Anionic peptides** are a subgroup that contains anionic antimicrobial peptides. Among these are small peptides present in surfactant extracts, bronchoalveolar lavage fluid and airway epithelial cells. They are required zinc as a cofactor for antimicrobial activity and are active against both Gram-positive and Gram-negative bacteria such as Maximin H5 and Dermcidin.

**2.6.2.2 Linear cationic α-helical peptides** are Cecropins A, andropin, moricin, ceratotoxin and melittin from insects. Cecropin P1 from *Ascaris* nematodes. Magainin 2, dermaseptin and buforin II from amphibians. Pleurocidin from skin mucous secretions of the winter flounder. LL37 from humans, CAP18 from rabbits.

**2.6.2.3 Cationic peptides enriched for specific amino acids** are proline- and arginine-containing peptides including apidaecins, drosocin, pyrrhocoricin and bactenecins. Proline- and phenylalanine-containing peptides are prophenin. Glycine- and proline-containing peptides are coleoptericin and holotricin. Tryptophan-containing peptides is indolicidin. Small histidine-rich salivary polypeptides are histatins.

**2.6.2.4 Anionic and cationic peptides that contain cysteine and form disulphide bonds** are peptides with 1 disulphide bond including brevinins. Peptides with 2 disulphide bonds are protegrin from pigs and tachyplesins. Peptides with 3 disulphide bonds include α-defensins. Peptides with more 3 disulphide bonds are drosomycin and antifungal defensins.



**2.6.2.5 Anionic and cationic peptide fragments of larger proteins** are lactoferricin from lactoferrin, casocidin I from human casein. Antimicrobial domains from bovine are α-lactalbumin, human hemoglobin, lysozyme and ovalbumin.

**2.6.3 Mechanisms of antimicrobial peptide**

**2.6.3.1 Extracellular mechanisms (Membrane targeting mechanisms)**

Each peptide has a unique structure, making the different polarities property classified on a cationic peptide and anionic peptide. The efficiency of peptides against pathogens has been reported. Since the particular structure and type of the amino acids, the element can damage pathogens by electrostatic interaction between the

peptides and pathogen surface (Lei et al. 2019). In cationic peptides, the positive charge of peptide binds with negative charge of lipopolysaccharide on the outer membrane of gram-negative bacteria or binds with teichoic acid on the cell wall of gram-positive bacteria. In the case of anionic peptides, they use the metal ion to form a cationic salt bridge bind with a negative charge in the bacterial cell wall. After that occurs, the peptide binds to the cytoplasmic membrane. They induce interaction between the peptide and phospholipid bilayer, causing pore at the cytoplasmic membrane by three models: the toroidal pore model, barrel-stave model, and carpet model, as shown in Figure 4.

- **Toroidal pore model**, the building of peptides with the bacterial membrane followed by cascade aggregation and insertion inside the cell membranes, results in an induced change of the bacterial phospholipid monolayer until a ring hole of 1 to 2 nm in diameter is formed, ultimately leads to bacterial cell damage and death. For example, melittin, magainin 2 and lacticin Q peptide.

- **Barrel-stave model,** the peptides bind to the bacterial membrane surface different from the toroidal model in that the monomer of peptide insert to the cell membrane in parallel arrange into phospholipid molecule of the membrane. The hydrophobic groups of peptides are embedded in the cell membranes to form a pore structure, which results in the bacterial cell contents overflowing and bacteria dying. Examples include alamethicin peptides.

- **Carpet model,** AMPs change the surface tension of the bacterial membranes to deform the membranes due to the cumulative effect of peptides interact with the outer membrane. Eventually, lead to the disintegration of the cell membranes such as aurein peptide.

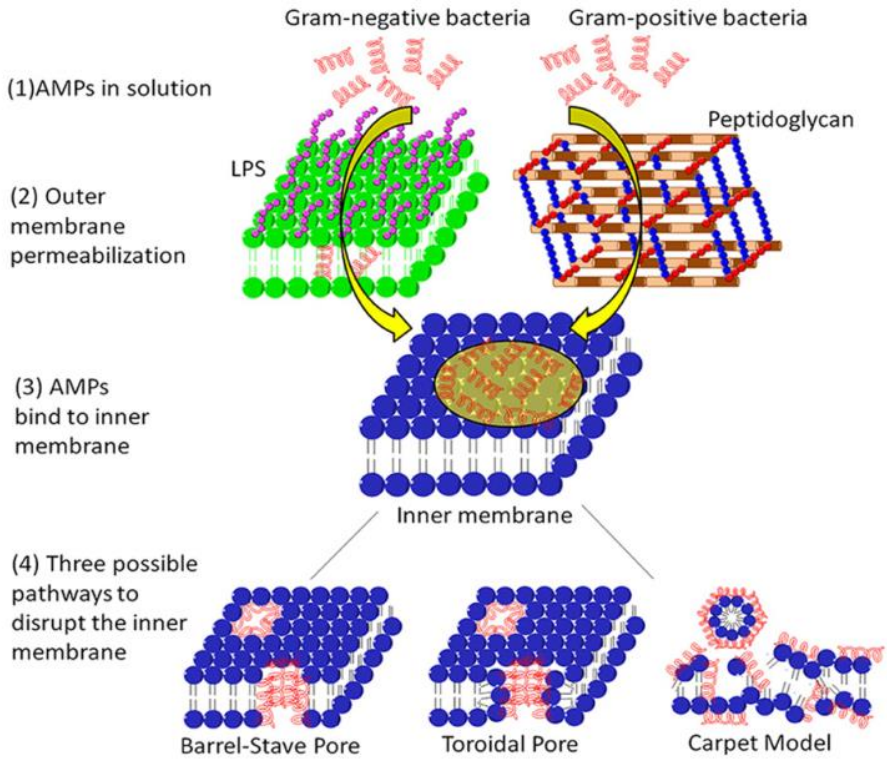



**Figure 4** Extracellular mechanisms of action of antimicrobial peptides (Lei et al. 2019)



**2.6.3.2 Intracellular mechanisms (Non-membrane targeting mechanisms)**

The cationic AMPs exhibit the potential to damage pathogen cells. Particularly, peptides in cathelicidins and the alpha-helical cationic peptides family are not only the cause of bacterial membrane holes but they also affect to intracellular mechanisms. The intracellular mechanisms are divided into 6 major mechanisms as indicated in Figure 5. They are (1) nucleic acid biosynthesis inhibitors by crosslink with single-stranded or double-stranded DNA or block the enzymes involved with DNA synthesis, (2) protein biosynthesis inhibitors by targeting at ribosomes cause inhibit protein translation, (3) protein-folding inhibitors by inactivation of chaperone pathway as heat shock protein 70 (HSP70) or DnaK, (4) cell division inhibitors by

inhibition of DNA replication, SOS induction, chromosome segregation or failure of septation process, (5) cell wall biosynthesis inhibitors by the peptides binding to lipid II as necessary precursor and then oppose the transglycosylation process in cell wall biosynthesis, (6) protease inhibitors by against protease secretion of the bacteria, which proteases present many physiological functions ranging from generalized protein degradation to more specific and regulated processes (Le, Fang, and Sekaran 2017).

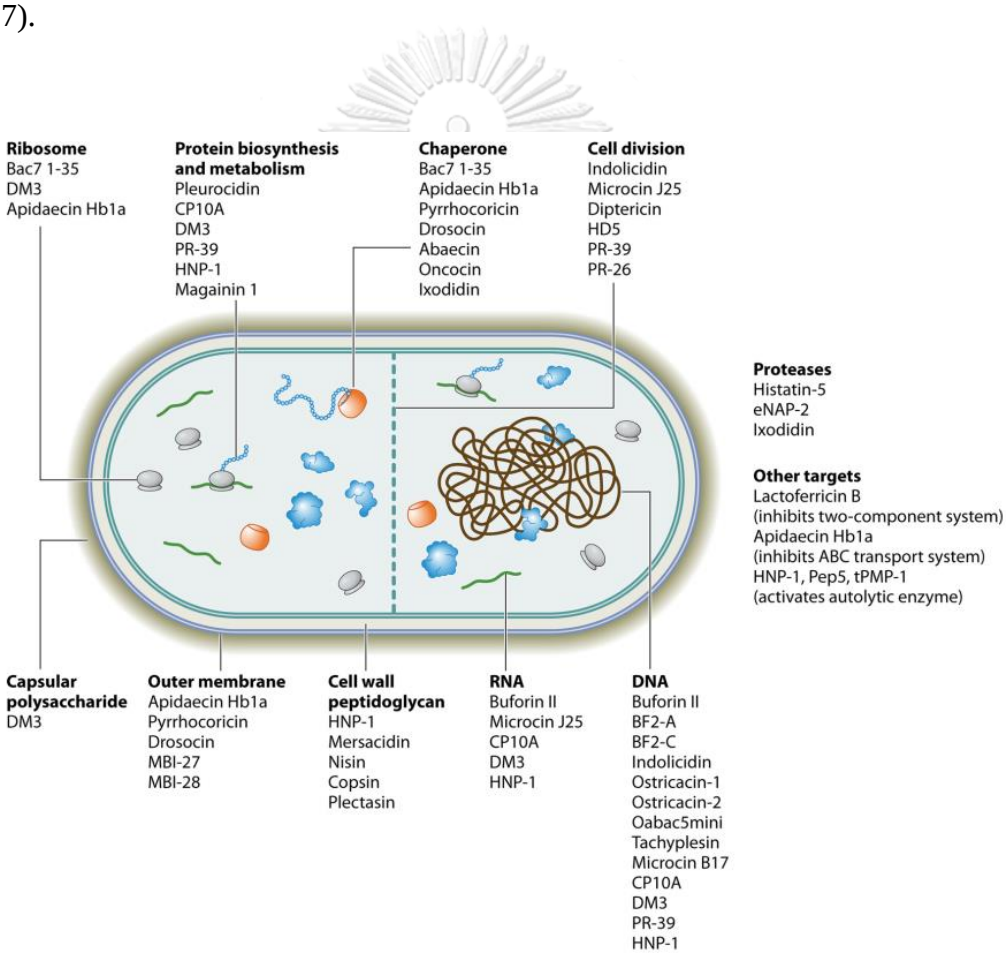



**Figure 5** Illustration showing the major pathways targeted by AMPs in bacterial cells.

(Oliver et al. 2020)

### 2.6.4 Peptide characteristics involving antimicrobial activity and specificity

**2.6.4.1 Size and sequence** of AMPs generally between 12 to 50 amino acids and short AMPs between 2 to 15 amino (Papagianni 2003; Ramesh et al. 2016). The sequence of AMPs often contains lysine or arginine amino acid residues and hydrophobic residues as leucine, alanine, phenylalanine or tryptophan. Other residues such as tyrosine, isoleucine and valine (Brogden 2005). The amino acid sequence with electrically charge side chain and hydrophobic side chain both affect the peptide's amphipathicity to pore formation in bacteria.

**2.6.4.2 Charge** of AMPs, cationic peptides are rich in lysine and arginine amino acids. The positive charge of peptides affects electrostatic interaction between AMPs and bacterial membrane. Anionic peptides are rich in aspartic and glutamic acids and complexed with zinc. The highly cationic peptides are often more active than neutral peptides or those with a lower charge.

**2.6.4.3 Conformation and structure** of AMPs have a variety of secondary structures that compose alpha-helices, relaxed coils, and antiparallel beta-sheet structures. Amphipathic alpha-helical peptides and peptides with gamma-core motifs are often more antimicrobial activity than peptides with less-defined secondary structures.



**2.6.4.4 Hydrophobicity** characteristic of peptides enables water-soluble antimicrobial peptides to partition into the membrane lipid bilayer. Moreover, amphipathicity characteristics of peptide contain hydrophilic amino acid residues aligned along one side and hydrophobic amino acid residues aligned along the opposite side of a molecule that affects antimicrobial activity.

### 2.6.5 Antimicrobial peptide production

**2.6.5.1 Expression of an antimicrobial peptide** by a generation of recombinant protein from the expression vector. The appropriate relationship between the recombinant protein and host should be a concern for transformation and expression. Mostly yeast is a candidate host eukaryotic protein expression. Moreover, the expression process should be concern about proper optimization of the host to produce recombinant protein.

**2.6.5.2 Synthesis of antimicrobial peptides**: To face global health concerns, synthetic antimicrobial peptides have arisen due to various advantages, such as potent activity and low production cost. Thus, many research groups have been seeking new synthetic antimicrobial peptides. As a consequence, it has been created to predict and characterize synthetic antimicrobial peptides that employing some criteria from natural antimicrobial proteins/peptides, such as positive net charge, hydrophobicity and helicity. The most important advantage of designing synthetic antimicrobial peptides from natural sequences is the gain of function. Since they can present activities that are not present in the original model sequence and reduce toxicity and allergenicity, it is possible to remove these specific sequences (Souza et al. 2020).



However, the production of peptide depends on the suitability of factors such as the type, length and characterization of the peptide and the cost-effectiveness of production.

### 2.6.6 Short antimicrobial peptide

The short antimicrobial peptides (SAMPs) are a small peptide with 2-15 amino acids in length (Ramesh et al. 2016) that also have ability to against the pathogens, whereas the study of the potential of the small peptides which can transmit

through microbial membranes has been reported, The smaller size may simplify access to target sites on the pathogen surface (Mishra et al. 2013) and SAMPs act as natural ligands for many cell surface receptors is the cause of action against of wind range of pathogen strains (Sharma et al. 2018). Furthermore, the SAMPs is a convenience to synthesize by chemical techniques and low cost.

**2.6.7 Cationic antimicrobial peptides (CAMPs)** are prominent peptides with the potential of antimicrobial activity due to the effectiveness of electrostatic interaction between the positive charge of the peptide and a negative charge of the bacterial membrane. The example of a cationic peptide with strong potential antimicrobial activities including

**2.6.7.1 Pleurocidin peptide** is a peptide from the alpha-helix cationic family found in skin mucous secretions of winter flounder (*Pleuronectes americanus*) (Cole, Weis, and Diamond 1997). It can cause bacterial membrane holes and plague intracellular mechanisms of pathogens by inhibit protein, DNA and RNA synthesis causing a reduction of thymidine, uridine and histidine integration. Pm11 is a derived peptide from pleurocidin peptide that has a strong potential against *in vitro Streptococcus* spp. causing oral disease (Le, Fang, and Sekaran 2017)



**2.6.7.2 Lactoferrin peptide** is the bovine neutrophil peptide from cathelicidins family. Lactoferrin affect the iron mechanism of bacteria by binding with free iron causing a limit amount of iron for bacteria. Lactoferrin peptide exhibit strong antimicrobial action to tacking pathogen membrane and inhibit the two-component process in intracellular bacteria that involved the perception and response of microorganisms to change many variation environmental conditions (Lönnerdal and Iyer 1995; Stock, Robinson, and Goudreau 2000). L10 peptide is the peptide-

derived from modification N-terminal of bovine lactoferrin with activities to kill multi-drug resistance gram-negative bacteria (Le, Fang, and Sekaran 2017)

**2.6.7.3 Lactoferricin peptide** is derived from the multifunctional protein. Lactoferrin (LF) through proteolysis by pepsin under acidic conditions. Lactoferricin encompasses a large portion of the functional domain of the intact protein, and in many cases, it not only retains the activities of lactoferrin but is more active. Lactoferricin possesses strong antimicrobial and potent antitumor and immunological properties (Gifford, Hunter, and Vogel 2005). Pep64 is the lactoferricin-based pentapeptide, which exhibits activities against *E. coli*, *B. subtilis* and *S. aureus* (Sharma et al. 2018).

## 2.7 The biological functions and safety of antimicrobial peptides

**2.7.1 Cytotoxicity** of cationic AMPs show low or non-toxicity in eukaryotes cell because of cationic AMPs interacting with the negative charge of the bacterial membrane leading to bacterial cell death but it does not interact with eukaryotes cell membrane, neutral cell membrane. Moreover, the cationic peptides mainly affect the survival of cancer cells via targeting cell membrane, organelle (mainly mitochondria), lysosome, nucleus, chromosomal DNA, and cytoskeleton. In many pieces of research, it has been demonstrated that cancer cells are more sensitive to AMPs than normal cells (Lei et al. 2019).



**2.7.2 Natural immunity** of the cationic antimicrobial peptides is the important role in the hosts. They not only kill the bacterial pathogens that invade the host, but also show their various functions of the natural immune responses. CAMPs are involved in the host defenses associated with acute inflammation. They can induce

bacterial lysis, promote phagocytosis of macrophages, prevent infection spreading, stimulate mitosis of fibroblasts and epithelial cells, and promote fibroblast growth to enhance wound healing. They can activate the lymphocytes of the host to eliminate the cells infected with viruses and bacteria and cancer cells. These AMPs also play a role in chronic inflammation (Lei et al. 2019).

**2.7.3 Broad spectrum of antimicrobial activities**, not only antimicrobial act to pathogens, but also the peptides are against viruses and fungal. The peptide antivirus directly interacts with virions. The peptides also inhibit the proliferation of viruses, so mimic the infectious process of the viruses. For example, peptides may result in the viral damage via interfering with the assembly process of the viruses. These cationic peptides can effectively destroy parasites that lead to parasitic diseases in humans and animals, such as malaria, dysentery, and other protozoa (Zanetti 2004; Mirski et al. 2017). Many AMPs also displayed their antifungal activities in addition to their antibacterial activity.

Moreover, the safety of AMPs are concern in clinical applications such as toxicity, immunogenicity, drug resistance, hemolytic activity and other side effects (Moravej et al. 2018). This is one of the reasons why they are rare to be used as drugs. Therefore, how to improve their activity and safety is highly concerned in AMP drug research and development. Scientists attempt to find new cationic peptides or modify and enhance the natural AMPs to obtain more effective and safer AMPs as potential drug candidates.

**2.8 The prospective of AMPs in clinical application**

More and more accumulated evidence shows that the long-term use of antibiotics which results in drug resistance of microbes. Many drug-resistant pathogenic strains have been identified to correspond to each of these traditional antibiotics. Thus, it is becoming much more difficult to find a new antibiotic. AMPs display their broad spectrum and high bactericidal activities. The appearance of these peptides provides us a golden opportunity to develop the potential antimicrobial peptide drug candidates instead of traditional antibiotics. The truth is that several AMPs have been approved by Food and Drug Administration (FDA) Table 4.

Cyclic lipopeptide, Daptomycin with 13 residues is also known as LY146032 are approved by FDA in 2003 to treat gram-positive bacteria infectious diseases. Daptomycin has potent to bind with the bacterial cell membrane and disrupt the cell membrane. Besides, lipoglycopeptides class that consists of oritavancin, telavancin, and dalbavancin are small lipoglycopeptide that derived from vancomycin (approved in 1983 by the FDA). They are efficient against vancomycin resistance – bacteria and inhibit bacterial cell wall formation. Moreover, telavancin and oritavancin also disrupt bacterial cell membranes and affect membrane permeability. Orbactiv (oritavancin), Dalvance (dalbavancin), and Vibativ (telavancin) are used for injection against complicated skin and skin structure infections (cSSSI) caused by *S. aureus*, as well as other Gram-positive bacterial infections, and are approved by the FDA in 2014, and 2009 respectively (Chen and Lu 2020).

**Table 4** The antimicrobial peptide drugs approved by Food and Drug Administration

| Drugs | Trade names | Antimicrobial activities | Administrations | In use |
|---|---|---|---|---|
| Bacitracin | Baciim | Gram-positive bacteria | Topical | Localized skin and eye infections, wound infections |
| Dalbavancin | Dalvance, Xydalba | Gram-positive bacteria | Intravenous | Acute bacterial skin infections |
| Daptomycin | Cubicin | Gram-positive bacteria | Intravenous | Bacterial skin infections |
| Enfuvirtide | Fuzeon | Virus | Subcutaneous | HIV-1 infection |
| Oritavancin | Orbactiv | Gram-positive bacteria | Intravenous | Bacterial skin infections |
| Teicoplanin | Targocid | Gram-positive bacteria | Intravenous and intramuscular | Bacterial infections |
| Telaprevir | Incivo, Incivek | Virus | Oral | Hepatitis C |
| Telavancin | Vibativ | Gram-positive bacteria | Intravenous | Bacterial skin infections |
| Vancomycin | Vancocin | Gram-positive bacteria | Oral and intravenous | Bacterial infections |

(Lei et al. 2019)

# CHAPTER III

# MATERIALS AND METHODOLOGY

## 3.1 Materials

### 3.1.1 Chemicals and reagent

| Chemicals and reagent | Company, Country |
|---|---|
| Absolute methanol | Merck, U.S.A. |
| Agar | Hi media, India |
| di-Sodium hydrogen phosphate | Merk, Germany |
| Dimethyl sulfoxide | Bio basic, Inc., Canada |
| 95% ethanol | Sacsci, Thailand |
| Hydrochloric acid | Merck, U.S.A. |
| Potassium chloride | Kemaus, Australia |
| Potassium dihydrogen phosphate | Merk, Germany |
| Sheep blood (Defibrinated) | Clinag co., Ltd, Thailand |
| Sodium chloride | Thermo Scientific, Australia |
| Sodium hydroxide | Merk, Germany |
| TritonX-100 | Fluka, Germany |
| Tryptic soy agar powder | Merk, Germany |
| Tryptic soy broth powder | Merk, Germany |
| Tryptone type I powder | Hi media, India |
| UHT milk | Thai Denmark, Thailand |
| Yeast extract powder | Hi media, India |

### 3.1.2 Equipment and supplies

| Equipment | Company, Country |
|---|---|
| Autoclave | Hirayama manufacturing crop., Japan |
| Bench-top centrifuge | MWP medical Instrument, Poland |
| Biological safety cabinet class II (Heal force®, HFsafe-1200) | Shanghai Lishen Scientific equipment Co., Ltd., China |
| Centrifuge tube 50 ml | Labcon, U.S.A. |
| Dish 90 mm | Clinag co., Ltd, Thailand |
| Freezer (-20°C) (SF-C697) | Sanyo Commerial Solution |
| Freezer (-80°C) | Spl life science, South Korea |
| Hard loops 10 µl | P intretrade, U.S.A. |
| Hot plate | Corning, U.S.A. |
| Incubator (MIR152) | Sanyo Electric Co., Ltd., Japan |
| Incubator shaker | New Brunswick Scientific Co., Inc., U.S.A. |
| Micro centrifuge tube | Labcon, U.S.A. |
| Micropipette | Eppendrof Co., Ltd., Germany |
| Microplate reader | BioTek Instruments, Inc., U.S.A. |
| Microwave oven | Matsushita Electric Industrial Co. Ltd., Japan |
| pH meter (Accumet® AB15) | Fisher Scientific, Singapore |
| Refrigerator | Panasonic Appliances Lights Action Alliance Co., Ltd., Thailand |
| Vortex mixture (KMC-1300V) | Vision scientific Co., Ltd., Korea |
| 96 well micro title plate | Labcon, U.S.A. |

### 3.1.3 Peptides and Antibiotic

| Peptides and Antibiotic | Company, Country |
|---|---|
| Ampicillin antibiotic | Sigma-Aldrich, |
| Oxacillin antibiotic | Sigma-Aldrich, |
| L10 peptide | GenScript Biotech, U.S.A. |
| Pep64 peptide | GenScript Biotech, U.S.A. |
| Pm11 peptide | GenScript Biotech, U.S.A. |

### 3.1.4 Media

- Luria-Bertani (LB) Agar medium was used as a enrich medium for bacterial lab strains and used for bacterial subculture every 3-4 weeks.
- Tryptic soy broth (TSB) medium was used as a enrich medium for bacterial lab strains and bovine mastitis pathogens growth. The medium was used for antimicrobial activities assay as minimum bactericidal concentration and time kill assay.
- Tryptic soy agar (TSA) medium was used as a enrich medium for bacterial lab strains and bovine mastitis pathogens growth. The medium was used for colony plate count in minimum bactericidal concentration assay, time kill assay and bacterial sub-cultured before antimicrobial activities step.

