ANTIVIRULENCE ACTIVITY OF EFFECTOR MOLECULES FROM *ENTEROCOCCUS FAECALIS* STRAIN R3 AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION

NATCHAYA PAKDEESIRIWONG

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การลดความรุนแรงของการเกิดโรคติดเชื้อจาก *Staphylococcus aureus* โดยโมเลกุลที่ผลิตจาก *Enterococcus faecalis* สายพันธุ์ R3

นางสาวณัฐชยา ภักดีศิริวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2566

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ณัฐชยา ภักดีศิริวงษ์ : การลดความรุนแรงของการเกิดโรคติดเชื้อจาก *Staphylococcus aureus* โดยโมเลกุลที่ผลิตจาก *Enterococcus faecalis* สายพันธุ์ R3 (ANTIVIRULENCE ACTIVITY OF EFFECTOR MOLECULES FROM *ENTEROCOCCUS FAECALIS* STRAIN R3 AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มัณฑนา แจ่มกลาง, 61 หน้า.

คำสำคัญ: เชื้อ *Staphylococcus aureus*, เชื้อ *Enterococcus faecalis*, การเกาะติด, การควบคุมโดยชีววิธี, เมตาโบโลมิกส์

เชื้อ *Staphylococcus aureus* (*S. aureus*) ซึ่งเป็นเชื้อแบคทีเรียที่ดำรงชีวิตแบบภาวะเกื้อกูล และยังเป็นเชื้อแบคทีเรียก่อโรคในบางโอกาส โดยทำให้เกิดโรคต่าง ๆ มากมาย ตั้งแต่การติดเชื้อที่ผิวหนังเล็กน้อยไปจนถึงแก่ชีวิตได้ การกำหนดเป้าหมายไปที่ปัจจัยความรุนแรงของเชื้ออาจเป็นกลยุทธ์ที่มีประสิทธิภาพในการต่อสู้กับการติดเชื้อและเสนอทางเลือกใหม่ให้กับยาปฏิชีวนะ ในการศึกษาก่อนหน้าพบ *Enterococcus faecalis* สายพันธุ์ R3 สามารถทำให้เม็ดเลือดแดงแตกจาก *S. aureus* ลดลง ดังนั้นวัตถุประสงค์ของงานวิจัยนี้จึงต้องการศึกษาความสามารถส่วนเหนือตะกอนของ *Enterococcus faecalis* สายพันธุ์ R3 (EFR3) ต่อปัจจัยที่ทำให้เกิดโรคของ *S. aureus* รวมถึงความเป็นพิษต่อเซลล์และความสามารถในการยึดเกาะกับเซลล์และการทำให้เซลล์เกิดความเสียหาย nuclear magnetic resonance (NMR) spectroscopy ถูกนำมาใช้เพื่อระบุ metabolomic profile ของ EFR3 และ ส่วนเหนือตะกอนของ *E. faecium* (EFC) จากผลการวิเคราะห์ metabolomic พบว่ามีการผลิตกรดแลกติกและกรดฟอร์มิกจาก EFR3 อย่างมีนัยสำคัญ และความเข้มข้นของ EFR3 ที่ 50% มีผลยับยั้งการยึดเกาะของ *S. aureus* กับเซลล์ Caco-2 นอกจากนี้การแสดงออกของยีนที่ก่อให้เกิดความรุนแรงของโรค เมื่อทำการทดสอบด้วยเทคนิคปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับ (RT-PCR) พบว่าทั้งใน *S. aureus* และ *S. aureus* ที่ถูกทดสอบกับ EFR3 มีการแสดงออกของยีน *hla* จากการผลศึกษาชี้ให้เห็นถึงแนวทางในการพัฒนาวิธีการรักษาด้วยปัจจัยการลดความรุนแรงที่มุ่งเน้นการติดเชื้อ *S. aureus* โดยไม่ต้องพึ่งยาปฏิชีวนะเพียงอย่างเดียว อย่างไรก็ตามการศึกษาต่อไปในอนาคตการสำรวจรูปแบบการแสดงออกของยีนโดยใช้ quantitative real-time PCR (qPCR) หรือการแสดงออกของโปรตีนโดยใช้การวิเคราะห์แบบ Western Blot เพื่อ