The medium compositions and preparation were described in appendix A.

### 3.1.5 Microorganisms

- Bacterial lab strains: *Escherichia coli* DH5alpha and *Bacillus subtilis* ATCC6633 were obtained from the Institute of Biotechnology and Genetic engineering, Chulalongkorn University and used for antimicrobial activities assay.

- Bovine mastitis pathogens: *Escherichia coli* strain (SCM1249), *Klebsiella* spp. strain (SCM1282), *Staphylococcus aureus* strain (CM967), *Streptococcus agalactiae* strain (SCM1084), *Streptococcus uberis* strain (SCM1310) were obtained from Faculty of Veterinary Science Chulalongkorn University which isolated from cows with mastitis and used for antimicrobial activities assay.

### 3.1.6 Synthesis and preparation of all synthesized peptides

Three peptides that derived from Lactoferrin (L10) 8 amino acid, WFRKQLKW, Pleurocidin (Pm11) 13 amino acid, WFKFFKKFFKKWK and Lactoferricin (Pep64) 5 amino acid, RRWWR were commercially synthesized by GenScript with purity >95%. The peptides were dissolved in solvent as stock solutions for all assays.



- L10 peptide: The concentrations of L10 peptide were performed by 2 mg/vial synthesized peptide powder was dissolved into DMSO 200 µl and then two-fold serially dilution by ultrapure water to final concentration of peptide at 2000.0, 1000.0, 500.0, 250.0 and 125.0 µM

- Pep64 peptide: The concentrations of Pep64 peptide were performed by 2 mg/vial synthesized peptide powder was dissolved into ultrapure water and then two-fold serially dilution by ultrapure water to final concentration of peptide at 2000.0, 1000.0, 500.0, 250.0,125.0, 62.5, 31.2 and 15.6 µM

- Pm11 peptide: The concentrations of Pm11 peptide were performed by 1 mg/vial synthesized peptide powder was dissolved into ultrapure water and then two-fold serially dilution by ultrapure water to final concentration of peptide at 80.0, 40.0, 20.0, 10.0, 5.0, 2,5, 1.2, 0.6, 0.3 µM

## 3.2 Methodology

### 3.2.1 Antimicrobial activity assay

#### 3.2.1.1 Determination of minimum bactericidal concentration (MBC)

Determination of minimum bactericidal concentration (MBC) assay was performed according to (Kaushal et al. 2016) with modifications. This method was followed from Figure 6.

The MBC defined as the lowest concentration of the peptide that killed 99.9% of the tested bacteria.

3.2.1.1.1 Bacterial cells preparation

- Calibration curve of bacterial strains



The single colony of bacteria was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loops of bacteria after incubation were suspended in 5 ml of 0.85% sodium chloride and mix by a vortex. The mixture was adjusted to optical density 1.00, 0.50, 0.25, 0.12 and 0.06 at 600 nm. At various optical density, the bacterial cells were ten-fold serial dilution in PBS and aliquot 100 µl of each dilution spread on tryptic soy agar. Then, the plates were incubated at 37°C, overnight. Then, colony forming unit was calculated and a graph between optical density at 600 nm and CFU/ml of bacteria was established (appendix C). The experiments were performed in triplicate and the mean values were interpreted.

- Preparation of $10^5$ CFU/ml of bacterial strains

The single colony of *E. coli* DH5alpha was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loops of bacteria after incubation were dissolved in 5 ml of 0.85% sodium chloride and the mixture was adjusted to optical density 0.1 at 600 nm After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.

The single colony of *B. subtilis* was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loops of bacteria after incubation were dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.2 at 600 nm and dilute $10^{-1}$ in tryptic soy broth in a test tube and mix by a vortex.

The single colony of *E. coli* (SCM1249) was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loop of bacteria after incubation was dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.1 at 600 nm. After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.



The single colony of *Klebsiella* spp. strain (SCM1282) was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loop of bacteria after incubation was dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.1 at 600 nm. After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.

The single colony of *S. aureus* strain (CM967) was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loop of bacteria after incubation was dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex.

The mixture was adjusted to optical density 0.05 at 600 nm. After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.

The single colony of *S. agalactiae* strain (SCM1084) was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loop of bacteria after incubation was dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.2 at 600 nm. After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.

The single colony of *S. uberis* strain (SCM1310) was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loop of bacteria after incubation was dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.1 at 600 nm. After that, the mixture was dilute to $10^{-2}$ in tryptic soy broth in a test tube and mix by a vortex.

3.2.1.1.2 Bacterial-Peptide Incubation

The serial two-fold dilutions of peptides solutions (form method 3.1.6) 100 µl were added to each well of 100 µl adjusted bacterial culture ($10^5$ CFU/ml) of bacterial strains (from method 3.2.1.1.1) in sterilized 96 well plate. Then, the mixtures were incubated for 3 h at 37°C, 100 rpm. After that ten-fold serial dilution was obtained in PBS and 100 µl of each dilution were spread on tryptic soy agar. The plates were incubated at 37°C, overnight. In this assay, TSB medium without bacteria and peptide was used as a negative control, bacterial culture without peptide was used as a untreated and bacterial culture with ampicillin was used as antibiotic control. The experiments were performed in triplicate and the mean values were interpreted.

3.2.1.1.3 Colony plate count

The plates after incubation (from method 3.2.1.1.2) were determined by colony plate count which count the plate between 30-300 colonies. Then, colony forming unit was calculated by the following formula according to (Fankhauser 1988) with modification.

$$\text{CFU/ml} = \frac{\Sigma C}{(0.1n1 + 0.01n2)d}$$

ΣC = Total of all countable colonies from plate range between 30-300 colonies

n1 = Number of plates that counted bacteria between 30-300 colonies at the first dilution

n2 = Number of plates that counted bacteria between 30-300 colonies at the second dilution

d = The first dilution that could be counted bacteria between 30-300 colonies

**3.2.1.2 Half maximal inhibitory concentration ($IC_{50}$)**

The half maximal inhibitory concentration ($IC_{50}$) indicates how much particular inhibitory substance is needed to inhibit a biological process by half. $IC_{50}$ was calculated by GraphPad prism5 program. The graph correlation between percent inhibitory of peptide to bacterial strains as y-axis (vertical line) and final concentration of peptide as x-axis (horizontal line) were established.

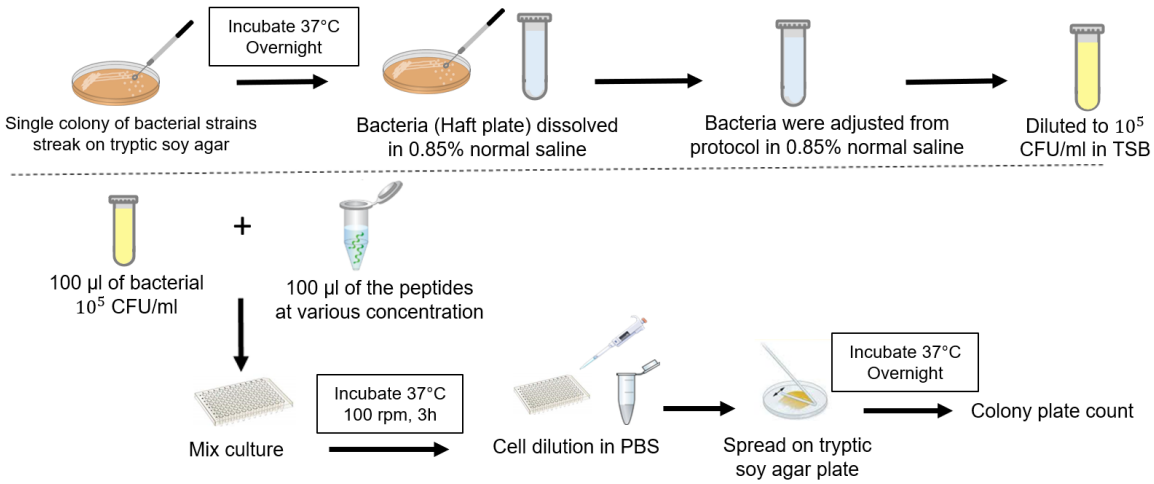



**Figure 6** Antimicrobial activity assay of peptides

### 3.2.2. Time kill assay of the peptide to bovine mastitis pathogens

Time kill assay of the peptide to bovine mastitis pathogens assay was performed according to (Li et al. 2017) with modification. This method was followed from Figure 7.

3.2.2.1 Peptide preparation

The concentration of Pm11 peptide was performed by 1 mg/vial synthesized peptide powder was dissolved into ultrapure water or PBS and then adjusted concentration of peptide at 2MBC ( Preparing peptide at 2MBC in order to give the final concentration in this method is 1MBC). The concentration of peptide at 2MBC was followed by MBC of each bovine mastitis pathogens.

3.2.2.2 Bacterial cells preparation

The single colony of bovine mastitis pathogens were streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loops of bacteria after incubation were dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The

mixture was adjusted to a suitable optical density at 600 nm and diluted to $10^5$ CFU/ml from the protocol of each pathogen follow method 3.2.1.1.1

3.2.2.3 Bacterial-Peptide Incubation

The peptide solutions at 2 MBC concentration, volume 100 µl, were added to each well of 100 µl adjusted bacterial culture ($10^5$ CFU/ml) of bovine mastitis pathogens (from method 3.2.1.1.1) in sterilized 96 well plate. Then, the mixtures were incubated for 0, 1, 2, 4, 8, and 12 h at 37°C, 100 rpm. Ten-fold serial dilution of the mixtures were obtained in PBS and 100 µl of each dilution bacteria cells were spread on tryptic soy agar. Then, the plates were incubated overnight at 37°C, for colony plate count. TSB medium without bacteria and peptide was used as a negative control, bacterial culture without peptide was used as a untreated control. The experiments were performed in triplicate and the mean values were interpreted.

3.2.2.4 Colony plate count

The plates after incubation (from method 3.2.2.3) were determined by colony plate count which count the plate between 30-300 colony. Then, colony forming unit was calculated by the formula in method 3.2.1.1.3



3.2.2.5 Time kill kinetic graph

The graph correlation between colony plate count (CFU/ml) of bovine mastitis pathogens as y-axis (vertical line) and the time as x-axis (horizontal line) were established. In this assay, medium without bacteria and peptide used as a negative control, bacterial culture without peptide was used as a untreated control.

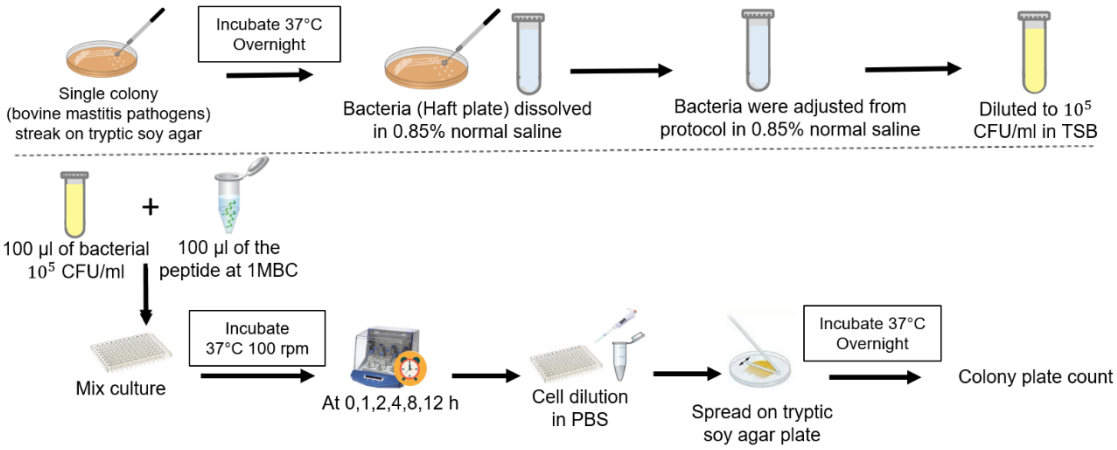



**Figure 7** Time kill assay of the peptide to bovine mastitis pathogens

### 3.2.3 Bactericidal activity of peptide to bacterial lab strain in UHT milk

Bactericidal activity of peptide in bovine milk was performed according to (Li et al. 2017) with modification. This method was followed from Figure 8.

#### 3.2.3.1 Activity of Pm11 peptide to E. coli DH5alpha in UHT milk

3.2.3.1.1 Peptide preparation

The concentration of Pm11 peptide was performed by 1 mg/vial synthesized peptide powder was dissolved into ultrapure water or PBS and then adjusted peptide concentration by two-fold serial dilution from 1056 to 33 µM

3.2.3.1.2 Bacterial cell preparation

The single colony of *E. coli* DH5alpha was streaked on tryptic soy agar and incubated at 37°C for 18 h. Then, the loops of bacteria after incubation were dissolved in 5 ml of 0.85% sodium chloride in a test tube and mix by a vortex. The mixture was adjusted to optical density 0.1 at 600 nm and diluted to $10^5$ CFU/ml in UHT milk.

3.2.3.1.3 Bacterial-Peptide Incubation

Twenty microliters of the various concentrations of Pm11 peptide were added to 180 µl of bacterial culture ($10^5$ CFU/ml) in UHT milk in sterilized 96 well plate. Then, the mixtures were incubated for 3 h at 37°C, 100 rpm. The mixtures after incubation were diluted by ten-fold serial dilution in PBS and 100 µl of each were spread on tryptic soy agar plate. Then, the plates were incubated at 37°C for overnight. Colony plate count were determined following method 3.2.1.1.3. UHT milk without bacteria and peptide was used as a negative control, bacterial culture in UHT milk without peptide was used as a untreated control. This experiment was compared with antimicrobial activity of Pm11 peptide to *E. coli* DH5alpha in TSB medium condition.

**3.2.3.2 The effect of protease inhibitor to bactericidal activity of Pm11 peptide**

3.2.3.2.1 Peptide preparation

The concentration of Pm11 peptide was performed by 1 mg/vial synthesized peptide powder was dissolved into ultrapure water or PBS and then the concentration of peptide was adjusted to 132 µM (10X of the final concentration).



3.2.3.2.2 Bacterial cell preparation

The *E. coli* DH5alpha cell preparation was described as 3.2.3.1.2.

3.2.3.2.3 Protease inhibitor preparation

Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was performed by dissolving 10 mg powder PMSF into 1 ml of absolute methanol to prepare the 10 mg/ml PMSF stock solution.

3.2.3.2.4 Bacterial-Peptide Incubation

The PMSF solution 4, 2, 1 and 0.5 µl of 10 mg/ml PMSF were added to each well of 176.0, 178.0, 179.0 and 179.5 µl adjusted bacterial culture ($10^5$ CFU/ml) in UHT milk respectively in sterilized 96 well plate. Then, 20 µl of the peptide solution from method 3.2.3.2.1 were added to each well. The final volume of each well is 200 µl. Then, the mixtures were incubated for 3 h at 37°C, 100 rpm. After incubation, the mixtures were diluted by ten-fold serial dilution in PBS and 100 µl of each dilution was spread on tryptic soy agar plate. The plates were incubated at 37°C, overnight. For colony plate count, the plates after incubation were determined followed from method 3.2.1.1.3. UHT milk without bacteria and peptide was used as a negative control, bacterial culture in UHT milk without peptide was used as a untreated control.

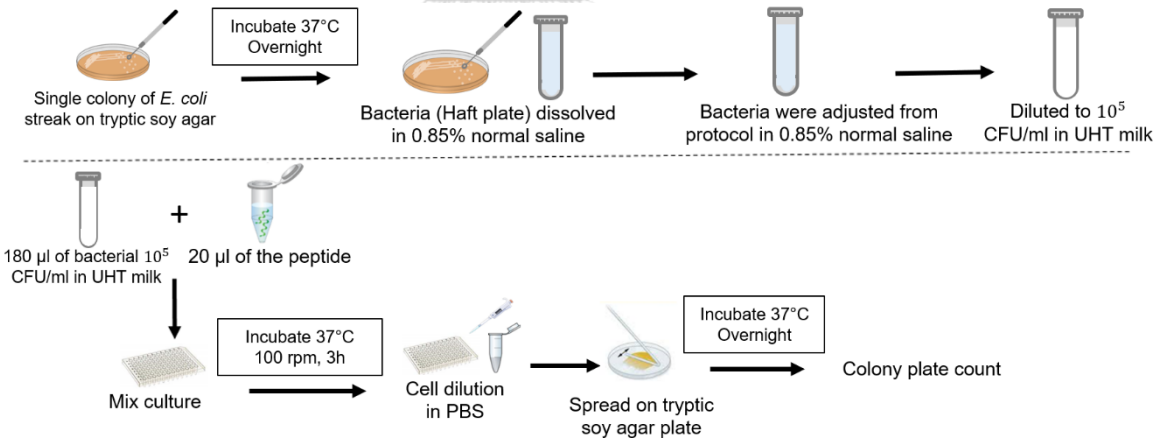



**Figure 8** Bactericidal activity of peptide to bacterial lab strain in UHT milk

### 3.2.4 Cytotoxicity of peptide

This method is evaluation the toxicity of peptide by measured the capability of peptide to lyse the sheep red blood cells, the suitable AMPs must show non or low

toxicity to sheep erythrocytes. The following three steps were carried out. This method was followed from Figure 9.

**3.2.4.1 Preparation of Pm11 peptide**

The concentration of Pm11 peptide performed by 1 mg/vial synthesized peptide powder was dissolved into phosphate buffer saline and then two-fold serial dilution at final concentration of peptide to 160, 80, 40, 20, 10, 5, 2,5, 1.2, 0.6, 0.3 µM

**3.2.4.2 Preparation of sheep red blood cell**

The fresh sheep red blood cell was performed by centrifugation of sheep blood to remove the buffy coat for 10 min at 3,500 rpm, 25°C. After centrifugation, the sheep blood was washed with phosphate buffer saline for 45 sec at 3000 rpm, 25°C until clear color. The supernatant was discarded and the pellet as sheep red blood cells was used for MHC assay in the next step (3.2.4.3).

**3.2.4.3 Measurement of minimum hemolytic concentration (MHC)**

Measurement of minimum hemolytic concentration (MHC) was performed according to (Mishra et al. 2013) with t modification. This method was followed from Figure 9.