เป็นข้อมูลในการอธิบายถึงอิทธิพลหรือวิถีทาง (pathways) ของ EFR3 ต่อการติดเชื้อ *S. aureus* ในระดับโมเลกุล

สาขาวิชาปรีคลินิก
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ลายมือชื่อนักศึกษา ณัฐชยา
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

NATCHAYA PAKDEESIRIWONG : ANTIVIRULENCE ACTIVITY OF EFFECTOR MOLECULES FROM *ENTEROCOCCUS FAECALIS* STRAIN R3 AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION THESIS ADVISOR : ASST. PROF. MANTANA JAMKLANG, Ph.D. 61 PP.

Keyword: *Staphylococcus aureus, Enterococcus faecalis,* adhesion ,biocontrol, Metabolomics

Staphylococcus aureus (*S. aureus*), a commensal bacterium and human pathogen, is a leading cause of numerous diseases, ranging from minor skin infections to potentially fatal conditions, targeting its virulence factors can be an effective strategy to combat infections and offer novel alternatives to conventional antibiotics. Our previous studies found *Enterococcus faecalis* strains R3 exhibited a positive result of the reverse-CAMP test attenuating from *S. aureus*. The aim of this study was to investigate the influence of the supernatant of *Enterococcus faecalis* strains R3 (EFR3) on factors contributing to the pathogenicity of *S. aureus*, including hemolysis and cytotoxicity. Nuclear magnetic resonance (NMR) spectroscopy was utilized to identify the metabolomic profile of EFR3 and *E. faecium* supernatant (EFC). The metabolomic analysis revealed significant production of lactic acid and formic acid by EFR3. Then, 50% EFR3 concentration exhibited inhibitory effects on *S. aureus* adhesion to Caco-2 cells. Moreover, the results revealed that presence of the *hla* virulence gene, in both *S. aureus* treated with EFR3 using reverse transcription polymerase chain reaction (RT-PCR) technique. The findings from this study suggest promising avenues for the development of antivirulence therapies targeting *S. aureus* infections without relying solely on conventional antibiotics. However, future research directions could involve exploring gene expression patterns using quantitative real-time PCR (qPCR) or

protein expression using western blot analysis to gain further insights into the effects or pathways of EFR3 on *S. aureus* infections at the molecular level.

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Natchaya Pakdeesiriwong

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LIST OF ABBREVIATIONS

A	Absorbance
ADAM10	A Disintegrin and Metalloprotease 10
CFU	Colony forming unit
CO_2	Carbon dioxide
°C	Degree Celsius
cm^3	Cubic centimeter
DNA	Deoxyribose nucleic acid
EFC	*Enterococcus faecium* supernatant
EFR3	*Enterococcus faecalis* strain R3 supernatant
g	Gram
g/l	Gram per liter
MALDI-TOF MS	Matrix assisted laser desorption/Ionization time of flight mass spectrometry
h	Hour
µg	Microgram
µl	Microliter
µm	Micrometer
mg/mL	Milligram per milliliter
min	Minute
mM	Millimolar
ng/mL	NanoGram per Milliliter
^1H NMR	Proton nuclear magnetic resonance
nm	Nanometer
nM	Nanomol

LIST OF ABBREVIATIONS (Continued)

NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ppm	Parts per million
rpm	Revolutions per minute
rDNA	Ribosomal deoxyribose nucleic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Second

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Staphylococcus aureus (*S. aureus*) is Gram-positive cocci in clusters being part of both pathogenic and non-pathogenic organisms. *S. aureus* colonizes on the nasal cavity, skin, and mucosal membranes of every system in humans (Wertheim et al., 2005). Previous publication reported that *S. aureus* can change its capability from a commensal to a pathogen becoming the most dangerous bacterial pathogen (Tong et al., 2015). Infections from pathogenic strains of *S. aureus* are often associated with the formation of abscesses, localized pus-producing lesions, and toxin production. In patients with low immunity, *S. aureus* can cause internal organ infections including pneumonia, osteomyelitis, endocarditis, staphylococcal enteritis, septicemia, and a life-threatening infection, staphylococcal toxic shock syndrome. Pathogenic strains of *S. aureus* produce a variety of virulence factors including exotoxins which are cytotoxic, enabling it to adhere, invade, and spread within the host cells (Sandel and McKillip, 2004). The exotoxins include a group of polypeptides capable with a function in damaging the host cell plasma membrane, such as pore-forming toxins α-hemolysin, serving their role in *S. aureus*-mediated diseases. Alpha hemolysin is the most important one that are cytotoxins in which they destroy the host cells including red blood cells and cause hemolysis leading to severe infections. Specifically, alpha-hemolysin is a key *S. aureus* virulence factor that leads to hemostasis disturbances, thrombocytopenia, and pulmonary lesions (Bhakdi and Tranum-Jensen, 1991 and Ortines et al., 2018) Non-pathogenic strains of *Enterococcus* spp. are Gram-positive cocci in chain which have been successfully used as probiotics to improve human or

animal health. The main effect of ingestion of enterococci probiotics against gastrointestinal disease, as a result of foodborne pathogens or from intestinal micropopulation imbalance, may contribute to the host by competing for microenvironments more effectively than pathogens. Enterococci can be isolated from inhabitants of diverse ecosystems, including the human gastrointestinal tract (Riaz et al., 2019). Enterococci produce and secrete compounds to communicate between cells and exclude competitive bacteria in ecological niches such as the gastrointestinal tract and oral cavity. One of these compounds is bacteriocin which have the ability to produce antimicrobial substances (Franz et al., 2011). Previous studies have demonstrated that *Enterococcus* spp. prevents colonization of pathogenic bacteria and possess some molecular factors act as antimicrobial activity against other bacterial pathogens (Özdemir et al., 2011).

Our previous studies have found several strains of *Enterococcus* spp. possess a capability in inhibition of hemolysins produced from *S. aureus*. Most enterococcal isolates that showed the hemolytic attenuating phenotype were identified as *Enterococcus faecalis* by MALDI-TOF MS. Our co-culture method has shown that the *E. faecalis* strain R3 (EFR3) was the most efficient strain in hemolytic activity attenuation. Therefore, in this study, we aimed to study the anti-virulence properties of EFR3 in the *in vitro* model of *S. aureus* infections. These properties include reduction of cytotoxicity, and cell adhesion damage induced by hemolysin produced from *S. aureus*. Moreover, we aimed to study the regulation of staphylococcal hemolytic activity by EFR3 whether the hemolysin (*hla*) gene is controlled in the gene expression levels. In addition, nuclear magnetic resonance (NMR) based metabolomics was used to compare bacterial culture supernatants between EFR3 and *E. faecium* supernatants (EFC) and explore key metabolite differences. The information received from our study will confirm the properties of effector molecule produced from EFR3 and this molecule could be applied as therapeutic agent for reduction of severity of systemic and local infections caused by *S. aureus* infections.

1.2 Research objectives

1.2.1 To evaluate the ability of EFR3 in attenuation of fibroblast cell damage by *S. aureus* supernatant infection

1.2.2. To investigate the ability of EFR3 in interference of the *S. aureus* adhesion to intestinal epithelial cells

1.2.3 To study the effect of EFR3 on α-hemolysin expression

1.2.4 To study metabolic profiles of EFR3 and EFC

1.3 Research hypothesis

EFR3 produces effector molecules that can interfere hemolytic activity of *S. aureus* hemolysin, thereby attenuating cell cytotoxicity and adhesion response induced by *S. aureus* infection.

1.4 Scope and limitations of the study

This research involved investigation of the influence of EFR3 on *S. aureus* infection. The study assessed the ability of EFR3 to mitigate fibroblast cell damage induced by *S. aureus* supernatants and evaluated the cytotoxicity of EFR3 on PMA-stimulated THP-1 cells. Additionally, the study evaluated the impact of EFR3 on *S. aureus* adhesion. The metabolic profiles of *E. faecalis* and *E. faecium* culture media were examined by proton nuclear magnetic resonance (^{1}H NMR)-based metabolomic characterization. Further investigation of the expression of the *hla* gene and α-hemolysin production in *S. aureus* was conducted to assess the effect of EFR3 in the hemolytic activity inhibition pathway in *S. aureus*.