The sheep red blood cells from method 3.2.4.2 were suspended in phosphate buffer saline by a ratio 4%(v/v). Then, 100 µl of suspended sheep red blood cells were added in 100 µl of Pm11 peptide at various concentrations (from method 3.2.4.1) in a sterilized 96 well plate. The mixture plate was incubated for 1 h, 37°C. After incubation, the mixture was centrifuged for 5 min at 1,000 g, 25°C. The supernatant after centrifugation, 100 µl was transferred to a new sterilized 96 well plate to measure hemoglobin released by a micro plate reader at optical density 414 nm. Then,

the percentage of hemolysis was calculated by the following formula. In this method, PBS was used as negative control (zero percent hemolysis) and 0.1% TritonX-100 was used as positive control (one-hundred percent hemolysis).

$$\% \text{ Hemolysis} = \{ ( A_{Sam} - A_{PBS}) / (A_{TX} - A_{PBS}) \} \times 100$$

$A_{Sam}$ = Absorbance value at 414 nm of reaction in the peptide solution

$A_{PBS}$ = Absorbance value at 414 nm of reaction in the PBS

$A_{TX}$ = Absorbance value at 414 nm of reaction in the TritonX-100

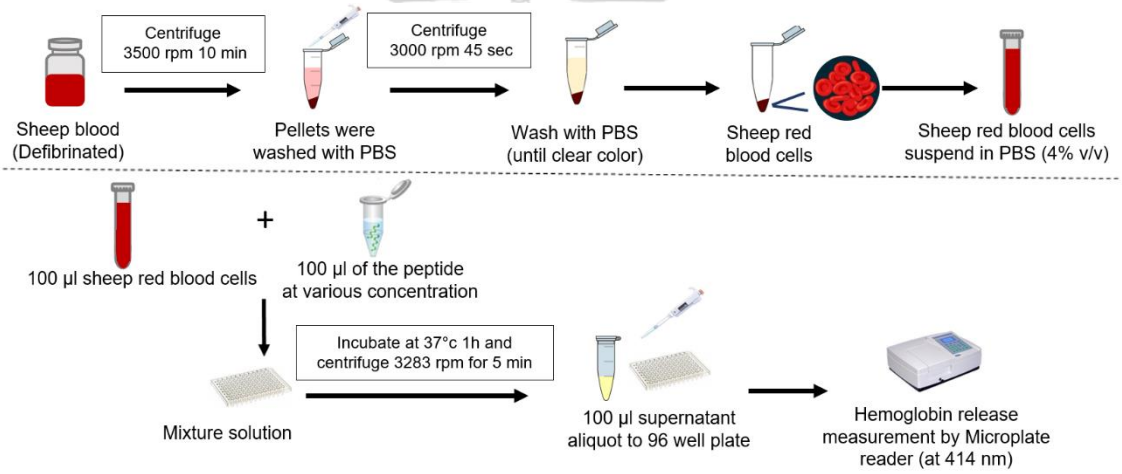



**Figure 9** Cytotoxicity of peptide: Measurement of minimum hemolytic concentration



### 3.2.5 Scanning electron microscopic study

The morphology change of bacterial cell damage was investigated when treated with the potential antimicrobial peptide, the following two steps were carried out. This method was followed from Figure 10.

**3.2.5.1 Preparation of bacterial cells sample**

The pathogens cells that represent Gram positive as *S. aureus* (CM967) ($10^7$ CFU/ml) and Gram negative as *E. coli* (SCM1249) ($10^6$ CFU/ml) were incubated with 1MBC of Pm11 peptide for 1.30 h. After that the mixtures were centrifuged for 10 min 10,000 rpm. Then, the supernatant was discarded. The cell pellets were fixed by mixing in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 and left at 4°C at least 4 h.

**3.2.5.2 Scanning electron microscopic observation**

The bacterial cells that suspended in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 (from method 3.2.5.1) were filtrated by used micro filter 0.2 µm diameter. The filtrated paper was dehydrated by the series concentration of ethanol as 25%, 50%, 75%, 95% and 100% ethanol, respectively. Then, the sample was drying by a critical point drying machine. After that mouth sample with glue or tape that was trapped on stap used as a base of sample. The sample that was trapped on stap was gold-coated by a sputter coater machine. Then, the sample was observed by scanning electron microscope. (This method was done by Scientific and Technological Research Equipment Centre)

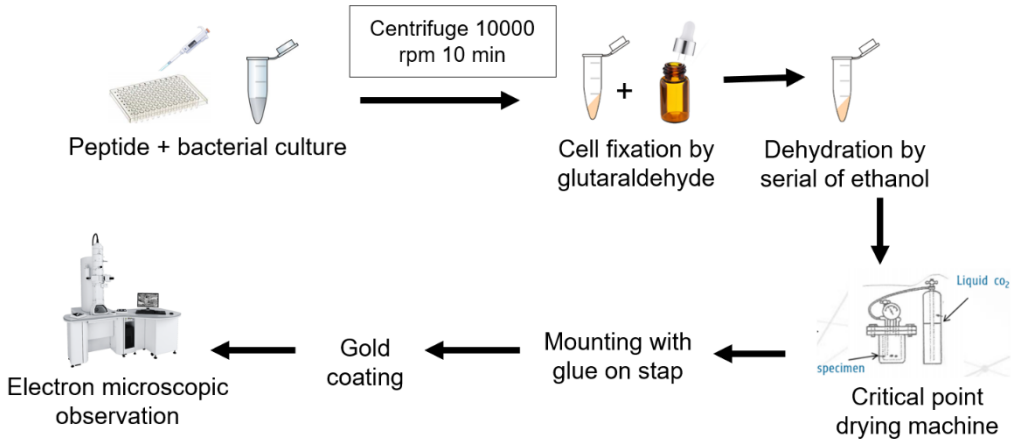



**Figure 10** Preparation of bacterial cells sample and Scanning electron microscopic observation

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Antimicrobial activity assay

### 4.1.1 Antimicrobial activity of peptides to bacterial lab strains

The antimicrobial of three selected peptides to bacterial lab strains were determined. The bacterial lab strains, *B. subtilis* ATCC6633 and *E. coli* DH5alpha were treated with the peptides as Pm11 (range 80.0 – 0.3 µM), L10 (range 2000.0 – 125.0 µM) and Pep64 (range 2000.0 – 15.6 µM). The percent bacterial inhibition of peptide was used for MBC value and $IC_{50}$ calculation by GraphPad Prism5 Program. Table 5 reported the antimicrobial activity consisting of MBC and $IC_{50}$ of three selected peptides to bacterial lab strains.

**Table 5** MBC and $IC_{50}$ of Pm11, Pep64, L10 peptides to bacterial lab strains

| Peptides | Sequence | *B. subtilis* ATCC6633 | | *E. coli* DH5alpha | |
|---|---|---|---|---|---|
| | | MBC (µM) | $IC_{50}$ (µM) | MBC (µM) | $IC_{50}$ (µM) |
| Pm11 | WFKFFKKFFKKWK | 5.0 | 1.05±0.07 | 2.5 | 1.08±0.14 |
| Pep64 | RRWWR | 2000.0 | 34.66±7.08 | 1000.0 | 42.38±2.74 |
| L10 | WFRKQLKW | 1000.0 | 178.60±11.35 | >2000.0 | 323.83±16.41 |

$IC_{50}$ value shown the mean ± standard error of the mean from three experiments.

Pm11 peptide was most effective against bacterial lab strains. The MBC of Pm11 peptide was 5.0 µM and $IC_{50}$ 1.05 µM in *B. subtilis*. The percent inhibition of Pm11 peptide against *B. subtilis* at various concentrations that were 4.49, 17.30, 61.07, 95.64% and 99.99% by 0.3, 0.6, 1.2, 2.5 and 5.0 µM, respectively (Figure 11). The MBC of Pm11 peptide to *E. coli* DH5alpha was 2.5 µM and $IC_{50}$ 1.08 µM. The

percent inhibition of Pm11 peptide to *E. coli* DH5alpha at various concentration are 55.65 and 99.95% by 1.2 and 2.5 µM respectively (Figure 12).

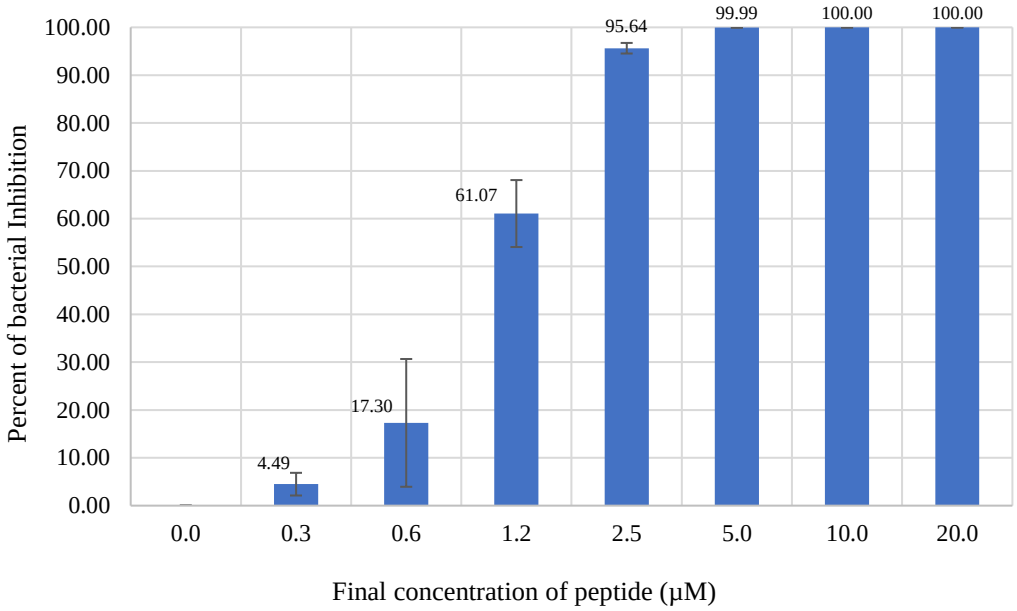



**Figure 11** Inhibition of Pm11 peptide at 0.3, 0.6, 1.2, 2.5, 5.0, 10.0, and 20.0 µM against B. subtilis ATCC6633 after 3 h incubation at 37$^{O}$C

The MBC of Pep64 peptide was 2000.0 µM and $IC_{50}$ 34.66 µM in *B. subtilis*. The percent inhibition of Pep64 peptide to *B. subtilis* at various concentrations were 37.29, 33.56, 43.82, 61.62, 84.17, 99.45 and 99.84% by 15.6, 31.2, 62.5. 125.0, 250.0, 500.0, and 1000.0 µM, respectively. The inhibition of Pep64 against *B. subtilis* 99.85% by 2000.0 µM (Figure 13). The MBC of Pep64 peptide to *E. coli* DH5alpha was 1000.0 µM and $IC_{50}$ 42.38 µM. The percent inhibition of Pep64 peptide to *E. coli* DH5alpha at various concentrations were 12.61, 55.96, 60.39, 59.60, 98.41, and 99.98% by 15.6, 31.2, 62.5. 125.0, 250.0, 500.0, and 1000.0 µM, respectively (Figure 14).

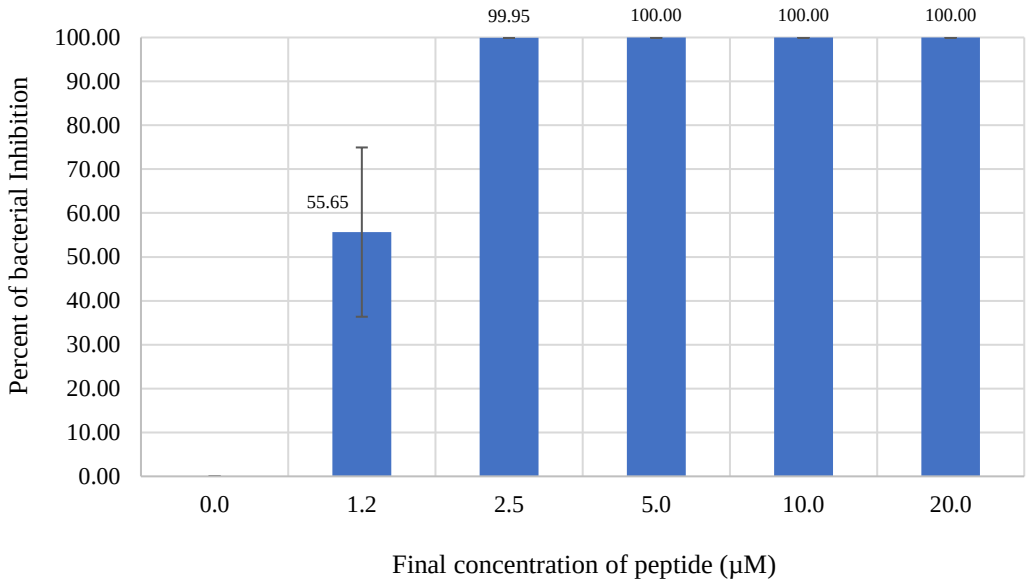



**Figure 12** Inhibition of Pm11 peptide at 1.2, 2.5, 5.0, 10.0, and 20.0 µM against E. coli DH5alpha after 3 h incubation at 37$^{O}$C

.

The MBC results of L10 peptide from Table 5 were 1000.0 µM and $IC_{50}$ 178.60 µM in *B. subtilis*. The percent inhibition of L10 peptide to *B. subtilis* at various concentrations were 19.90, 81.18, 99.83 and 99.98% by 125.0, 250.0, 500.0 and 1000.0 µM, respectively (Figure 15). Nevertheless, L10 peptide was dissolved in DMSO that toxic to bacterial cell, resulting in deactivate bacteria. Therefore, the inhibition rate of DMSO to bacterial lab strains were observed. The percents of DMSO at 1.5, 3.0, 6.0 and 11.9% were presented in 125.0, 250.0, 500.0 and 1000.0 µM of L10 peptide. moreover, the percents inhibition of DMSO at 1.5, 3.0, 6.0 and 11.9% were presented in 13.26, 36.42, 79.99 and 99.08%, respectively. *E. coli* DH5alpha was inhibited by L10 peptide with MBC more than 2000.0 µM and $IC_{50}$ 323.83 µM. The percent inhibition of L10 peptide against *E. coli* DH5alpha at various

concentrations that were 4.10, 38.54, 71.63, 93.71 and 97.41% by 125.0, 250.0, 500.0, 1000.0 and 2000.0 µM, respectively (Figure 16). However, the MBC of L10 peptide to *E. coli* DH5alpha was higher than 2000.0 µM, more than 90.0 % inhibition which was presented since 1000.0 µM. The percents of DMSO at 6.0, 11.9 and 23.8% were presented in 500.0, 1000.0 and 2000.0 µM of L10 peptide and the inhibition of DMSO at 6.0, 11.9 and 23.8% was 41.84, 93.32 and 96.69%, respectively.

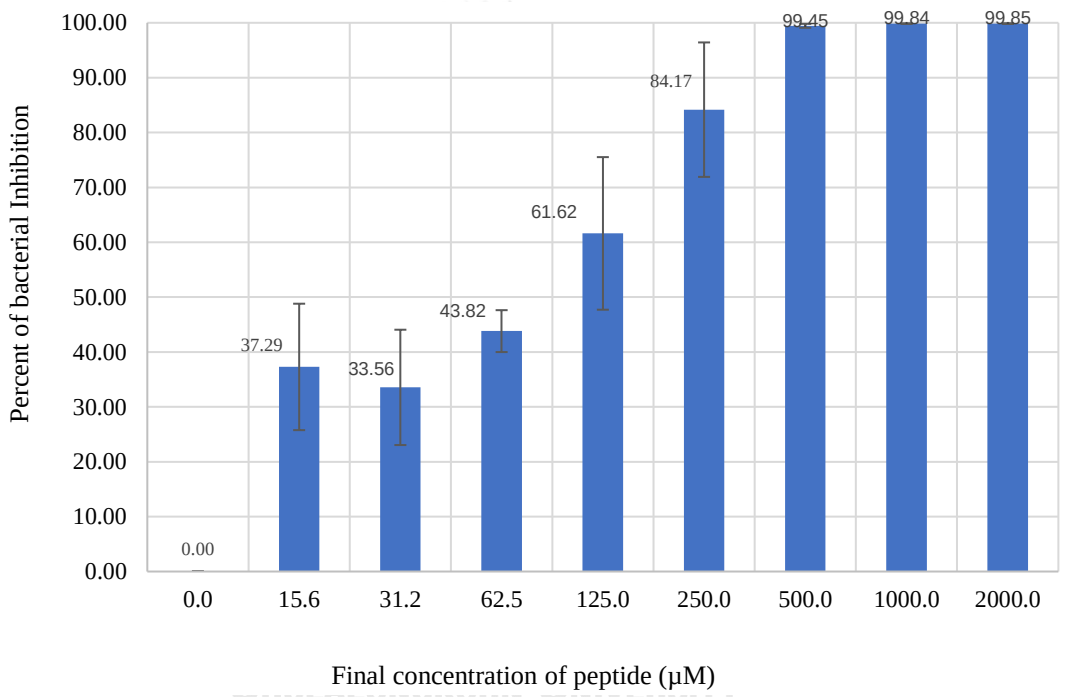



**Figure 13** Inhibition of Pep64 peptide at various concentration against B. subtilis ATCC6633 after 3 h incubation at 37$^{O}$C

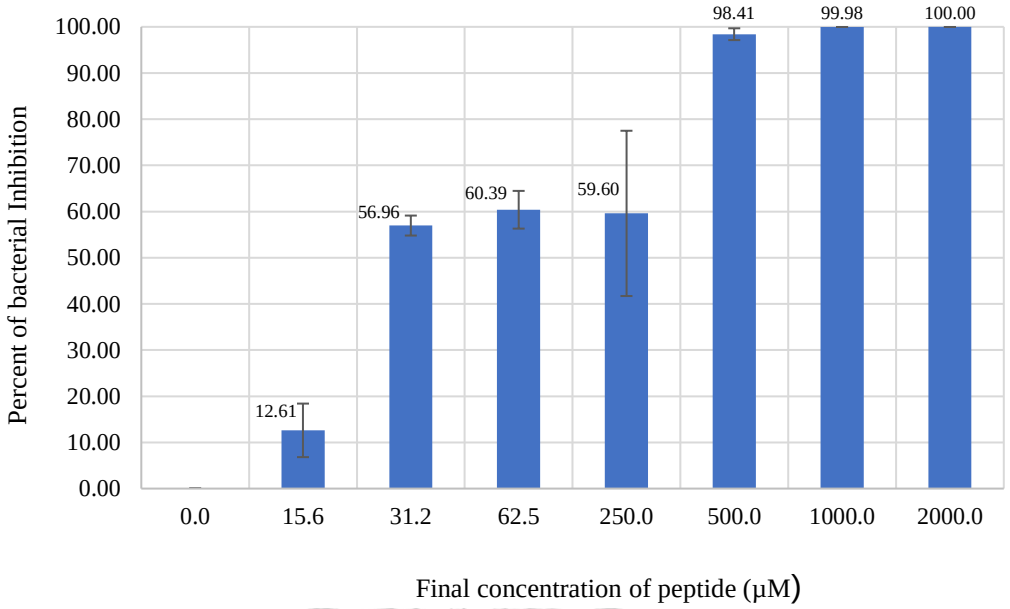



**Figure 14** Inhibition of Pep64 peptide at various concentration against E. coli DH5alpha after 3 h incubation at 37$^{O}$C

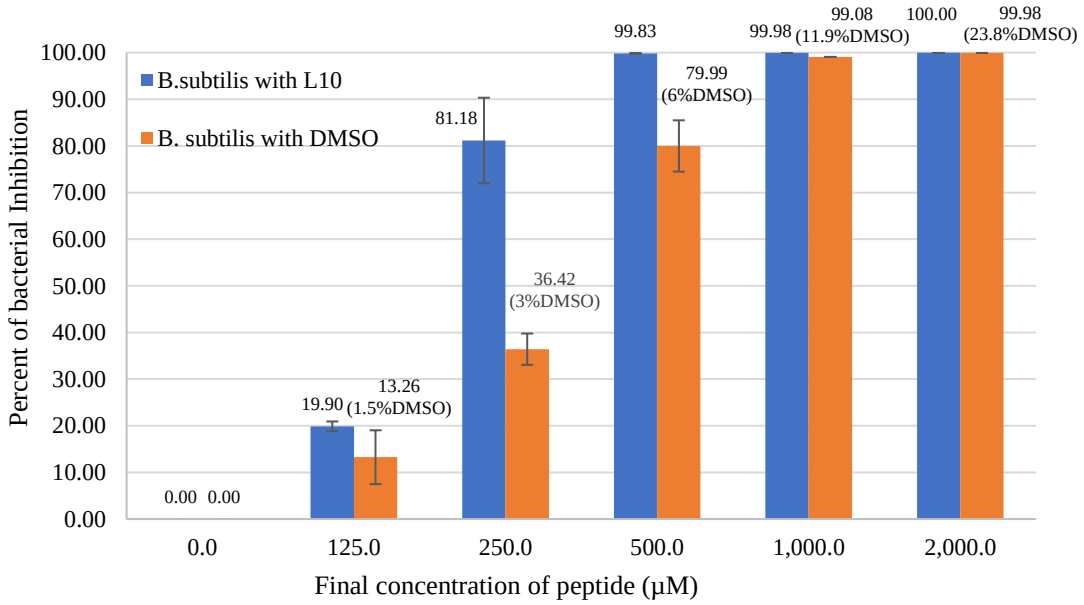



**Figure 15** Inhibition of L10 peptide that was dissolved with DMSO at various concentration against B. subtilis ATCC6633 after 3 h incubation at 37$^{O}$C.

The blue bars represent percent the effect of Pm11 peptide and orange represent of DMSO to *B. subtilis* ATCC6633.

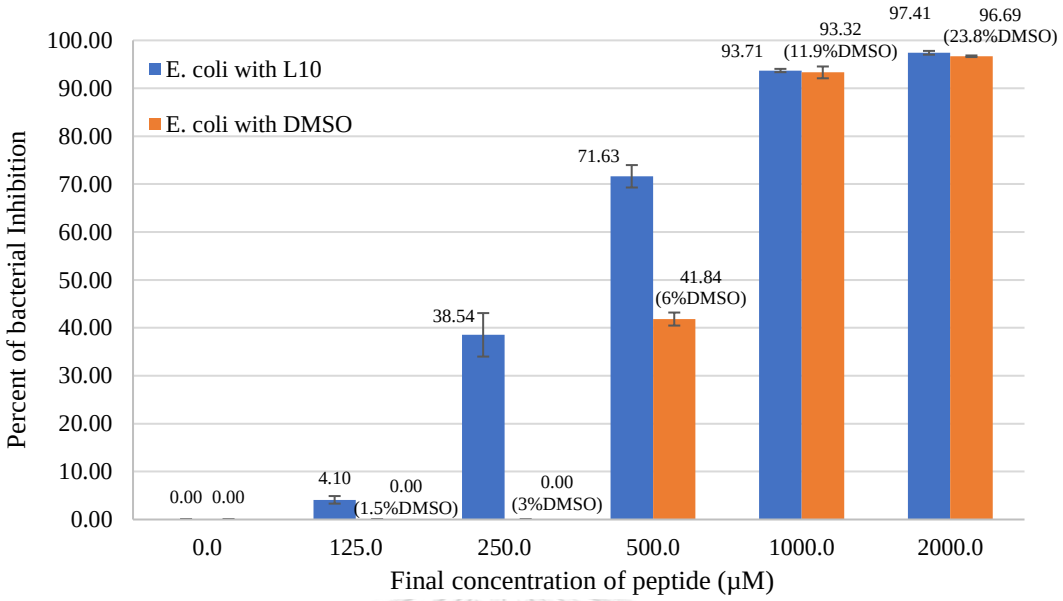



**Figure 16** Inhibition of L10 peptide that was dissolved with DMSO at various concentrations against E. coli DH5alpha after 3 h incubation at 37$^{O}$C.

The blue bars represents the percent of effect of Pm11 peptide and orange represents of DMSO to *E. coli* DH5alpha.

The percent inhibition of DMSO to deactivate bacterial lab strains demonstrates that *B. subtilis* ATCC6633 was inhibited by 1.5% DMSO and *E. coli* DH5alph was inhibited by 6.0% DMSO. The results correspond with Wadhwani et al. (2008) reported that the effect of DMSO on decreasing bacterial survival due to the fact that bacteria being exposed to stress by these solvents. All bacteria as *Staphylococcus epidermidis* MTCC 435, *Pseudomonas oleovorans* MTCC 617, Vibrio cholerae MTCC 3906, *Shigella flexneri* MTCC 1457 and *Salmonella paratyphi* exhibit significant decrease in growth when they exposed to DMSO at a concentration of 4% (Wadhwani et al. 2009). Moreover, Dyrda et al. (2019) reported that the viable count in cultures (%) of *B. subtilis* and *E. coli* were obviously inhibited when they exceeded the DMSO concentration of 4.8% v/v (Dyrda et al. 2019).

From the results, all of the selected peptides having cationic peptides that is reported that there is a strong antimicrobial activity with electrostatic interaction between a charge of peptide molecule and negative charge of the bacterial membrane. Moreover, Pm11 peptide has alpha-helix structure with +6 net charged, which is the highest net charge and hydrophobicity compared with Pep64 and L10 peptides, shown strong antimicrobial activity against lab strains. Pm11 peptide with lysine represented cationic and phenylalanine and tryptophan represented hydrophobic amino acid with important characteristic, amphipathic molecule of antimicrobial peptide. The effect of hydrophobicity of peptide was confirmed by Saint et al. (2018) reported that the peptide that composed of leucine, phenylalanine and isoleucine amino acid shown significantly greater ability to disrupt lipid vesicles and bacterial membranes (Saint Jean et al. 2018). Therefore, Pm11 peptide is the best candidate for further antimicrobial activity testing with bovine mastitis pathogens.

### 4.1.2 Antimicrobial activity of Pm11 peptide to bovine mastitis pathogens



Antimicrobial activity of Pm11 peptide to bovine mastitis pathogens were conducted after determining the MBC of the three selected peptides to bacterial lab strains. The Pm11 peptide was the most efficient activity to lab strains and would be used to determine MBC to bovine mastitis pathogens. The bovine mastitis pathogens consist of Gram-negative bacteria as *E. coli* (SCM1249) and *Klebsiella* spp. (SCM1282) and Gram-positive as *S. aureus* (CM967), *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) were treated with Pm11 peptide (range 80.0 – 0.3 µM).

From Table 6 represented the antimicrobial activity of Pm11 peptide consists of MBC and $IC_{50}$ of Pm11 peptide to the bovine mastitis pathogens. The strong

activity of Pm11 peptide was shown in *E. coli* (SCM1249) with MBC 2.5 µM and $IC_{50}$ 0.32 µM. The percent inhibition of Pm11 peptide to *E. coli* (SCM1249) at various concentration were 44.47, 88.42, 99.28 and 100.00% by 0.3, 0.6, 1.2 and 2.5 µM, respectively (Figure 17). Figure 18 shown a drop plate of *E. coli* (SCM1249) treated with Pm11 peptide at various concentrations. The results were demonstrated that Pm11 peptide at 2.5 µM complete elimination of bacterial colony that was inhibited when compared with untreated control. The pathogens, *Klebsiella* spp. (SCM1282) was inhibited by Pm11 peptide with 10.0 µM and $IC_{50}$ 2.07 µM. From Figure 19 represented the bacterial inhibition of Pm11 peptide against *Klebsiella* spp. (SCM1282) at various concentration were 44.07, 99.88 and 100.00% by 2.5, 5.0 and 10.0 µM, respectively. Figure 20 shown a drop plate of *Klebsiella* spp. (SCM1282) was treated with Pm11 peptide at various concentrations. The result displayed that Pm11 at 10.0 µM complete elimination of colony of bacteria that was inhibited when compared with untreated control. The antimicrobial activity against Gram-positive bovine mastitis pathogens, the MBC of Pm11 peptide against *S. aureus* (CM967) by 5.0 µM and $IC_{50}$ 1.33 µM with the percent bacterial inhibition 13.76, 97.15 and 99.98% by 0.6, 2.5 and 5.0 µM, respectively (Figure 21). Figure 22 shown a drop plate of *S. aureus* (CM967) treated with Pm11 peptide at various concentrations, at 5.0 µM of Pm11 showed complete elimination of the bacterial colony. Similarly, the MBC of Pm11 peptide against *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) were 5.0 µM and $IC_{50}$ 0.87 and 0.97, respectively. Figures 23 and 25 represented the percent bacterial inhibition of Pm11 peptide against *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) at various concentrations, *S. agalactiae* (SCM1084) was inhibited 36.77, 57.21, 99.66 and 100.00 % by 0.6, 1.2, 2.5 and 5.0 µM of Pm11, respectively.

*S. uberis* (SCM1310) was inhibited 34.88, 55.41, 99.56 and 100.00 % by 0.6, 1.2, 2.5 and 5.0 µM of Pm11, respectively. The Figure 24 and 26 shows drop plate of *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) were treated with Pm11 peptide at various concentration, at 5.0 µM of Pm11 shown complete inhibition of colony formation of bacteria.

**Table 6** MBC and $IC_{50}$ of Pm11 peptide to bovine mastitis pathogens

| **Bovine mastitis pathogens** | **MBC (µM)** | **$IC_{50}$ (µM)** |
|---|---|---|
| *Escherichia coli* (SCM1249) | 2.5 | 0.32 ± 0.03 |
| *Klebsiella* spp. (SCM1282) | 10.0 | 2.07 ± 0.47 |
| *Staphylococcus aureus* (CM967) | 5.0 | 1.33 ± 0.19 |
| *Streptococcus agalactiae* (SCM1084) | 5.0 | 0.87 ± 0.18 |
| *Streptococcus uberis* (SCM1310) | 5.0 | 0.97 ± 0.15 |

$IC_{50}$ value shown the mean ± standard error of the mean from three experiments.

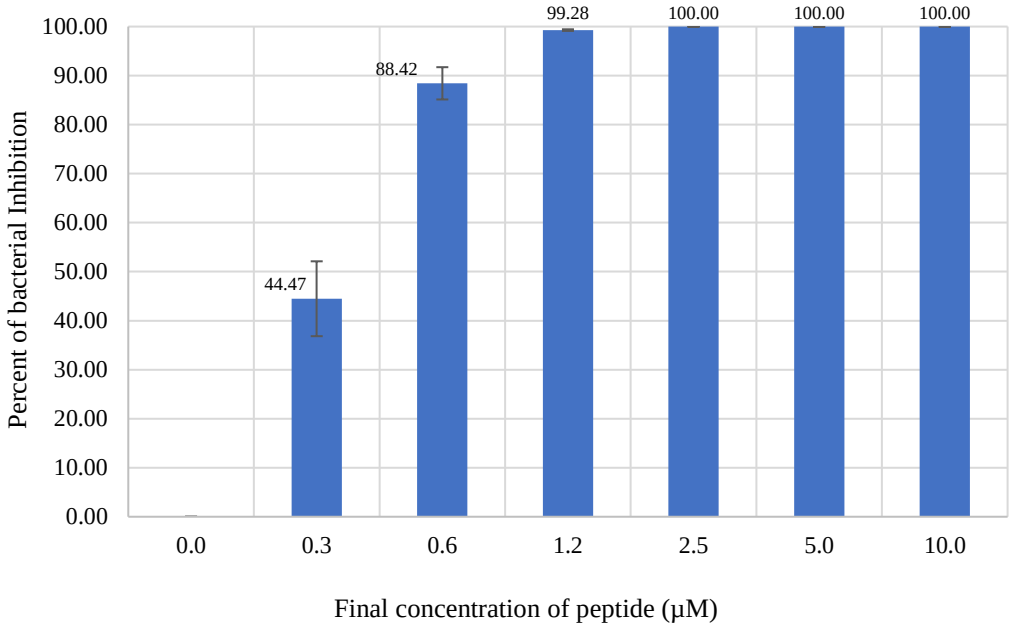



**Figure 17** Inhibition of Pm11 peptide at various concentrations against E. coli (SCM1249) after 3 h incubation at 37$^{O}$C

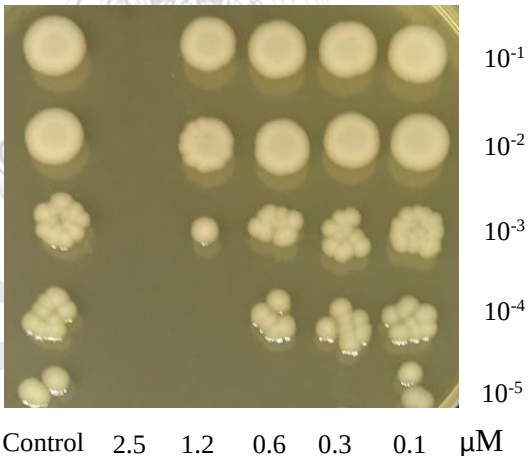



**Figure 18** Drop plate assay of Pm11 peptide against E. coli (SCM1249)

Pm11 was serially diluted and incubated with *E. coli* (SCM1249) for 3 h. The three microliters of 10 fold serial dilution of the treated bacteria were dropped on TSA plates and incubated at 37$^{O}$C overnight.

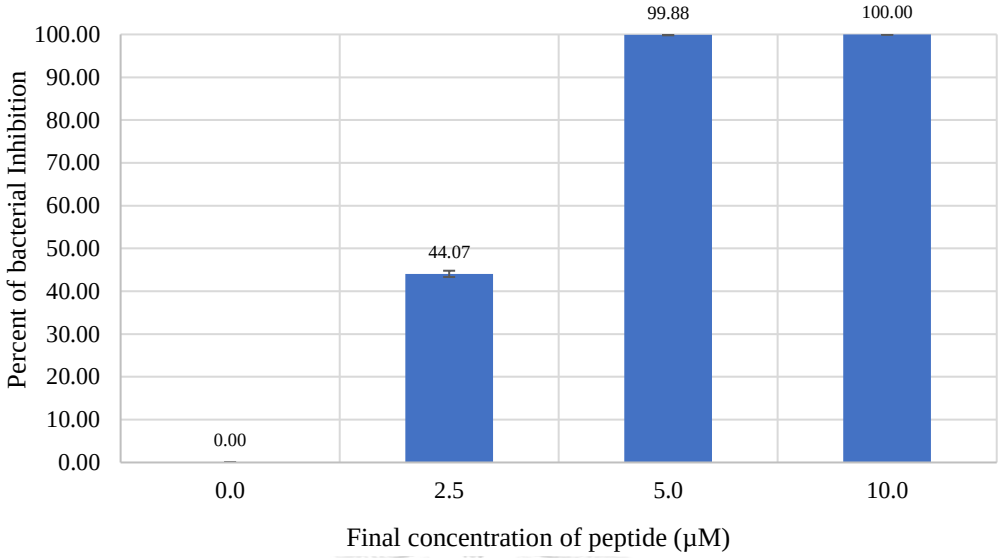



**Figure 19** Inhibition of Pm11 peptide at various concentrations against Klebsiella spp. (SCM1282) after 3 h incubation at 37$^{O}$C

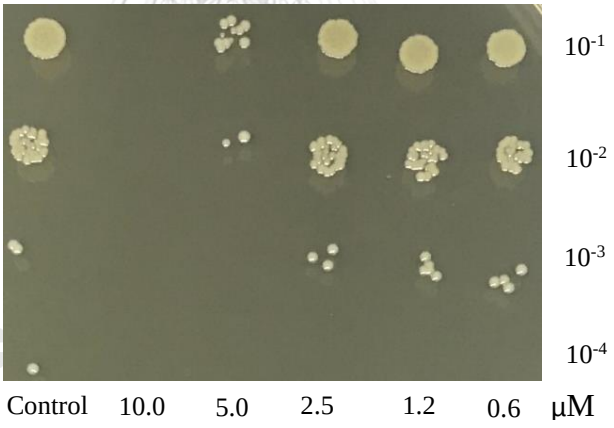



**Figure 20** Drop plate assay of Pm11 peptide against Klebsiella spp. (SCM1282)

Pm11 was serially diluted and incubated with *Klebsiella* spp. (SCM1282) for 3 h. The three microliters of 10 fold serial dilution of the treated bacteria were dropped on TSA plates and incubated at 37$^{O}$C overnight.

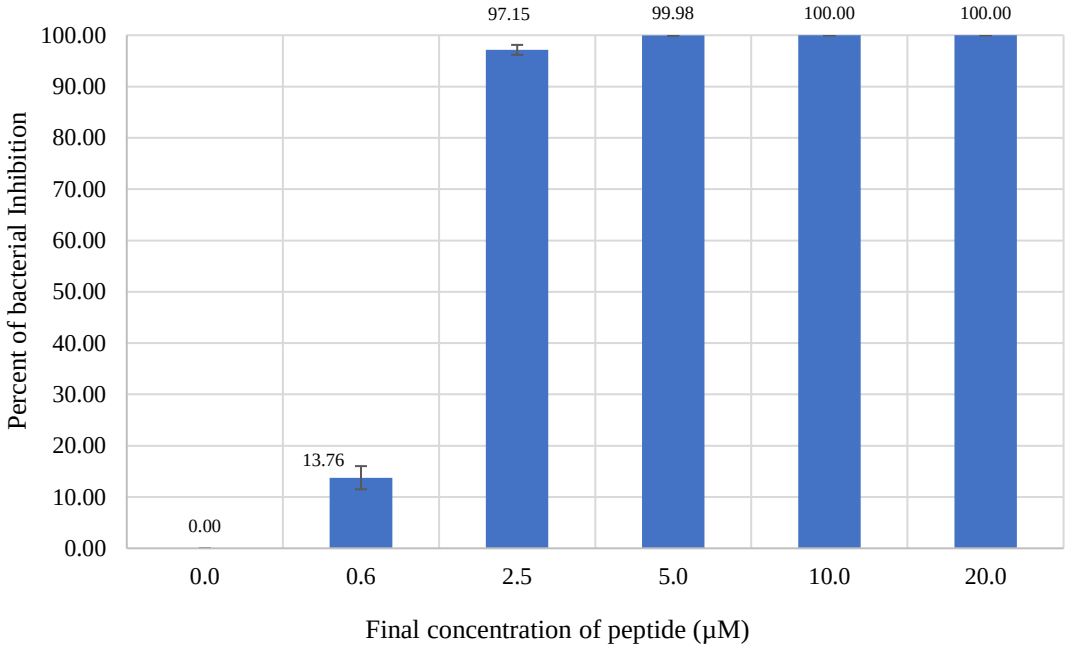



**Figure 21** Inhibition of Pm11 peptide at various concentrations against S. aureus (CM967) after 3 h incubation at 37$^{O}$C

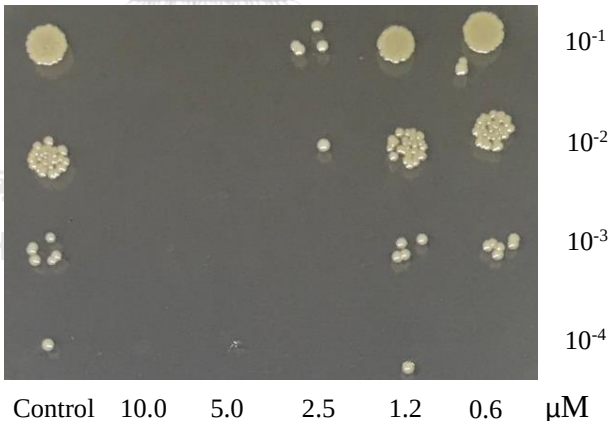



**Figure 22** Drop plate assay of Pm11 peptide against S. aureus (CM967)

Pm11 was serially diluted and incubated with *S. aureus* (CM967) for 3 h. The three microliters of 10 fold serial dilution of the treated bacteria were dropped on TSA plates and incubated at 37$^{O}$C overnight.

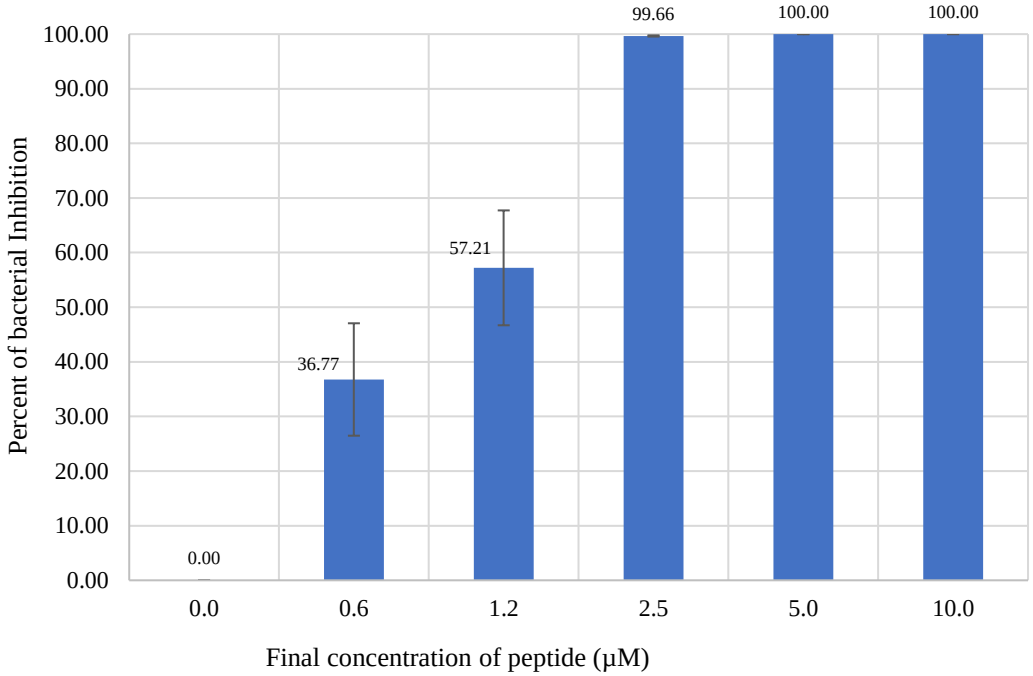



**Figure 23** Inhibition of Pm11 peptide at various concentrations against S. agalactiae (SCM1084) after 3 h incubation at 37$^{O}$C

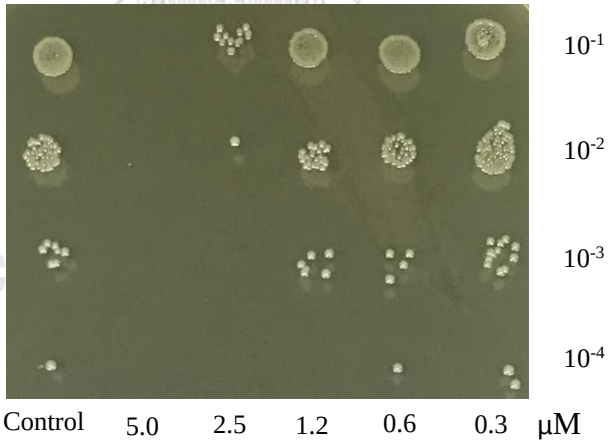



**Figure 24** Drop plate assay of Pm11 peptide against S. agalactiae (SCM1084)

Pm11 was serially diluted and incubated with *S. agalactiae* (SCM1084) for 3 h. The three microliters of 10 fold serial dilution of the treated bacteria were dropped on TSA plates and incubated at 37$^{O}$C overnight.