CHAPTER II

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

S. aureus, a Gram-positive coccus bacterium that forms clusters, is a highly dangerous pathogen (Figure 2.1) that causes various infections and diseases in humans. Its virulence factors contribute to the development of various types of infections, ranging from skin infections to life-threatening diseases (Balasubramanian et al., 2017). The enormous health burden of *S. aureus* is consistent with a mortality rate of up to 57% in adult patients with *S. aureus* bacteremia (Van et al., 2012).

In addition, the rapid spread of multidrug-resistant and highly virulent *S. aureus* strains is increasing, leading to increased morbidity and mortality that could diminish the major economic benefits worldwide. According to recent studies, the annual number of *S. aureus* infections in the United States is a major problem, with 119,000 infections and nearly 20,000 deaths. The dangerous pathogens need to be brought under control (Kavanagh, 2019). The overuse of antibiotics contributes to the development of antibiotic resistance and this problem could favour the spread of *S. aureus* (Sampedro et al., 2014)

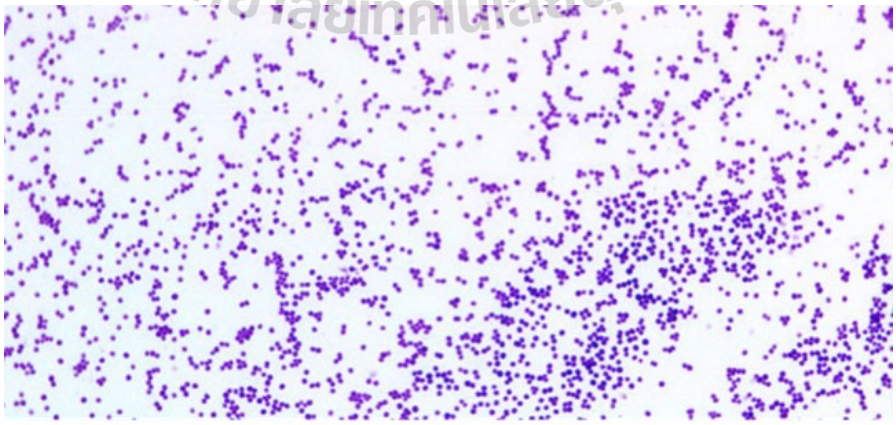

Figure 2.1 Gram staining of *S. aureus* shows Gram-positive cocci in cluster (Jumaah et al., 2014).

2.1.1 Pathogenesis of *S. aureus*

This bacterium, commonly found on the skin and mucous membranes of humans and animals, can cause a wide range of infections, varying from mild skin issues to severe, life-threatening conditions. Thus, acquiring a profound comprehension of *S. aureus*' pathogenesis is crucial for developing effective treatment strategies (Becker, 2018). Infections primarily occur through contiguous spreading in the skin and its associated structures. The natural habitats of *S. aureus* are the skin and mucous surfaces of humans and other mammals, making it easily transmissible and causing skin and soft tissue infections due to its ubiquitous presence on the skin (Linz et al., 2023).

Skin infections caused by *S. aureus* commonly start with invasion through a hair follicle, leading to folliculitis. This can progress to furuncles (boils) and potentially develop into carbuncles. Superficial infections affecting the outer layers of the epidermis are known as impetigo, while deeper infections involving the dermis or subcutaneous tissues result in cellulitis (Del Giudice, 2020) Collectively, these infections are referred to skin infections. During *S. aureus* skin infections, tissue damage occurs triggering inflammation,an immune response characterized by increased temperature, redness, swelling, and pain in the affected area. In some cases, abscess formation may occur. If the infection spreads further particularly to adjacent areas, it can infiltrate deeper muscle tissues, resulting in pyomyositis. *S. aureus* also possesses the ability to reach bone tissue, causing osteomyelitis, and can affect joints, leading to septic arthritis (Urish and Cassat, 2020). *S. aureus* infections predominantly results in skin and soft tissue infections. Moreover, mastitis which is an inflammation of the breast, is now known to be reproductive system and responsible for lactating women. *S. aureus* can enter the breast tissue through cracks or fissures in the nipple, which can occur during breastfeeding. The bacteria can also enter through the milk ducts or from the surrounding skin. The breast pain and inflammation can make breastfeeding uncomfortable and painful for the mother. The infection can also affect milk