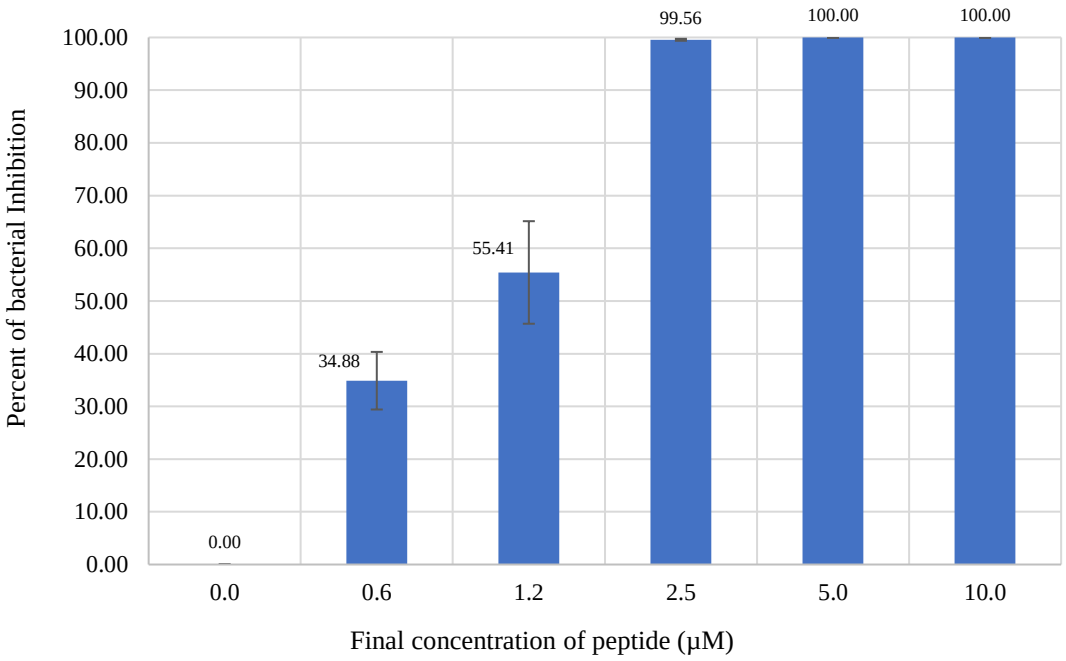



**Figure 25** Inhibition of Pm11 peptide at various concentration against S. uberis (SCM1310) after 3 h incubation at 37$^{O}$C

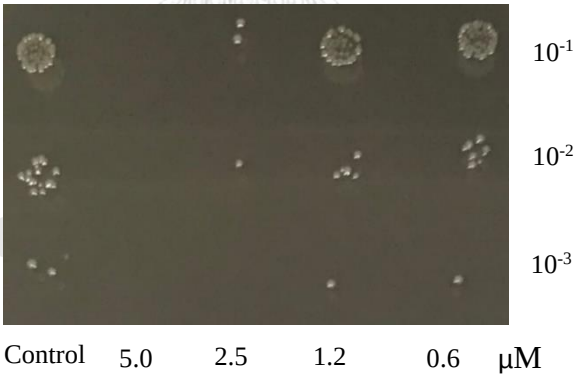



**Figure 26** Drop plate assay of Pm11 peptide against S. uberis (SCM1310)

Pm11 was serially diluted and incubated with *S. uberis* (SCM1310) for 3 h. The three microliters of 10 fold serial dilution of the treated bacteria were dropped on TSA plates and incubated at 37$^{O}$C overnight.

Pm 11 peptide with positive charged (+6) and alpha-helix structure exhibited strong antimicrobial activity that is corresponds with Wang et al. (2019). The optimal number of cationic residues, usually +4 to +6 would improve the antimicrobial activity of AMPs (Wang et al. 2019). The result showed Pm11 peptide against Gram negative bacteria with MBC 2.5 and 10.0 µM in *E.coli* (SCM1249) and *Klebsiella* spp. (SCM1282). Pm 11 peptide against Gram positive cocci, *S. aureus* (CM967), *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) by 5.0 µM. The results from above may be the result of the different cells wall of Gram positive and Gram negative bacteria. Gram positive cocci has a similar cell envelope with rich phosphatidylglycerol (Malanovic and Lohner 2016), resulting in the similar activity of Pm11 peptide to Gram positive cocci bacteria. In the case of Gram-negative bacteria have an outer membrane and external layers such as capsules, S-layers that are diverse (Beveridge 1999), resulting in the antimicrobial activity of peptide specific to each pathogen.



**4.2 Time kill kinetic of the Pm11 peptide to bovine mastitis pathogens**

Time kill assay of Pm11 peptide on bovine mastitis pathogens was conducted after determining the MBC of the five bovine mastitis pathogens. The Pm11 peptide at 1MBC final concentration specified for each bovine mastitis pathogen, as *E. coli* (SCM1249) (MBC = 2.5 µM), *Klebsiella* spp. (SCM1282) (MBC = 10.0 µM), *S. uberis* (SCM1310) (MBC = 5.0 µM), *S. aureus* (CM967) (MBC = 5.0 µM), and *S. agalactiae* (SCM1084) (MBC = 5.0 µM), was treated for 0, 1, 2, 4, 8 and 12 h. The colony plate count in each time was determined and the graph of time kill kinetic of peptide to pathogens were generated.

The result from Figure 27 shown time kill kinetic of 2.5 µM (1MBC) of Pm11 peptide to the growth of *E. coli* (SCM1249) by using *E. coli* (SCM1249) without Pm11 peptide as a untreated control. The strong antimicrobial activity of Pm11 peptide was shown at the first hour after the assay. The survival of *E. coli* (SCM1249) was obviously decreased from 5.84 to 3.95 $\log_{10}$ CFU/ml and 6.61 to 3.21 $\log_{10}$ CFU/ml within 1 and 2 h, respectively. After 4 h incubation, *E. coli* (SCM1249) was completely killed by Pm11 peptide.

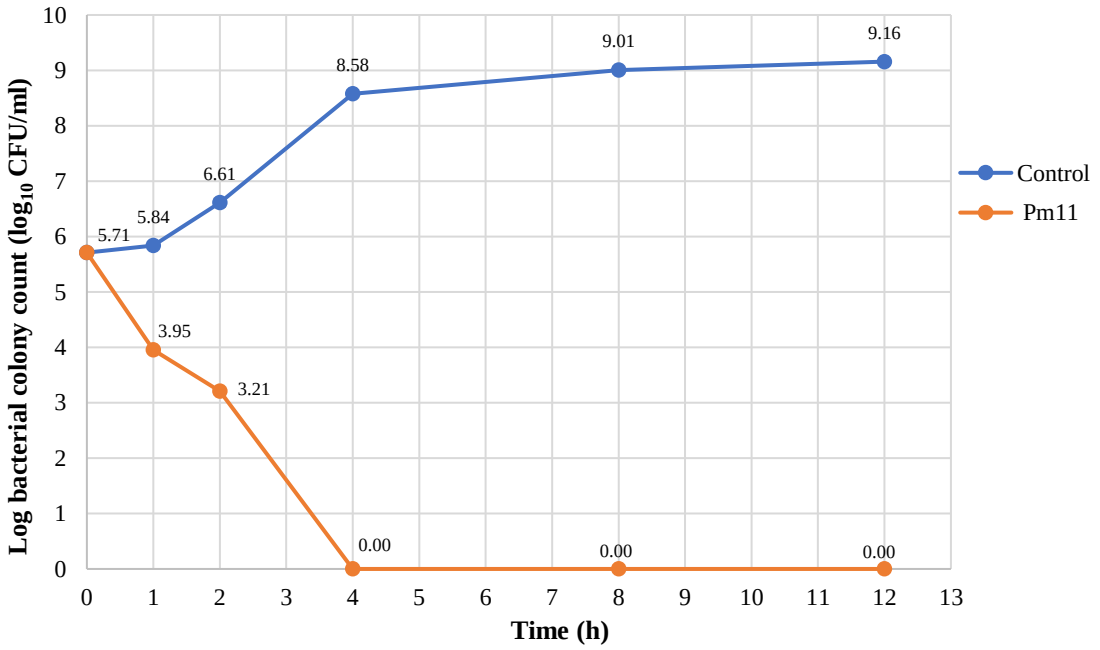



**Figure 27** Time kill kinetic of Pm11 peptide against E. coli (SCM1249)

Time kill kinetic of 2.5 µM (1MBC) Pm11 peptide to the growth of *E. coli* (SCM1249) after incubated for 0, 1, 2, 4, 8 and 12 h by using *E. coli* (SCM1249) without Pm11 peptide as a untreated control. Each value shown is the mean ± standard error of the mean from three experiments.

Figure 28 shown time kill kinetic of 10.0 μM (1MBC) Pm11 peptide against *Klebsiella* spp. (SCM1282) was slightly decreased from 5.78 to 5.27 $\log_{10}$ CFU/ml within 1 h and 6.61 to 3.73 $\log_{10}$ CFU/ml within 2 h. However, after 8 h incubation, the pathogen's survival dramatically increases and the return of the bacterial survival to 8.54 $\log_{10}$ CFU/ml within 12 h. The results demonstrated that the Pm11 peptide incomplete killing of *Klebsiella* spp. The finding corresponded with Fleeman et al. (2020) reported that the penetration of antimicrobial peptide was resisted by extracellular polysaccharide capsule of *Klebsiella pneumoniae* resulting in low efficiency of antimicrobial peptide. *Klebsiella pneumoniae* is also known as hypermucoviscous, which is composed of a mucoviscous exopolysaccharide bacterial coating capsule that is tough than other Gram-negative capsules. They promote infection by hiding the pathogen from immune recognition and provide an especially potent barrier against peptide-based antimicrobials (Fleeman et al. 2020; Campos et al. 2004; Paczosa and Mecsas 2016). Interaction with the bacterial capsule is thought to induce structural changes that cause sequestration of antimicrobial peptides to prevent them from reaching their pathogen membrane target (Chan, Burrows, and Deber 2005; Kuo et al. 2007).

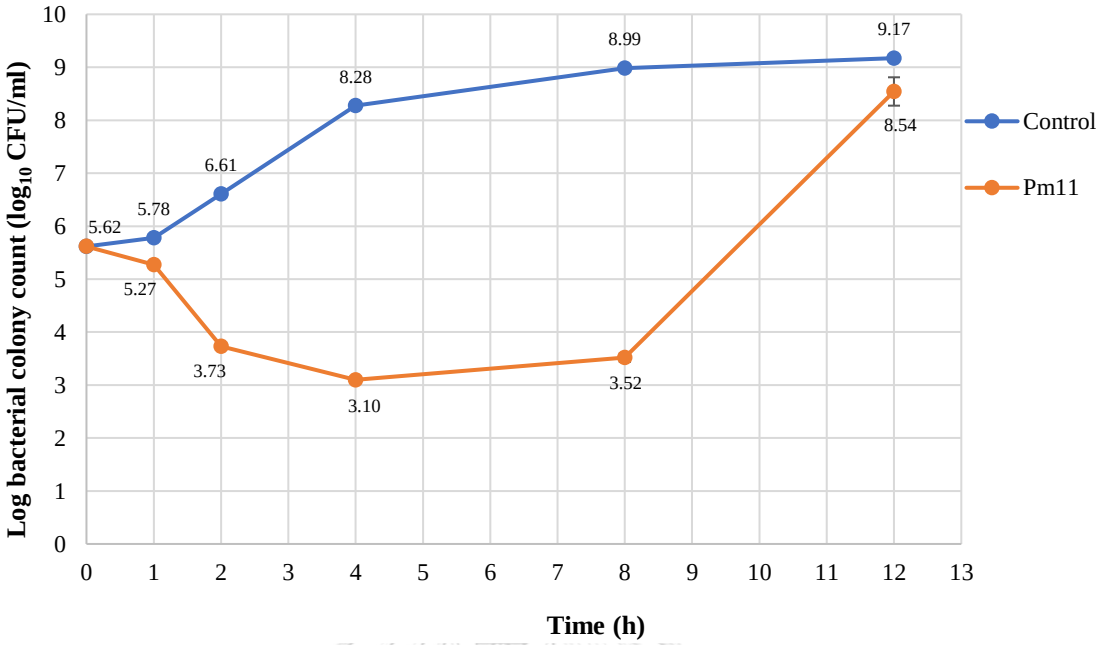



**Figure 28** Time kill kinetic of Pm11 peptide against Klebsiella spp. (SCM1282)

Time kill kinetic of 10.0 µM (1MBC) Pm11 peptide to the growth of *Klebsiella* spp. (SCM1282) after incubated for 0, 1, 2, 4, 8 and 12 h by using *Klebsiella* spp. (SCM1282) without Pm11 peptide as a untreated control. Each value shown is the mean ± standard error of the mean from three experiments.

Figure 29 shown time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to *S. aureus* (CM967) after incubated for 0, 1, 2, 4, 8 and 12 h by using *S. aureus* (CM967) without Pm11 peptide as a untreated control. The antimicrobial activity of Pm11 peptide was exhibited at the early time of assay. The survival of *S. aureus* (CM967) was obviously decreased from 5.48 to 4.25 $log_{10}$ CFU/ml and 5.50 to 3.40 $log_{10}$ CFU/ml within 1 and 2 h, respectively. The Pm11 peptide completely killed *S. aureus* (CM967) within 4 h. The survival of the pathogen when treated with 2 µM

oxacillin antibiotic (that the MBC of oxacillin against *S. aureus* (Alalaiwe et al. 2018)), the results appear to be obviously decreased from 5.48 to 4.25 $\log_{10}$ CFU/ml that closely with Pm11 within 1 h. After that, the survival of *S. aureus* (CM967) was slightly decreased and completely killed within 8 h.

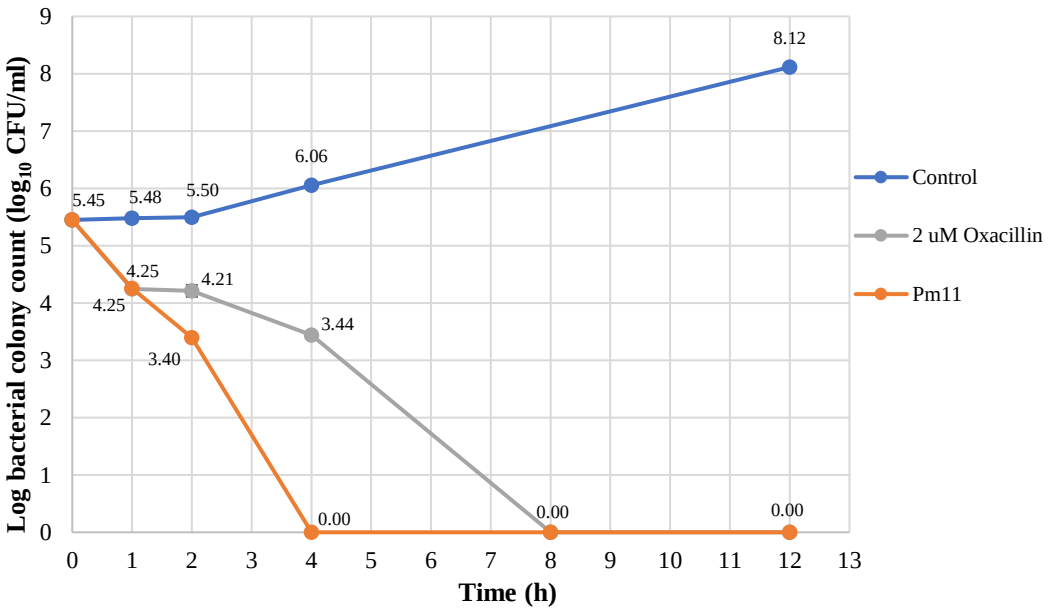



**Figure 29** Time kill kinetic of Pm11 peptide against S. aureus (CM967)

Time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to the growth of *S. aureus* (CM967) after incubated for 0, 1, 2, 4, 8 and 12 h by using *S. aureus* (CM967) without Pm11 peptide as a untreated control and 2.0 µM oxacillin as an antibiotic control. Each value shown is the mean ± standard error of the mean from three experiments.

Figure 30 shown time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to *S. agalactiae* (SCM1084) after incubated for 0, 1, 2, 4, 8 and 12 h by using *S. agalactiae* (SCM1084) without Pm11 peptide as a untreated control. The strong antimicrobial activity of Pm11 peptide was exhibited to completely killing *S. agalactiae*

(SCM1084) from 5.40 $log_{10}$ CFU/ml within the first hour of the assay. The survival of the pathogen, when treated with 180 µM ampicillin (which the general antibiotic for *Streptococcus* infected treatment (Boonyayatra and Pata 2016)) was decreased from 5.40 to 4.39 $log_{10}$ CFU/ml and 6.10 to 3.60 $log_{10}$ CFU/ml within 1 and 2 h, respectively. After 4 h, *S. agalactiae* (SCM1084) was completely killed by ampicillin.

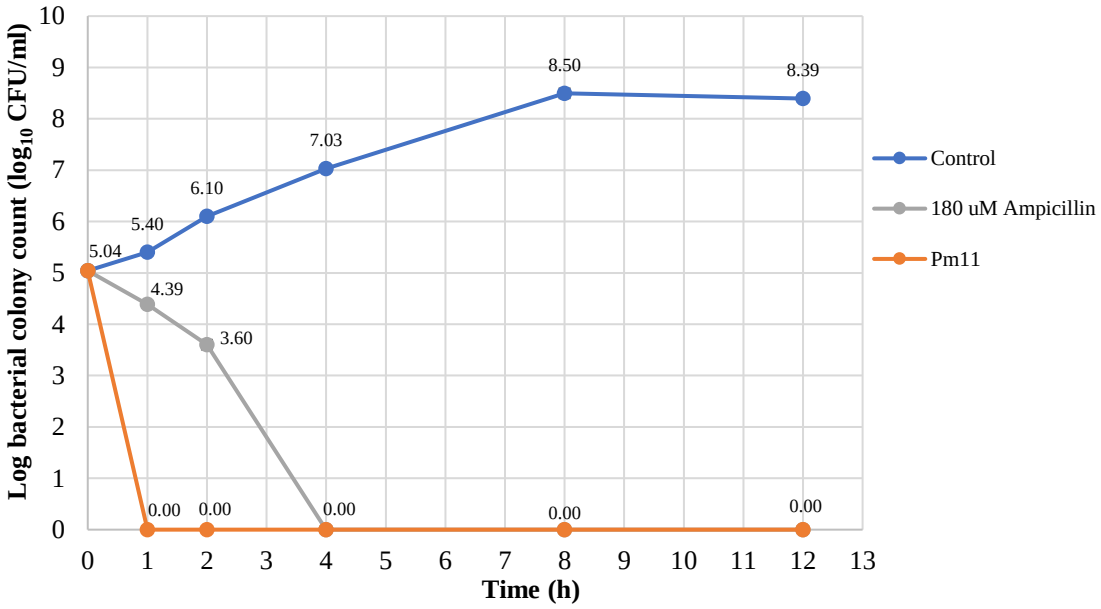



**Figure 30** Time kill kinetic of Pm11 peptide against S. agalactiae (SCM1084)



Time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to the growth of *S. agalactiae* (SCM1084) after incubated for 0, 1, 2, 4, 8 and 12 h by using *S. agalactiae* (SCM1084) without Pm11 peptide as a untreated control and 180.0 µM ampicillin as an antibiotic control. Each value shown is the mean ± standard error of the mean from three experiments.

Figure 31 shown the time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to *S. uberis* (SCM1310) after incubating for 0, 1, 2, 4, 8 and 12 h using *S. uberis* (SCM1310) without Pm11 peptide as a untreated control. The results were similar to

*S. agalactiae* (SCM1084), the strong antimicrobial activity of Pm11 peptide was shown at the first hour after the assay. The survival of *S. uberis* (SCM1310) was completely killed by Pm11 peptide from 5.78 $\log_{10}$ CFU as a control within 1 h.

Zhang et al. (2016) reported that the strong antimicrobial activity of Pm11 peptide inhibit *S. mutans* within 20 min by 64 µg/ml (33.8 µM) of Pm11 peptide (Zhang et al. 2016b) that correspond with these results (time kill kinetic of Pm11 peptide on *S. agalactiae* and *S. uberis*), confirmed that Pm11 exhibited strong and rapidly antimicrobial activity against *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) within 1 h by only 5.0 µM

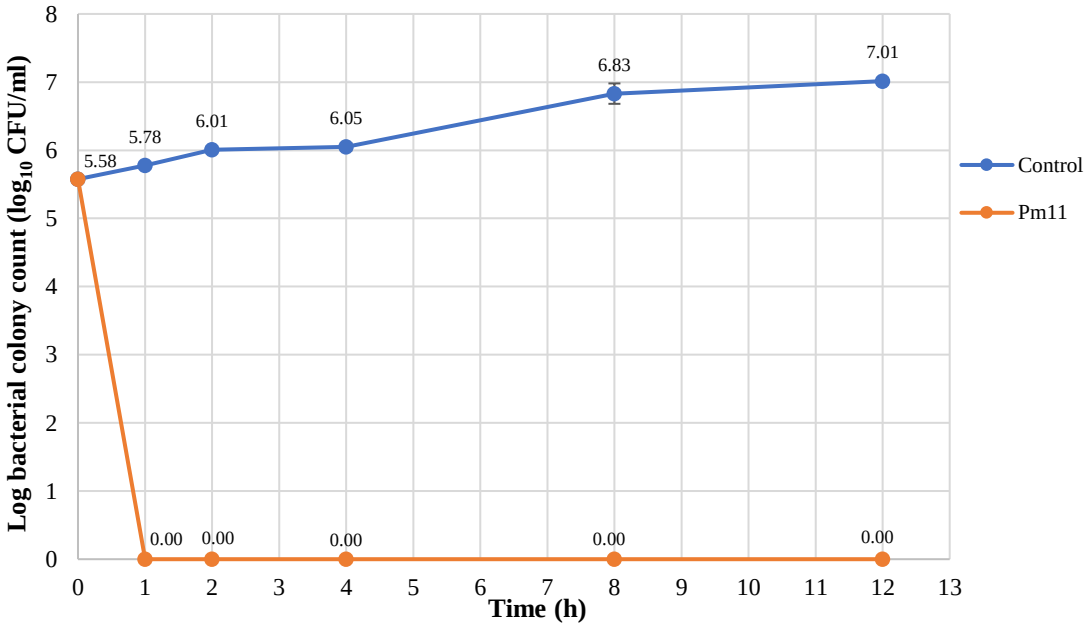



**Figure 31** Time kill kinetic of Pm11 peptide against S. uberis (SCM1310)

Time kill kinetic of 5.0 µM (1MBC) Pm11 peptide to the growth of *S. uberis* (SCM1310) after incubated for 0, 1, 2, 4, 8 and 12 h by using *S. uberis* (SCM1310) without Pm11 peptide as a untreated control. Each value shown is the mean ± standard error of the mean from three experiments.

### 4.3 Bactericidal activity of peptide to bacterial lab strain in UHT milk

#### 4.3.1 Activity of Pm11 peptide to E. coli DH5alpha in UHT milk

Bactericidal activity of Pm11 peptide against *E. coli* DH5alpha was observed in ultra-high-temperature (UHT) milk condition after the MBC test in TSB medium. The $10^5$ CFU/ml bacteria in UHT milk were incubated with Pm11 peptide at 2.6 µM (final concentration), which is approximately MBC value for 3 h. The bacterial survival of *E. coli* DH5alpha in the UHT milk condition was observed compared with the TSB medium condition (control).

Table 7 shown that Pm11 peptide did not exhibit bactericidal activity against *E. coli* DH5alpha in UHT milk condition compared with the control. In addition, the results demonstrated that Pm11 peptide was loss of antimicrobial activity when applied to UHT milk condition.

**Table 7** Bactericidal activity of Pm11 peptide 2.6 µM against E. coli DH5alpha in UHT milk and tryptic soy broth condition incubated for 0 and 3 h



| The experiments | Colony plate count |
|---|---|
| $10^5$ CFU/ml *E. coli* in TSB + ultrapure water at 0 h | $6.20*10^5$ |
| $10^5$ CFU/ml *E. coli* in TSB + ultrapure water at 3 h (Untreated control) | $7.63*10^6$ |
| $10^5$ CFU/ml *E. coli* in TSB + 2.6 µM Pm11 peptide | 0.00 (100.00% inhibition) |
| $10^5$ CFU/ml *E. coli* in UHT + ultrapure water at 0 h | $7.17*10^5$ |
| $10^5$ CFU/ml *E. coli* in UHT + ultrapure water at 3 h (Untreated control) | $>3.00*10^6$ |
| $10^5$ CFU/ml *E. coli* in UHT + 2.6 µM Pm11 peptide | $>3.00*10^6$ |

According to the lost activity of Pm11 peptide in UHT milk condition, the bactericidal activity of increasing Pm11 peptide concentrations up to 105.6 µM, which approximately 40 times of MBC was conducted. The results shown in Figure 32 demonstrated that Pm11 peptide was not exhibited antimicrobial activity against *E. coli* DH5alpha even concentration of Pm11 peptide was increased to 105.6 µM. On the other hand, the bactericidal activity of Pm11 in TSB medium displayed strong antimicrobial activity at all of the concentrations which obviously reduces the survival rate of *E. coli* DH5alpha as shown in Figure 33. The survival of *E. coli* DH5alpha was decreased from 7.22 to 3.99 $\log_{10}$ CFU/ml by 3.3 µM of Pm11 peptide (99.94% inhibition) and 7.22 to 3.00 $\log_{10}$ CFU/ml by 6.6 µM of Pm11 peptide (99.99% inhibition). Moreover, the Pm11 peptide completely inhibits *E. coli* DH5alpha by 13.2 to 105.6 µM (100.00% inhibition).

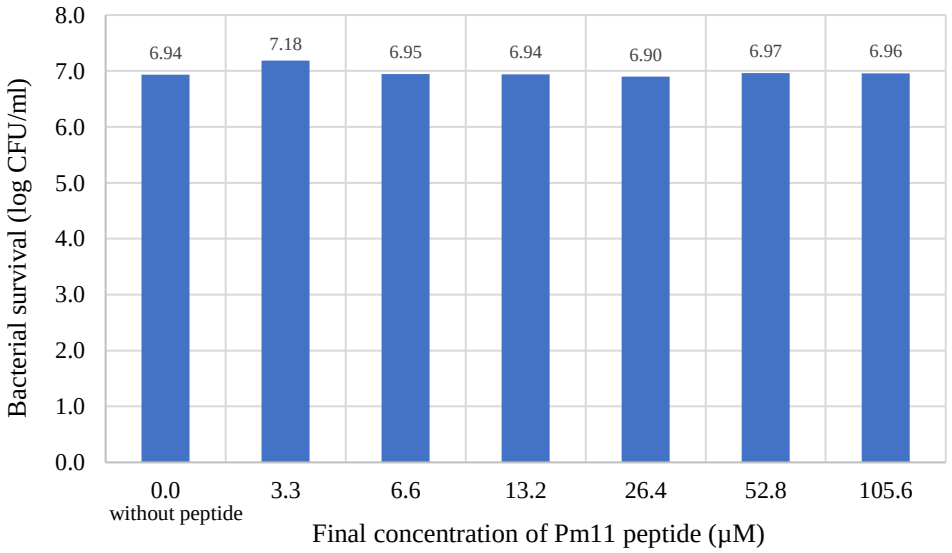



**Figure 32** The bactericidal activity of Pm11 at 105.6, 52.8, 26.4, 13.2, 6.6 and 3.3 µM when treated with E. coli DH5alpha in UHT milk condition incubated for 3 h.

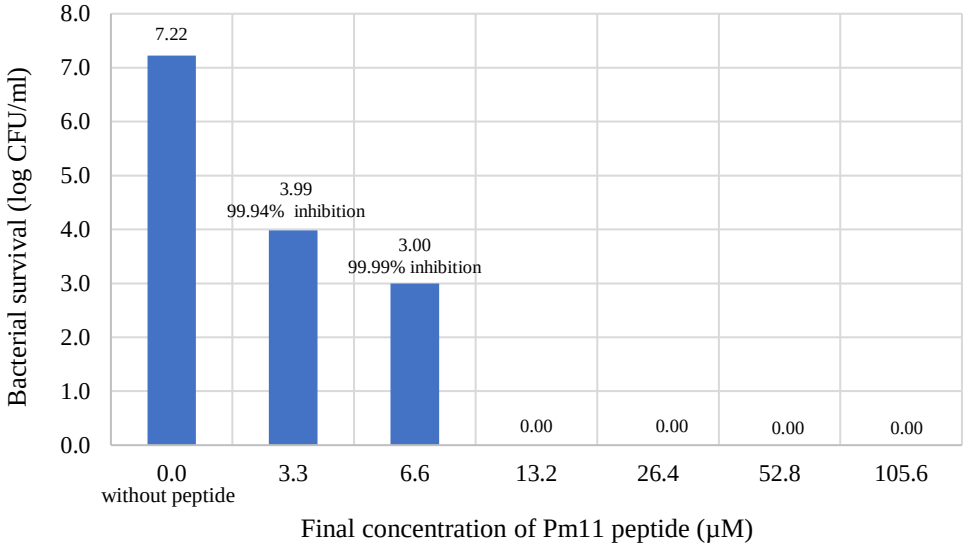



**Figure 33** The bactericidal activity of Pm11 at 105.6, 52.8, 26.4, 13.2, 6.6 and 3.3 µM when treated with E. coli DH5alpha in TSB medium condition incubated for 3 h.

According to the decadence of antimicrobial activity of Pm11 peptide when applying in the presence of UHT milk, therefore protease existence in UHT milk was concerned, resulting in peptide bond cleavage. Plasmin is an industrially important alkaline serine proteinase enzyme indigenous in milk (Garcia, López-Hernandez, and Hill 2011). Moreover, Chavan et al. (2011) reported that plasmin proteinase still a presence in milk even the milk was passed the UHT process, which may subsequently cause age gelation in UHT milk (Chavan et al. 2011; Rauh et al. 2014). This enzyme has optimum activity at pH 7.5 and 37 °C and preferentially cleaves Lys-X and Arg–X bonds (Garcia, López-Hernandez, and Hill 2011). The property of plasmin included amino acid cleaves site, optimum pH and temperature that possibly could affect to Pm11 peptide molecule due to the amino acid sequence of Pm11 peptide mainly composed of Lys amino acid. Besides, the pH and temperature of the experiment were similar to the plasmin optimum condition.

### 4.3.2 The effect of a protease inhibitor on the bactericidal activity of Pm11 peptide

A serine protease inhibitor, or serpins, comprises a family of proteins that antagonize the activity of serine proteases. These proteins inhibit protease activity by a conserved mechanism involving a profound conformational changes (Almonte and Sweatt 2011). Phenylmethylsulfonyl fluoride (PMSF) is the one of the serine protease inhibitors selected for inhibiting plasmin protease activity in UHT milk.

Bactericidal activity of Pm11 peptide at 13.2 µM (final concentration) was observed when it was incubated for 3 h with *E. coli* DH5alpha in UHT milk added with 200.0, 100.0, 50.0, 25.0 µg/ml of PMSF protease inhibitor in methanol (final concentration). The bacterial survival was observed by colony plate count. The result of UHT milk condition supplement with PMSF was compared with antimicrobial activity of Pm11 peptide to *E. coli* DH5alpha in TSB medium condition.

The results from Figure 34 shown the bactericidal activity of Pm11 at 13.2 µM treated with *E. coli* DH5alpha in UHT milk condition supplement with 200.0, 100.0, 50.0, 25.0 µg/ml of PMSF protease inhibitor and in TSB medium condition demonstrated that the antimicrobial activity property of Pm11 peptide was recovered when PMSF inhibitor was added into the UHT milk for the test of bactericidal activity of peptide. The survival of *E. coli* DH5alpha was slightly decreased from 6.72 to 6.26, 6.52, 6.68 and 6.68 $\log_{10}$ CFU/ml by 200.0, 100.0, 50.0, 25.0 µg/ml PMSF protease inhibitor, respectively, and has bacterial inhibition at 65.20, 36.33, 8.22 and 7.65 %, respectively.

A common weakness in the drug ability of antimicrobial peptides is their poor pharmacokinetics, as they may be degraded by a multitude of proteases inside the

organism. Therefore, a lot of research has been reported about preventing peptides being degraded or inactivated before reaching its target at therapeutic concentrations. In 2016, Santos et al. reported that antimicrobial activity of nisin as MIC, MBC and minimum biofilm inhibitory concentration (MBIC) were decreased when applying the cooperation of natural polysaccharide guar gum gel with nisin peptide compared with nisin peptide alone (Santos et al. 2016). Makowski et al. (2019) reported about advanced antimicrobial peptide delivery system by lipid and metal nanoparticles for effectiveness pharmacokinetics (Makowski et al. 2019).

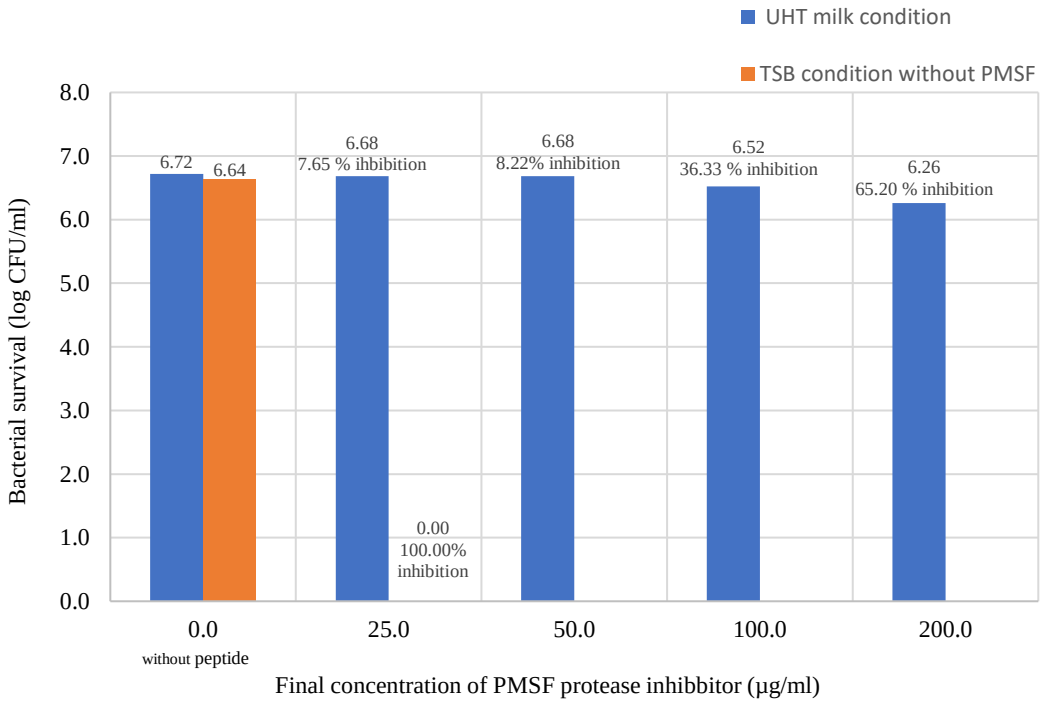



**Figure 34** The bactericidal activity of Pm11 to E. coli DH5alpha in UHT milk condition

The bactericidal activity of Pm11 at 13.2 µM treated with *E. coli* DH5alpha in UHT milk condition supplement with 200.0, 100.0, 50.0, 25.0 µg/ml of PMSF protease inhibitor and the bactericidal activity of Pm11 at 13.2 µM treated with *E. coli* DH5alpha in tryptic soy broth medium condition incubated for 3 h.

Besides, the improvement of antimicrobial peptides for protease resistance has been reported, whether it is a chemical modification of peptide template as N-acetylation or C-amidation, convert enantiomer and designed appropriate formulations. In 2018, Hirt et al. reported that GL13K, peptide derived from the human salivary protein BPIFA2, the results were shown the strong activity and resistance to bacterial metalloprotease by changing the L-form of a peptide to D-form (Hirt et al. 2018). Moreover, in 2019, Wang et al. reported that the improvement of the novel antimicrobial peptide was specifically designed and synthesized based on an antitrypsin hydrolytic peptide structure. The unit $(XYPX)_n$ was used for a formula since X for variation of hydrophobic amino acids and Y represents for variation of cationic amino acids. The results demonstrated that X represented R (arginine) and Y represented I (isoleucine) amino acid with 7 repeat units has potential to high proteolytic resistance (Wang et al. 2019).

**4.4 Minimum hemolytic concentration (MHC) measurement**



Hemolysis represented the most commonly employed initial toxicity assessment. For AMPs are interesting in systemic applications, they must show low toxicity against erythrocytes. Thus, the capability of Pm11 peptide to lyse the sheep red blood cells was observed after incubation of various peptide concentrations from 0.0 to 160.0 µM with the sheep red blood cells. The results were shown in Table 8 that Pm11 peptide at 0.3 - 40.0 µM was closely non-hemolytic. Pm11 at 10.0 µM that the highest MBC of Pm11 peptide with pathogens, was shown 0.65% hemolysis that very low hemoglobin release. Even at the highest concentration, 16 times of the highest MBC of Pm11 peptide, 160.0 µM, the hemolysis was only 10.19%. Figure 35

shown the supernatant of Pm 11 peptide and sheep red blood cells mixtures after incubation. The color of the supernatant indicated that at 0.3 - 80.0 µM of Pm11 peptide did not affect the hemolytic of sheep red blood cells compared with negative control. Pm11 peptide at 160.0 µM shown that the slightest red color indicated that low hemoglobin releases appears when compared with positive control.

The *in vitro* hemolysis guidance for the pharmaceutical scientist reported that the hemolytic cutoff for the various species is approximate and represented in percent hemolysis: humans 10% or higher, dogs between 10% and 29%, and for rabbits between 0% and 37%. In general, 10% and 25% hemolysis are the relative boundaries. In other words any hemolysis value below 10% considered to be non-hemolytic while values above 25% to be at risk for hemolysis (Amin and Dannenfelser 2006). Moreover, Greco et al. (2020) reported that the *in vitro* hemolysis of seven peptides which is different levels of hemolytic activity shown there is the corresponding with *in vivo* cytotoxicity. The results suggested that the peptides with percent hemolytic less than 23% in rat, bovine and canine erythrocytes result in high percent viability of HeLa, HaCaT and HepG2 cells compared with another peptide with high hemolytic activity (Greco et al. 2020).



Ruiz et al. (2014) reported the hemolytic activity of peptides that it affected hemoglobin release in mammalian red blood cells was reported that associated with charge and hydrophobicity (Ruiz et al. 2014). The ability to form amphipathic molecules of peptides not only has been linked to an improved antimicrobial activity but also to increased hemolytic activity once a threshold hydrophobicity has been reached (Chen et al. 2007; Hong, Oren, and Shai 1999). The amino acids which is strong effect on hemolytic activity through hydrophobicity or charge are Trp, Lys, and

Arg. Tryptophan residues have involved with the binding of peptides to cholesterol present in biological membranes through the indole moiety (Kruijff 1990). However, even if the amino acid sequence of Pm11 shown the containing more than 50% of hydrophobic residues and strong basic charge; however the results suggested that Pm11 peptide has exhibits low hemolytic activity to lyse sheep red blood cells. from the *in vitro* hemolysis guidance for the pharmaceutical scientist.

**Table 8** The hemolytic activity at various concentrations of Pm11 peptide to sheep red blood cells.

| **The final concentration of peptide (μM)** | **Average Percent Hemolysis** |
|---|---|
| 160.0 | 10.19 ± 2.29 |
| 80.0 | 1.96 ± 0.13 |
| 40.0 | 0.44 ± 0.44 |
| 20.0 | 0.15 ± 0.23 |
| 10.0 | 0.65 ± 0.22 |
| 5.0 | 0.34 ± 0.31 |
| 2.5 | 0.06 ± 0.08 |
| 1.2 | 0.22 ± 0.20 |
| 0.6 | 0.13 ± 0.23 |
| 0.3 | 0.09 ± 0.16 |
| 0.0 | 0.00 ± 0.00 |

Each value shown is the mean ± standard error of the mean from three experiments.

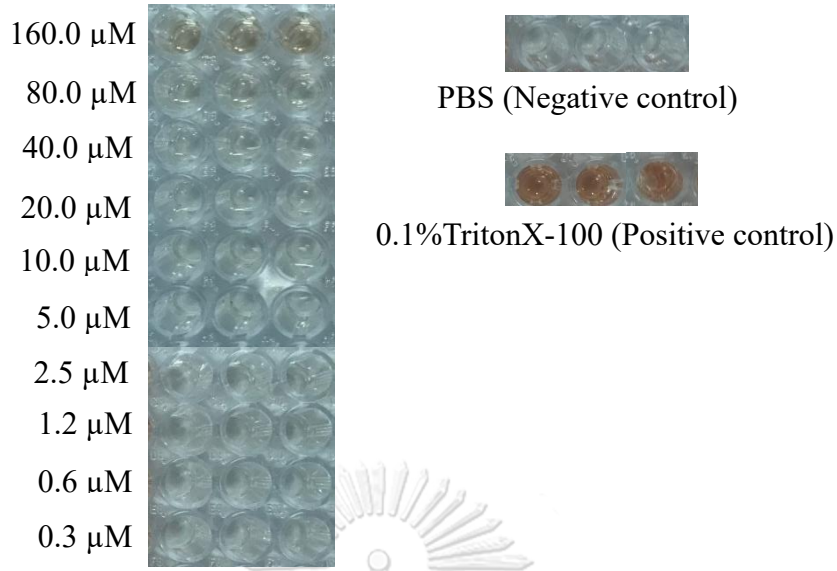


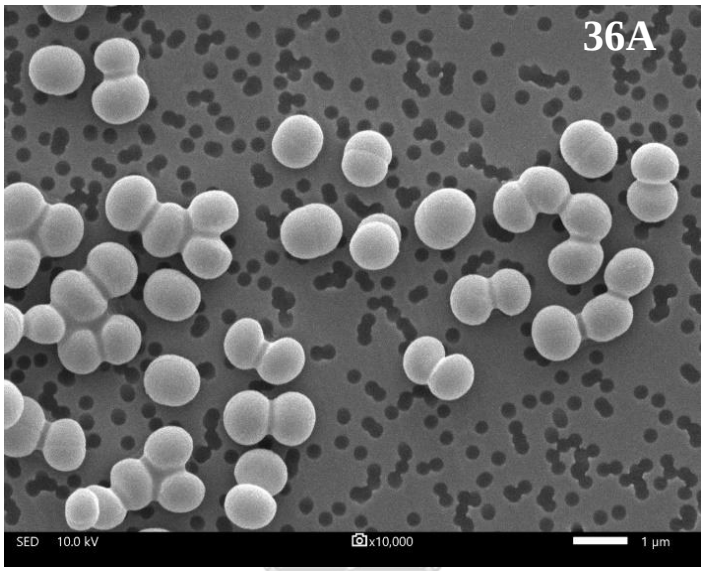

**Figure 35** The supernatant of peptide-sheep red blood cells mixtures at various concentration of PM11 peptide

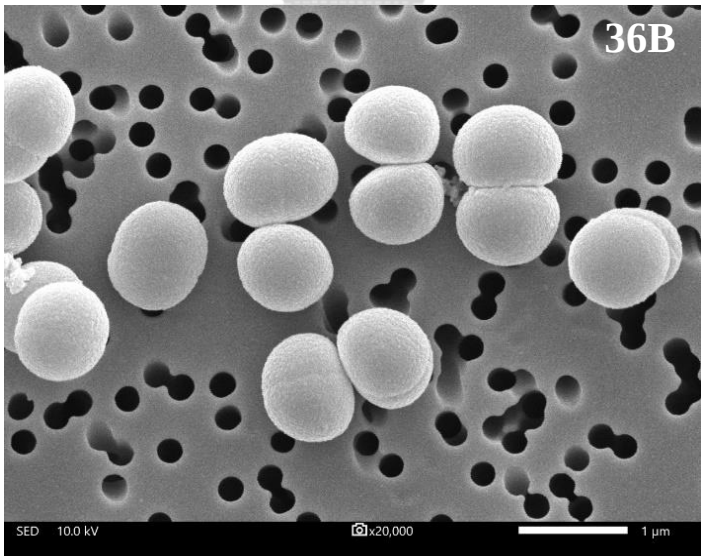
The supernatant of peptide-sheep red blood cells mixtures after 1h incubation, 37 °C. PBS as a negative control and 0.1%TritonX-100 with surfactant activity as a positive control