production, leading to a decrease in milk supply. If the infection is transmitted to infants during the birth process, it can cause fatal illness (Rimoldi et al., 2020).

Apart from being skin and soft tissue infections, *S. aureus* has an ability to enter the bloodstream through various pathways, including wounds, surgical sites, or through localized infections that spread. However, when *S. aureus* strains that manage to evade other immune defenses can be transported in the bloodstream, allowing them to disseminate throughout the body. *S. aureus* can disseminate to various organs and tissues, leading to metastatic infections. These disseminations include abscesses in organs such as the heart, brain, lungs, liver, or kidneys. Metastatic infections can invade and interfere the functions of surrounding normal tissues and contribute to the severity of the septicemic condition (Corey, 2009).

In addition, acute endocarditis caused by *S. aureus* is a rapidly progressive disease that impair heart valve. *S. aureus* from another body site of infection is transported to the heart. Then it can form vegetations on the heart valves, in which exposed collagen fibers on damaged valvular surfaces trigger fibrin deposition. These vegetations are clumps of infected material that can grow, causing further decrease their flexibility, and prevent heart valves from closing completely. Blood flows backward from ventricles into atria when the ventricles contract, decreasing the pumping efficiency of the heart. Congestive heart failure, an accumulation of fluids around the heart, is the most common complication and direct cause of death from bacterial endocarditis. Fragments of the vegetations then break off into the bloodstream and form clots, which travel through the bloodstream and can lodge in other organs, leading to diseases such as strokes. As a result, *S. aureus* infection is a very dynamic process that spreads widely and metastasizes regularly (Fowler et al., 2005).

If the abnormalities are not eliminated, *S. aureus* may progress to septicemia or blood poisoning. Septicemia results when bacteria can rapidly multiply and disseminate to different organs and tissues. The presence of *S. aureus* in the bloodstream causes a release of inflammatory mediators, such as cytokines and chemokines. This immune response is essential for combating infections. Although the

inflammatory process is usually beneficial, it can sometimes be harmful. Excessive release of pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-alpha), can lead to a state of widespread inflammation throughout the body. This systemic inflammation can affect multiple organ systems and contribute to tissue damage and organ dysfunction (Söderquist et al., 1995).

It is necessary to distinguish between two different types of diseases caused by *S. aureus*: first, systemic infections, which can affect virtually all organs and organ systems, and second, toxin-mediated diseases.

2.2 α-hemolysin as a major virulence factor

There has been ongoing and increasing interest in *S. aureus* virulence determinants to *S. aureus* infection. Several virulence factors of *S. aureus* have roles in the host, ranging from mediating immune suppression, and degrading host tissues to obtain space for spreading or to acquire nutrients for bacterial growth (Divyakolu et al., 2019).

The growth of *S. aureus* is able to produce an extensive array of virulence factors. All *S. aureus* produce secreted exotoxin virulence factors including several cytolytic, pore-forming toxins. Systemic *S. aureus* infection is always dependent on bacterial breach through the epithelial protective layer. For example, skin infections can develop from minor scratches of the skin and may become invasive. Finally, *S. aureus* accomplishes this by distributing through the bloodstream. However, *S. aureus* can also actively promote epithelial breach, for which α-hemolysin has a protein that injures the cell by forming a damaging pore across the cell membrane (Cheung et al., 2021).