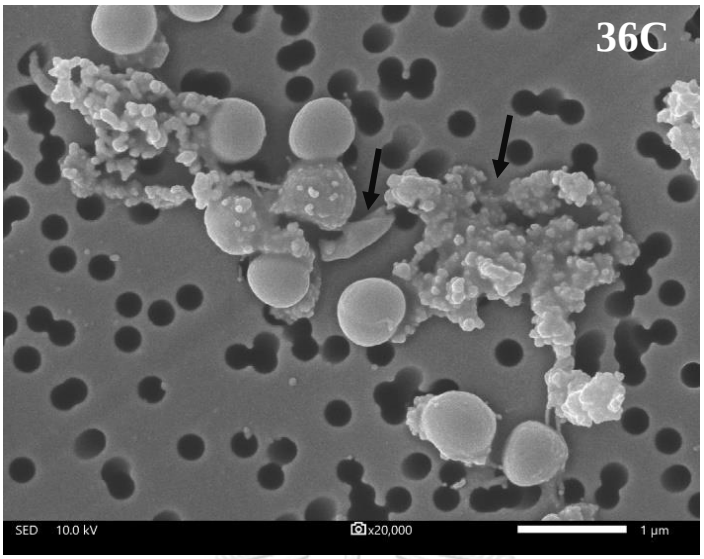
**4.5 Scanning Electron microscopic (SEM) study**

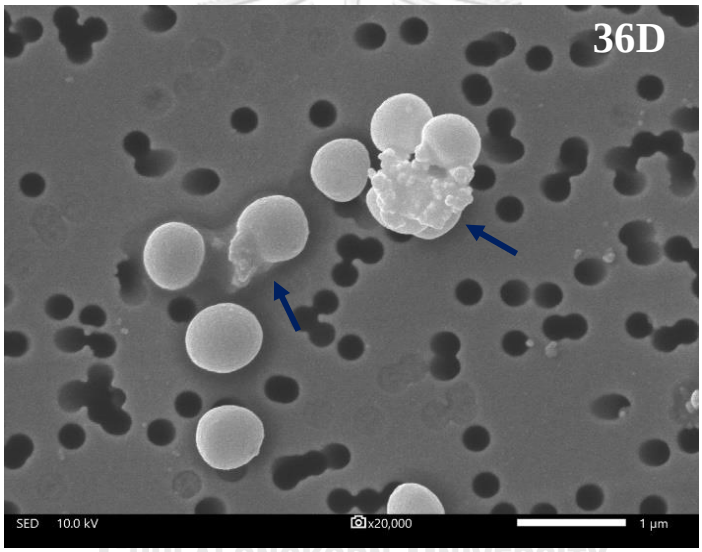
*S. aureus* (CM967) and *E. coli* (SCM1249), the representative of gram-positive and gram-negative pathogens were treated with Pm11 peptide and morphological change of the pathogens were observed under SEM. Figure 36 and 37 display the effect of the Pm11 peptide attacked to *S. aureus* (CM967) and *E. coli* (SCM1249) and the control pathogens without Pm11 peptide. The results shown that the untreated control pathogen cells were undamaged and cell membrane appears smooth without cell lysis (Figure 36A, B and 37 A, B), whereas the surface of pathogen cells that were treated with Pm11 peptide induced dramatic morphological

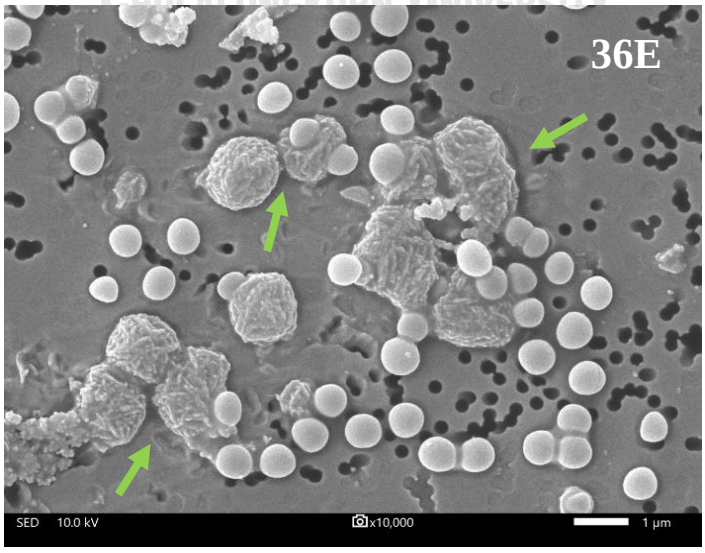
changes; debris, ghost cell, roughening, blebbing and small cells (Figure 36 C - F and 37 C - H). Indeed, these morphological alterations of pathogens surface indicated a membrane-damaging activity of these Pm11, in agreement with data obtained in MBC assays.




36C
SED 10.0 kV
x20,000
1 µm


36D
SED 10.0 kV
x20,000
1 µm


36E
SED 10.0 kV
x10,000
1 µm

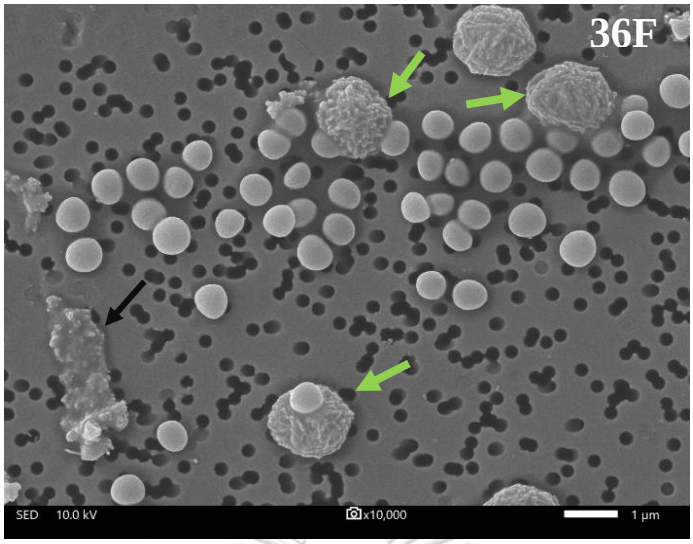
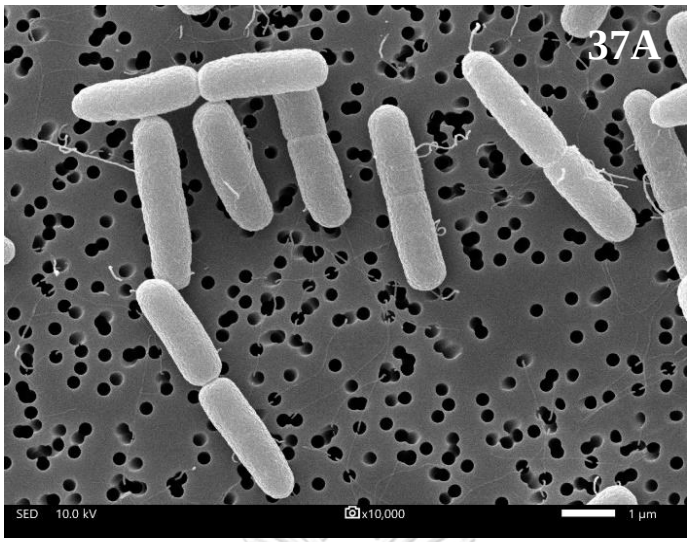
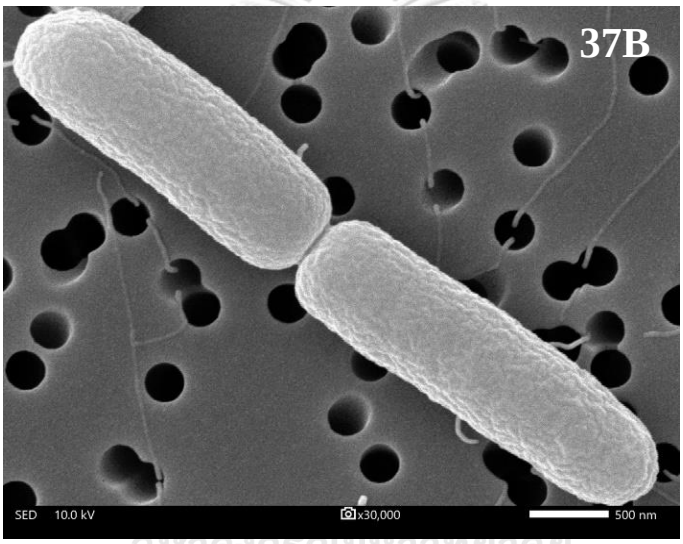
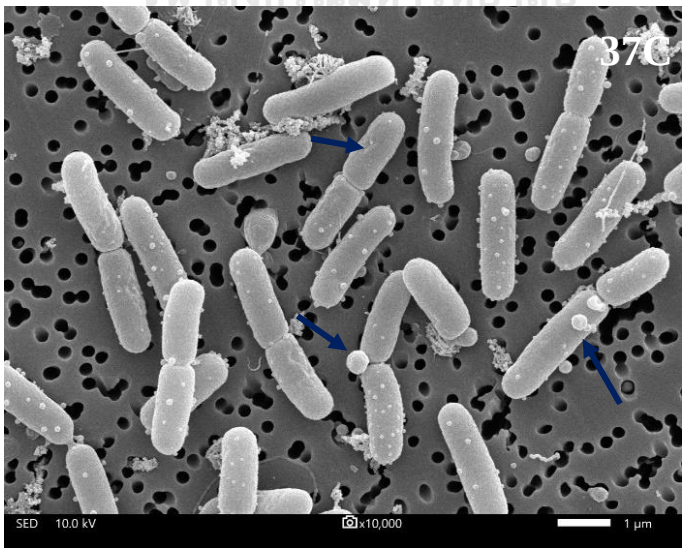


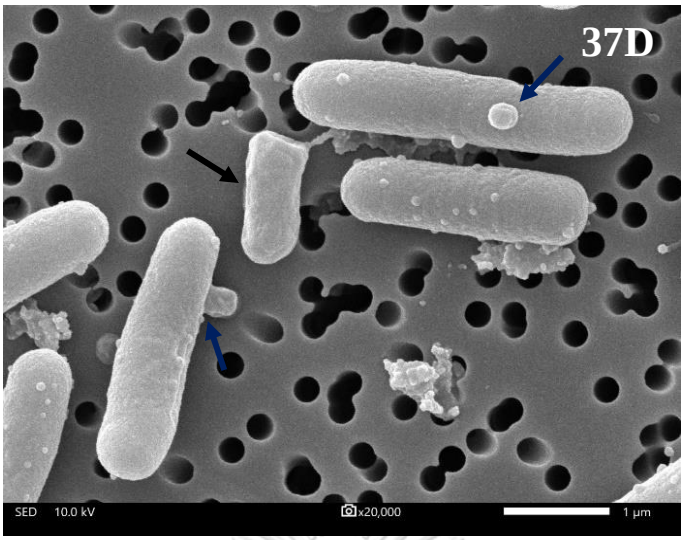

**Figure 36** The effect of the Pm11 peptide treatment on S. aureus (CM967) by using SEM

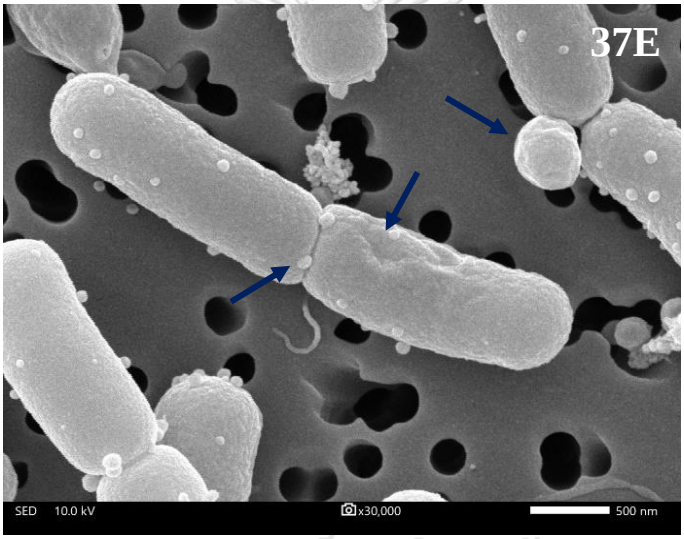
Pm11 non-treated control magnification ×10,000 (A) and ×20,000 (B), Pm11 (5.0 µM) treated magnification ×20,000 (C and D) and magnification ×10,000 (E and F). The black arrows indicated shape loss and cell debris. The blue arrows indicated cell ruptures. The green arrows indicated accumulation of cell debris.

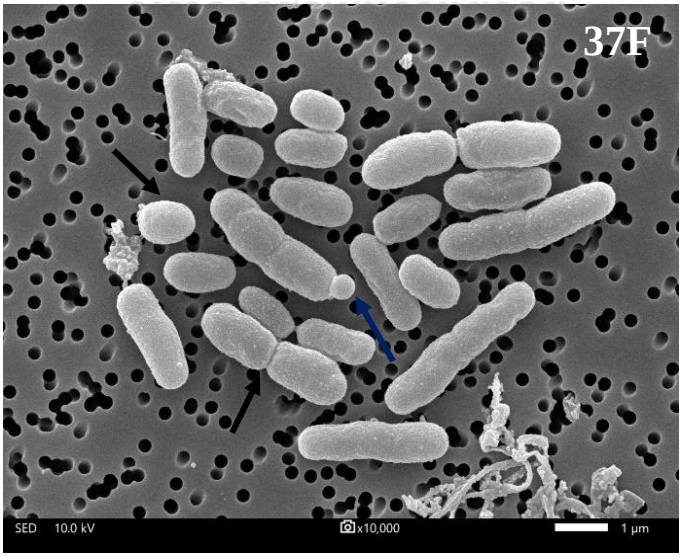

37A
SED 10.0 kV
x10,000
1 µm


37B
SED 10.0 kV
x30,000
500 nm


37C
SED 10.0 kV
x10,000
1 µm


37D
SED 10.0 kV
x20,000
1 µm


37E
SED 10.0 kV
x30,000
500 nm


37F
SED 10.0 kV
x10,000
1 µm

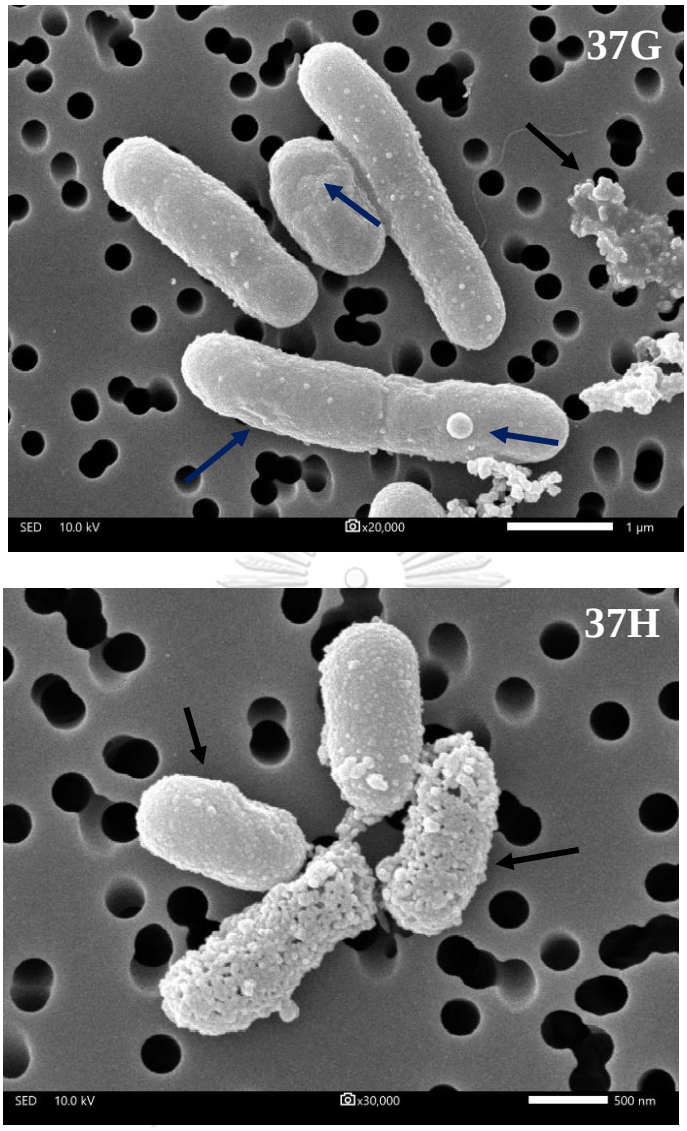





**Figure 37** The effect of the Pm11 peptide treatment on E. coli (SCM1249) by using SEM

Pm11 non-treated control magnification ×10,000 (A) and ×30,000 (B), Pm11 (2.5 µM) treated (C, D and E), Pm11 (5.0 µM) treated (F, G and H) magnification ×10,000 ×20,000 and ×30,000 respectively. The black arrows indicated shape loss, cell debris and ghost cell. The blue arrows indicated roughening, nicks and blebs.

As the SEM micrographs, the characteristics of Pm11 as cationic peptide and alpha-helix structure with amphipathic molecule exhibited the strong antimicrobial

due to electrostatic interaction between positive charge of a peptide with negative charge of bacterial membrane resulting in pathogen membrane damaged. The several models of action for cytoplasmic membrane disruption by alpha-helix AMPs were reported including carpet model by accumulation of peptide until reaching to a critical concentration, The barrel stave model by peptides interact laterally, resulting in the formation of a structure that behaves like a protein ion channel, toroidal pore formations by peptides interact only with the head groups of the lipids leading to the formation of high curvature peptide-lipid toroids (Sato and Feix 2006). All of these reasons result in bacterial membrane disintegration. The NMR of mechanically aligned samples and differential scanning calorimetry (DSC) were used to study peptide-containing lipid bilayers for determined the models of action by peptide (Hallock, Lee, and Ramamoorthy 2003). Moreover, self-assembly of AMPs with diphenylalanine motif was reported to a correlation with antimicrobial activity. The self-assembly model of AMPs plays a key role in their interactions with bacteria and bacterial membrane components. Peptide self-assembly may either boost membrane interaction and destabilization, such as for amyloid-forming peptides (Cardoso et al. 2021). The self-assembly of peptide model corresponding with Pm11 peptide (WFKFFKKFFKKFK) with diphenylalanine amino acid may induce amyloid oligomer interaction with the polar head group on the bacterial membrane as known lipid co-aggregation in Figure 36 E and F. Moreover, the interaction of diphenylalanine on Gram negative membrane causes outer membrane permeation and inner membrane depolarization, resulting in severe changes to membrane morphology, such as the appearance of nicks and tears (Schnaider et al. 2017). The appearance of bleb morphology including bleb at division plane, bleb at cell pole and

multiple blebs found on *E. coli* (SCM1249) with Pm11 peptide treated in Figure 37 C-H indicated that the membrane damage and periplasmic leakage. This result was alike to *E. coli* that treated with human defensin 5 that causes bleb formation, cellular elongation, and clumping (Chileveru et al. 2015).

# CHAPTER V

# CONCLUSION AND SUGGESTION

The three cationic antimicrobial peptides as Pm11, Pep64 and L10 peptides were tested for the antimicrobial activity against bacterial lab strains, *B. subtilis* ATCC6633 and *E. coli* DH5alpha. The results were reported by minimum bactericidal concentration (MBC) and half minimal inhibitory concentration ($IC_{50}$). Pm11 peptide exhibited strong antimicrobial activity against *B. subtilis* ATCC6633 and *E. coli* DH5alpha with low MBC value 5.0 and 2.5 µM, while Pep64 and L10 peptides have to use high concentration (1000 to 2000 µM) for MBC. The MBC and $IC_{50}$ value of Pm11, Pep64, L10 peptides against bacterial lab strains demonstrated that Pm11 peptide is the best candidate and was chosen for investigating the activity against bovine mastitis pathogens. Further process antimicrobial activity of Pm11 peptide to bovine mastitis pathogens isolated from cows with mastitis as *E. coli* (SCM1249), *Klebsiella* spp. (SCM1282), *S. aureus* (CM967), *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) was conducted. The pathogens were inhibited 99.9% by 2.5, 10.0 and 5.0 µM of Pm11 peptide. The result demonstrated that Pm11 peptide remain the strong activity with low MBC even in pathogens. Time kill kinetic of Pm11 peptide against pathogens, *E. coli* (SCM1249) and *S. aureus* (CM967) was completely killed within 4 h incubation. The dramatically decrease of *S. agalactiae* (SCM1084) and *S. uberis* (SCM1310) with completed killing within 1 h. While the survival of *Klebsiella* spp. (SCM1282) was decreased around 3 $\log_{10}$ CFU/ml within 2 h. However, after 8 h the survival dramatically increased because it was not completely killed, resulting in pathogens was returning to grow up. The time kill kinetic of Pm11 peptide against the

mastitis pathogens demonstrated that Pm11 peptide showed strong bactericidal activity rapidly within first hour of incubation compared with antibiotic control. The activity of Pm11 peptide to *E. coli* DH5alpha in UHT milk demonstrated that the antimicrobial activity of Pm11 peptide was reduced in UHT milk condition. Nevertheless, the use of PMSF protease inhibitor could be reactivation of Pm11 antimicrobial activity. The result was confirmed that protease in UHT milk affects to the antimicrobial activity of Pm11 peptide.