One of the most prominent virulence factors produced from *S. aureus*, is α-hemolysin. It is also known as alpha toxin which contributes to pathogenesis in a wide variety of experimental infections, particularly during epithelial infections such as skin abscesses, pneumonia and sepsis. These toxins are produced and secreted during late post exponential phase of bacterial growth as monomers. After secretion as a

soluble monomer, α-hemolysin oligomerizes on the targeted host cell surface via interactions with its high-affinity metalloprotease receptor, a disintegrin and metalloprotease 10 (ADAM10), forming a 1–3-nm pore that spans the cellular membrane lipid bilayer. Pore formation on susceptible host cell membranes triggers alterations in ion gradients, loss of membrane integrity, activation of inflammasome, and cell death (Husmann et al., 2006). In addition, α-hemolysin causes epithelial and endothelial break by breaching adherens junctions and compromising the cytoskeleton. ADAM10 is essential for tissue morphogenesis and remodeling, and acts on a multitude of extracellular substrates, one of which is the adherens junction protein E-cadherin. It has been proposed that α-hemolysin enhanced ADAM10 cleavage of E-cadherin demolishes the adherens junctions to disrupt the integrity of cell–cell contacts in epithelial tissues during infection to contribute to *S. aureus* pathogenesis (Figure 2.2) (Popov et al., 2015).

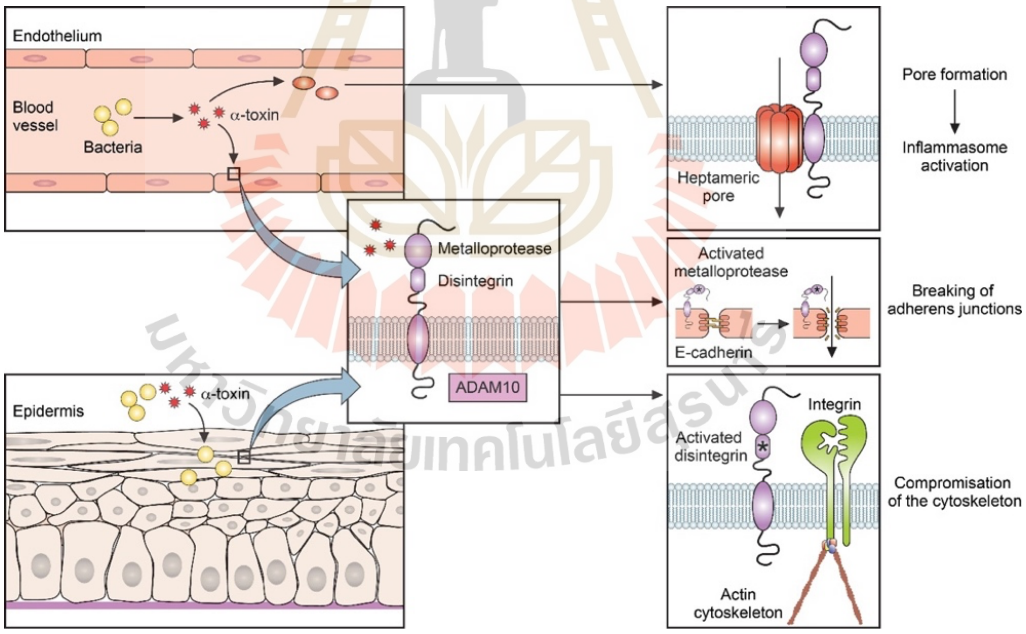

Figure 2.2 Role of α-hemolysin in *S. aureus* infection (Husmann et al., 2006).

This toxin is described for its ability to induce lysis of erythrocytes, which is a key feature of *S. aureus* and is one of the best characterized pore-forming toxins encoded by the *hla* gene. The expression of virulence factors is controlled in a

coordinated fashion by a network of regulatory systems. Multiple regulatory factors of *hla* revealed 3 regulatory systems, comprising the accessory gene regulator (*agr*), staphylococcal accessory protein effector (saeRS) and staphylococcal accessory gene regulator (*sarA*) systems (Jenul et al., 2019). The *agr* locus activates *hla* expression directly and positively, while *sarA* exerts a positive impact on *hla* expression by both *agr*-dependent and *agr*-independent pathways. In addition, the *sae* locus includes a two-component signal-transduction system encoded by *saeS* and *saeR* that positively regulates the expression of *hla* at the transcriptional level (Xiong et al., 2006).