The cytotoxicity of Pm11 peptide was proven by observing the effect of Pm11 peptide on the lyse of sheep red blood cells. The highest MBC of Pm11 at 10 µM was close to non-hemoglobin release below 1% hemolysis. Even at the highest concentration, 160 µM of Pm11 peptide, the hemolysis was only 10.19%. By the *in vitro* hemolysis guidance for the pharmaceutical scientist, hemolysis value below 10% considered to be non-hemolytic. The result demonstrated that Pm11 peptide exhibited very-low toxicity to sheep red blood cells.

The observations of pathogens morphological change by scanning electron microscope were established. *S. aureus* (CM967) and *E. coli* (SCM1249) were treated with 1MBC of Pm11 peptide. The results demonstrated that Pm11 peptide shown strong activity against bovine mastitis pathogens and affected pathogens membrane damage. The micrographs show the obviously cell debris, roughening, blebbing and small cells compared to the untreated control pathogens.

Our findings revealed the offer of selected antimicrobial peptide and their antimicrobial activities on bovine mastitis pathogens. The potential peptide in this study is Pm11.

## SUGGESTION

We suggested our findings will be the useful information for an antimicrobial peptide that has the potential for mastitis treatment instead of antibiotics.

# APPENDIX A

## Culture Media

1. Luria-Bertani (LB) agar medium

| | | |
|---|---|---|
| - Tryptone | 1 | g |
| - Yeast extract | 0.5 | g |
| - NaCl | 1 | g |
| - Agar | 1.5 | g |
| - Distilled water | 100 | mL |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes. Store at 4°C

2. Tryptic soy broth (TSB) medium

| | | |
|---|---|---|
| - Tryptic soy broth (TSB) powder | 3 | g |
| - Distilled water | 100 | mL |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes. Store at 4°C



3. Tryptic soy agar (TSA) medium

| | | |
|---|---|---|
| - Tryptic soy broth (TSA) powder | 4 | g |
| - Distilled water | 100 | mL |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes. Store at 4°C

# APPENDIX B

## Chemical Solutions Preparation

1. 0.85% Normal Saline

| | | |
|---|---|---|
| - NaCl | 0.85 | g |
| - Distilled water | 100 | mL |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes.

2. Phosphate buffer saline for bacteria dilution

| | | |
|---|---|---|
| - NaCl | 8 | g |
| - KCl | 0.2 | g |
| - $KH_2PO_4$ | 0.14 | g |
| - $Na_2HPO_4$ | 0.9 | g |
| - Distilled water | 1000 | mL |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes.



3. Phosphate buffer saline for peptide dissolved and MHC assay

| | | |
|---|---|---|
| - NaCl | 150 | mM |
| - $Na_2HPO_4$, pH 7.0 | 35 | mM |

Sterilized by autoclave at 121°C, 15 $lb.in^2$ for 15 minutes.

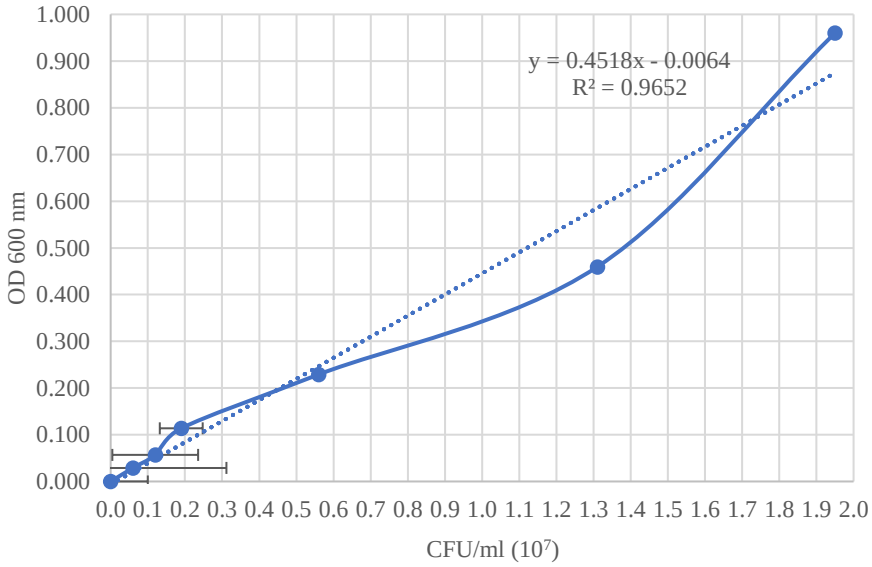
4. 0.2% TritonX-100 in PBS (MHC assay)

| | | |
|---|---|---|
| - TritonX-100 | 0.2 | mL |
| - Phosphate buffer saline for MHC assay | 99.8 | mL |

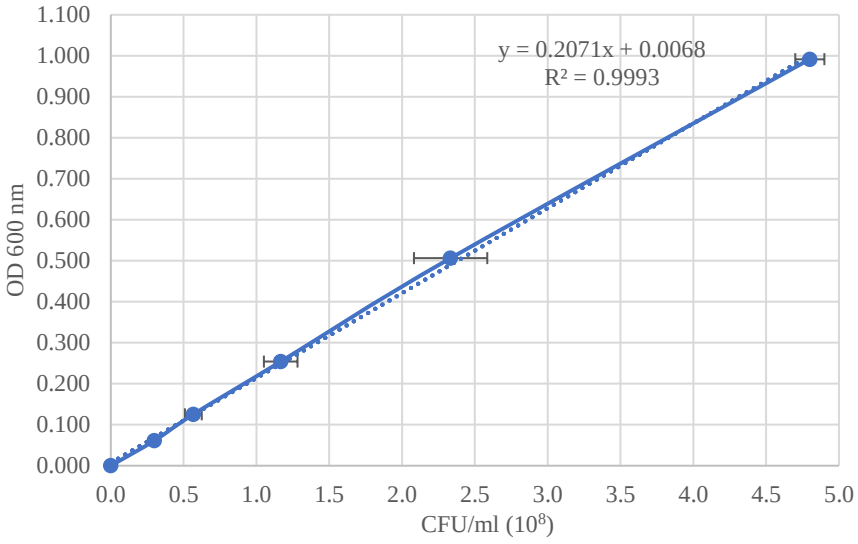
# APPENDIX C

## Determination of minimum bactericidal concentration (MBC)

1. Calibration curve of *B. subtilis* ATCC6633 (Gram-positive bacteria) for bacterial cells preparation




2. Calibration curve of *E. coli* DH5alpha (Gram-negative bacteria) for bacterial cells preparation



# APPENDIX D

## Determination of minimum bactericidal concentration (MBC)

1. Antimicrobial activity of peptides against *B. subtilis* ATCC6633

| Final conc. (μM ) | Type of peptides | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pm11 | | L10 | | DMSO | Pep64 | |
| | %Inhibit | %Survival | %Inhibit | %Survival | %Inhibit | %Inhibit | %Survival |
| 2000.0 | | | 100.00±0.00 | 0.00±0.00 | 99.98±0.01 | 99.85±0.05 | 0.15±0.05 |
| 1000.0 | | | 99.98±0.00 | 0.02±0.00 | 99.08±0.01 | 99.84±0.04 | 0.16±0.04 |
| 500.0 | | | 99.83±0.05 | 0.17±0.05 | 79.99±5.50 | 99.45±0.36 | 0.55±0.36 |
| 250.0 | | | 81.18±9.16 | 18.82±9.16 | 36.42±3.36 | 84.17±12.25 | 15.83±12.25 |
| 200.0 | | | | | | | |
| 100.0 | | | | | | | |
| 125.0 | | | 19.90±1.04 | 80.11±1.04 | 13.26±5.77 | 61.62±13.90 | 38.38±13.90 |
| 62.5 | | | | | | 43.82±3.82 | 56.18±3.82 |
| 50.0 | | | | | | | |
| 31.2 | | | | | | 33.56±10.51 | 66.44±10.51 |
| 25.0 | | | | | | | |
| 20.0 | 100.00±0.00 | 0.00±0.00 | | | | | |
| 15.6 | | | | | | 37.29±11.52 | 62.71±11.52 |
| 12.5 | | | | | | | |
| 10.0 | 100.00±0.00 | 0.00±0.00 | | | | | |
| 5.0 | 99.99±0.00 | 0.01±0.00 | | | | | |
| 2.5 | 95.64±1.11 | 4.36±1.11 | | | | | |
| 1.2 | 61.07±7.00 | 38.93±7.00 | | | | | |
| 0.6 | 17.30±13.36 | 82.70±13.36 | | | | | |
| 0.3 | 4.49±2.37 | 95.51±2.37 | | | | | |
| 0.0 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 |
| MBC | 5.0 μM | | 1000.0 μM | | | 2000.0 μM | |
| $IC_{50}$ | 1.05±0.07 μM | | 178.60±11.35 μM | | | 34.66±7.08 μM | |

2. Antimicrobial activity of peptides against *E. coli* DH5alpha

| Final conc. (μM ) | Type of peptides | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pm11 | | L10 | | DMSO | Pep64 | |
| | %Inhibit | %Survival | %Inhibit | %Survival | %Inhibit | %Inhibit | %Survival |
| 2000.0 | | | 97.41±0.40 | 2.59±0.40 | 96.69±0.17 | 100.00±0.00 | 0.00±0.00 |
| 1000.0 | | | 93.71±0.34 | 6.29±0.34 | 93.32±1.23 | 99.98±0.01 | 0.02±0.01 |
| 500.0 | | | 71.63±2.34 | 28.37±2.34 | 41.84±1.36 | 98.41±1.28 | 1.59±1.28 |
| 250.0 | | | 38.54±4.54 | 65.77±4.54 | -4.93±9.22 | 59.60±17.90 | 40.40±17.90 |
| 125.0 | | | 4.10±0.79 | 95.90±0.79 | -37.92±11.23 | 42.92±8.95 | 57.08±8.95 |
| 100.0 | | | | | | | |
| 62.5 | | | | | | 60.39±4.08 | 39.61±4.08 |
| 50.0 | | | | | | | |
| 31.2 | | | | | | 56.96±2.16 | 43.04±2.16 |
| 25.0 | | | | | | | |
| 20.0 | 100.00±0.00 | 0.00±0.00 | | | | | |
| 15.6 | | | | | | 12.61±5.80 | 87.39±5.80 |
| 12.5 | | | | | | | |
| 10.0 | 100.00±0.00 | 0.00±0.00 | | | | | |
| 5.0 | 100.00±0.00 | 0.00±0.00 | | | | | |
| 2.5 | 99.95±0.04 | 0.05±0.04 | | | | | |
| 1.2 | 55.65±19.29 | 44.35±19.29 | | | | | |
| 0.0 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 |
| MBC | 2.5 μM | | >2000.0 μM | | | 1000.0 μM | |
| $IC_{50}$ | 1.08±0.14 μM | | 323.83±16.41 μM | | | 42.38±2.74 μM | |

3. Antimicrobial activity of Pm11 peptide against bovine mastitis bacteria

| Final conc. of Pm11 (μM) | *E. coli* (SCM1249) | | *Klebsiella* spp. (SCM1282) | | *S. aureus* (CM967) | | *S. agalactiae* (SCM1084) | | *S. uberis* (SCM1310) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | %Inhibit | %Survival | %Inhibit | %Survival | %Inhibit | %Survival | %Inhibit | %Survival | %Inhibit | %Survival |
| 80.0 | 100.00±0.00 | 0.00±0.00 | | | | | | | | |
| 40.0 | 100.00±0.00 | 0.00±0.00 | | | | | | | | |
| 20.0 | 100.00±0.00 | 0.00±0.00 | | | 100.00±0.00 | 100.00±0.00 | | | | |
| 10.0 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 100.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 |
| 5.0 | 100.00±0.00 | 0.00±0.00 | 99.88±0.08 | 0.12±0.08 | 99.98±0.01 | 99.98±0.01 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 |
| 2.5 | 100.00±0.00 | 0.00±0.00 | 44.07±0.74 | 55.93±0.74 | 97.15±0.97 | 97.15±0.97 | 99.66±0.11 | 0.34±0.11 | 99.56±0.19 | 0.41±0.19 |
| 1.2 | 99.28±0.16 | 0.72±0.16 | 1.85±29.33 | 98.16±29.33 | -29.83±44.28 | 129.83±44.28 | 57.21±10.52 | 42.79±10.52 | 55.41±9.73 | 35.83±9.73 |
| 0.6 | 88.42±3.29 | 11.58±3.29 | -15.89±10.16 | 115.89±10.16 | 13.76±2.26 | 13.76±2.26 | 36.77±10.29 | 63.23±10.29 | 34.88±5.47 | 70.59±5.47 |
| 0.3 | 44.47±7.64 | 55.53±7.64 | -27.10±12.53 | 127.10±12.53 | -9.20±21.50 | 109.20±21.50 | | | | |
| 0.0 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 | 0.00±0.00 | 100.00±0.00 |
| MBC | 2.5 μM | | 10.0 μM | | 5.0 μM | | 5.0 μM | | 5.0 μM | |
| $IC_{50}$ | 0.32±0.03 μM | | 2.07±0.47 μM | | 1.33±0.19 μM | | 0.87±0.18 μM | | 0.97±0.15 μM | |

# APPENDIX E

## Time kill kinetic of the Pm11 peptide to bovine mastitis pathogens

1. Time kill kinetic of Pm11 peptide to *E. coli* (SCM1249)

| Time (h) | Colony plate count (CFU/ml) Replicate1 | Colony plate count (CFU/ml) Replicate2 | Colony plate count (CFU/ml) Replicate3 | Average Colony plate count (CFU/ml) |
|---|---|---|---|---|
| Control *E. coli* + Ultrapure water | | | | |
| 0 | $5.03*10^5$ | $5.13*10^5$ | $5.23*10^5$ | $5.13*10^5$ |
| 1 | $6.87*10^5$ | $7.23*10^5$ | $6.67*10^5$ | $6.92*10^5$ |
| 2 | $3.30*10^6$ | $3.67*10^6$ | $5.67*10^6$ | $4.21*10^6$ |
| 4 | $3.80*10^8$ | $3.67*10^8$ | $3.90*10^8$ | $3.79*10^8$ |
| 8 | $1.07*10^9$ | $1.05*10^9$ | $0.93*10^9$ | $1.02*10^9$ |
| 12 | $1.35*10^9$ | $1.41*10^9$ | $1.56*10^9$ | $1.44*10^9$ |
| *E. coli* + Pm11 peptide (final concentration 2.5 μM) | | | | |
| 1 | $9.03*10^3$ | $9.57*10^3$ | $8.37*10^3$ | $8.99*10^3$ (98.70%inhibit) |
| 2 | $1.50*10^3$ | $1.97*10^3$ | $1.45*10^3$ | $1.64*10^3$ (99.96%inhibit) |
| 4 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |

2. Time kill kinetic of Pm11 peptide to *Klebsiella* spp. (SCM1282)

| Time (h) | Colony plate count (CFU/ml) Replicate1 | Colony plate count (CFU/ml) Replicate2 | Colony plate count (CFU/ml) Replicate3 | Average Colony plate count (CFU/ml) |
|---|---|---|---|---|
| Control *Klebsiella* spp. + 1X PBS | | | | |
| 0 | $3.90*10^5$ | $4.80*10^5$ | $3.80*10^5$ | $4.17*10^5$ |
| 1 | $6.77*10^5$ | $5.20*10^5$ | $6.27*10^5$ | $6.08*10^5$ |
| 2 | $3.45*10^6$ | $4.60*10^6$ | $4.17*10^6$ | $4.07*10^6$ |
| 4 | $1.65*10^8$ | $1.94*10^8$ | $2.13*10^8$ | $1.91*10^8$ |
| 8 | $8.87*10^8$ | $1.03*10^9$ | $9.87*10^8$ | $9.68*10^8$ |
| 12 | $1.43*10^9$ | $1.52*10^9$ | $1.51*10^9$ | $1.49*10^9$ |
| *Klebsiella* spp. + Pm11 peptide (final concentration 10.0 μM) | | | | |
| 1 | $1.92*10^5$ | $1.88*10^5$ | $1.79*10^5$ | $1.86*10^5$ (69.41%inhibit) |
| 2 | $4.87*10^3$ | $5.27*10^3$ | $6.10*10^3$ | $5.41*10^3$ (99.87%inhibit) |
| 4 | $1.50*10^3$ | $1.10*10^3$ | $1.20*10^3$ | $1.27*10^3$ (100.00%inhibit) |
| 8 | 0.00 | $2.73*10^3$ | $4.00*10^3$ | $3.37*10^3$ (100.00%inhibit) |
| 12 | $1.02*10^8$ | $6.08*10^8$ | $6.87*10^8$ | $4.66*10^8$ (68.72%inhibit) |

3. Time kill kinetic of Pm11 peptide to *S. aureus* (CM967)

| Time (h) | Colony plate count (CFU/ml) Replicate1 | Colony plate count (CFU/ml) Replicate2 | Colony plate count (CFU/ml) Replicate3 | Average Colony plate count (CFU/ml) |
|---|---|---|---|---|
| Control *S. aureus* + 1X PBS | | | | |
| 0 | $3.35*10^5$ | $2.62*10^5$ | $2.54*10^5$ | $2.84*10^5$ |
| 1 | $4.23*10^5$ | $2.37*10^5$ | $2.78*10^5$ | $3.13*10^5$ |
| 2 | $3.23*10^5$ | $2.33*10^5$ | $4.10*10^5$ | $3.22*10^5$ |
| 4 | $1.30*10^6$ | $1.00*10^6$ | ND | $1.15*10^6$ |
| 8 | ND | ND | ND | ND |
| 12 | $1.40*10^8$ | $1.33*10^8$ | $1.20*10^8$ | $1.31*10^8$ |
| *S. aureus* + Pm11 peptide (final concentration 5 µM) | | | | |
| 1 | $1.91*10^4$ | $1.70*10^4$ | $1.78*10^4$ | $1.80*10^4$ (94.25%inhibit) |
| 2 | $2.27*10^3$ | $2.37*10^3$ | $2.93*10^3$ | $2.52*10^3$ (99.22%inhibit) |
| 4 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| *S. aureus* + Oxacillin antibiotic (final concentration 2 µM) | | | | |
| 1 | $1.58*10^4$ | $1.67*10^4$ | $2.08*10^4$ | $1.78*10^4$ (94.31%inhibit) |
| 2 | $1.54*10^4$ | $2.53*10^4$ | $1.11*10^4$ | $1.73*10^4$ (94.63%inhibit) |
| 4 | $3.05*10^3$ | $2.73*10^3$ | $2.50*10^3$ | $2.76*10^3$ (99.76%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |

4. Time kill kinetic of Pm11 peptide to *S. agalactiae* (SCM1084)

| Time (h) | Colony plate count (CFU/ml) Replicate1 | Colony plate count (CFU/ml) Replicate2 | Colony plate count (CFU/ml) Replicate3 | Average Colony plate count (CFU/ml) |
|---|---|---|---|---|
| Control *S. agalactiae* + 1X PBS | | | | |
| 0 | $1.04*10^5$ | $1.26*10^5$ | $1.02*10^5$ | $1.10*10^5$ |
| 1 | $2.86*10^5$ | $2.52*10^5$ | $2.22*10^5$ | $2.53*10^5$ |
| 2 | $1.48*10^6$ | $1.07*10^6$ | $1.27*10^6$ | $1.27*10^6$ |
| 4 | $9.27*10^6$ | $1.05*10^7$ | $1.25*10^7$ | $1.08*10^7$ |
| 8 | $2.29*10^8$ | $2.82*10^8$ | $4.77*10^8$ | $3.29*10^8$ |
| 12 | $2.60*10^8$ | $2.33*10^8$ | $2.47*10^8$ | $2.47*10^8$ |
| *S. agalactiae* + Pm11 peptide (final concentration 5 µM) | | | | |
| 1 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 2 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 4 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| *S. agalactiae* + Ampicillin antibiotic (final concentration 180 µM) | | | | |
| 1 | $2.55*10^4$ | $2.37*10^4$ | $2.43*10^4$ | $2.45*10^4$ (90.32%inhibit) |
| 2 | $3.43*10^3$ | $6.13*10^3$ | $3.03*10^3$ | $4.20*10^3$ (99.67%inhibit) |
| 4 | 0.00 | 0.00 | $1.30*10^3$ | 0.00 (100.00%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |

5. Time kill kinetic of Pm11 peptide to *S. uberis* (SCM1310)

| Time (h) | Colony plate count (CFU/ml) Replicate1 | Colony plate count (CFU/ml) Replicate2 | Colony plate count (CFU/ml) Replicate3 | Average Colony plate count (CFU/ml) |
|---|---|---|---|---|
| Control *S. uberis* + 1X PBS | | | | |
| 0 | $3.97*10^5$ | $3.65*10^5$ | $3.70*10^5$ | $3.77*10^5$ |
| 1 | $5.60*10^5$ | $6.43*10^5$ | $6.00*10^5$ | $6.01*10^5$ |
| 2 | $1.15*10^6$ | $9.30*10^5$ | $1.00*10^6$ | $1.03*10^6$ |
| 4 | $8.93*10^5$ | $1.39*10^6$ | $1.15*10^6$ | $1.14*10^6$ |
| 8 | $9.57*10^6$ | $6.80*10^6$ (1 rep) | $4.80*10^6$ | $7.18*10^6$ |
| 12 | $1.11*10^7$ | $3.50*10^6$ (1 rep) | $9.63*10^6$ | $1.04*10^7$ |
| *S. uberis* + Pm11 peptide (final concentration 5 µM) | | | | |
| 1 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 2 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 4 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 8 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 (100.00%inhibit) |

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