2.3 Inflammatory response

Inflammation is a protective response by the body that aims to remove invading pathogens, neutralize noxious stimuli, and initiate tissue repair. Inflammation is triggered when innate immune cells sense to pathogens (pathogen-associated molecular patterns, PAMPs) or endogenous stress signals (damage-associated molecular patterns, DAMPs) recognition by germline-encoded pattern recognition receptors (PRRs). PRR activation triggers a complex array of inflammatory processes through the release of proinflammatory cytokines that further have the ability to induce the release of a broad range of cytokines (Figure 2.3).

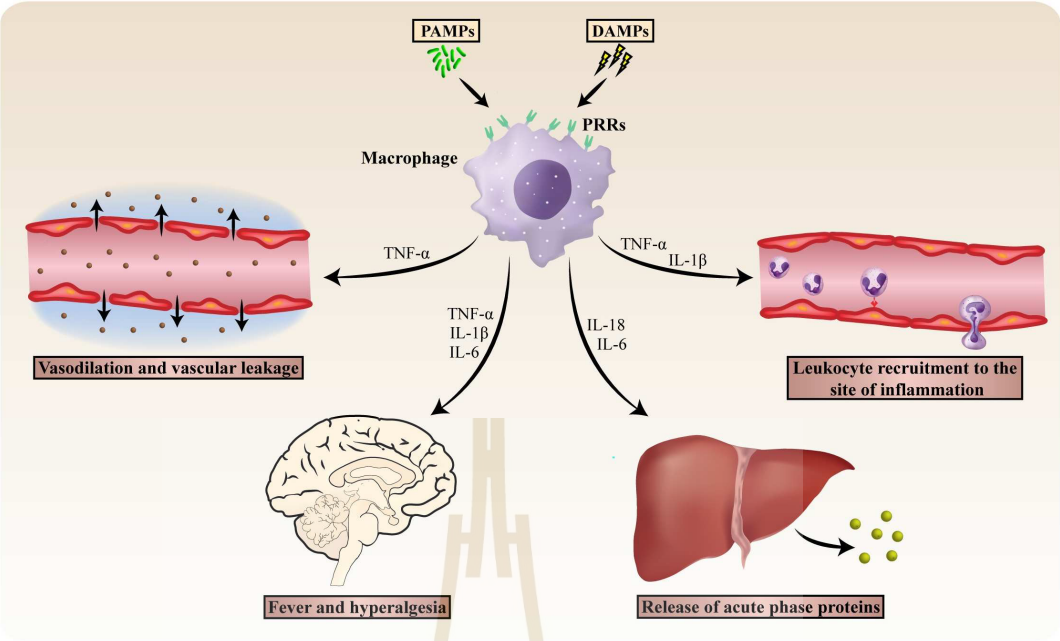

Figure 2.3 The acute inflammatory response mediated by stimuli (Slaats et al., 2016).

Physiologic inflammatory signaling can be triggered by pathogen and host derived molecules. Activation of the NLRP3 inflammasome by α-hemolysin is a critical pathway that induces an inflammatory response and can lead to cell damage. Staphylococcal α-hemolysin is capable of inducing activation of caspase-1 through the NLRP3 inflammasome. The NLRP3-inflammasome is activated in response to a multitude of pro-inflammatory stimuli, including interleukin-1β and interleukin-18. Additionally, the α-hemolysin activate the NLRP3 inflammasome to further induce a caspase-independent pro-inflammatory program of necrotic cell death. Finally, the α-hemolysin has also been shown to induce some cytokine secretion, including IL-6 in mouse peritoneal macrophages (Bhakdi et al., 1991 and Onogawa, 2002).

2.4 Antivirulence therapy

Antivirulence therapy is an approach in disarming key virulence factors involved in disease progression rather than killing bacteria. Unlike traditional antimicrobials, antivirulence do not inhibit bacterial growth (Totsika, 2016). Toxin neutralization constitutes a useful strategy to decrease the virulence of pathogens, as secretion of

toxins is used by pathogens to colonize the host as well as to get away host immune system response (Fleitas et al., 2019). This recent research on *S. aureus* antivirulence strategies, with pore-forming toxins virulence factors. Therapeutics developed for direct inhibition of pore-forming toxins have thus far exploited two naturally occurring mechanisms neutralizing antibodies and decoy membrane receptors. An alpha-hemolysin neutralizing monoclonal antibody (mAb) was previously developed. Alpha hemolysin binds the metalloprotease ADAM10 to promote oligomerization and pore formation. Exploiting this mechanism, MEDI4893 inhibits α-hemolysin interactions with ADAM10 and α-hemolysin self-oligomerization by binding a highly conserved region of α-hemolysin. Importantly, mutants resistant to neutralization show reduced dermatonecrosis and mortality in murine models of *S. aureus* skin infection and pneumonia, respectively, likely reflecting impair α-hemolysin function in the mutants (Surewaard et al., 2018). As of November 2020, MEDI4893 has completed Phase 2 clinical trials for prevention of *S. aureus* pneumonia in high-risk ICU patients. The data demonstrate a non-statistically significant trend toward prevention (Ford et al., 2021).

2.5 Non-pathogenic strains of *Enterococcus faecalis*

Enterococcus spp. is a Gram-positive, non-spore-forming facultative anaerobic organism that is arranged individually, in pairs, or short chains. *Enterococcus* spp. are opportunistic pathogens that are likely to be highly intrinsically virulent. It's necessary to ensure that it is a safe strain before using it in probiotics. Strains are opportunistic pathogens that are likely to be highly intrinsically virulent. It's necessary to ensure that it is a safe strain before using it in probiotics (Oliveira, et al., 2007). However, they are successfully used as probiotics to improve human or animal health. These are known for their ability to survive harsh conditions, such as high temperatures, high salt concentrations, and they can be found in various environments including the intestines of humans and animals, soil, water, and food. *E. faecalis* has been studied for its potential health benefits, such as its ability to inhibit the growth of harmful bacteria. The main effect of ingestion of enterococci probiotics in against to gastrointestinal

disease, as a result of foodborne pathogens or from intestinal micro population imbalance, may contribute the host by competing for microenvironments more effectively than pathogens (Franz et al., 2011). Some strains showed antimicrobial potential including methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Özdemir et al., 2011 and Riaz et al., 2018). The presence of *E. faecalis* in additional biopreservative leads to prevention of pathogen growth in fermented food. These bacteria may play an important beneficial role in the production of various traditional such as dairy products and sausages. Some species of *Enterococcus* have been used as probiotics in many countries because of the high ability to produce bacteriocins (Franz et al., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains and cell lines

The bacterial strains utilized in this study include *S. aureus* ATCC 25923, which was acquired from the american type culture collection (ATCC, USA), *E. faecium* and *E. faecalis* strains R3, obtained from our previous studies. Especially the remarkable strain of *Enterococcus* which is *E. faecalis* strains R3, which exhibited a hemolytic attenuating phenotype. The bacterial isolates were cultured in tryptic soy at 37°C for 18-24 hours (h) before proceeding to all experiments.

The cell lines that were used in this study as follows:

Human leukemia monocytic cell line (THP-1 monocytic cells ATCC®TIB-202™) was purchased from the ATCC. This cell line was used as a model for studying the function and biology of human macrophages. The viability of THP-1 macrophages treated with EFR3 after 24 h of culture was assessed. The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA), 1% penicillin/streptomycin (Gibco, NY, USA), and 2-mercaptoethanol to a final concentration of 0.05 mM.

Human fibroblast cell line (OUMS-36 JCRB®) which was purchased from the Japanese Collection of Research Bioresources (JCRB, cell bank, Japan) provided by Assoc. Prof. Dr. Pathanin Chantree. This cell line was maintained in culture according to Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% FBS (Gibco, NY, USA), and 1% penicillin/streptomycin (Gibco, NY, USA).

Human colon cancer (Caco-2 cell line ATCC ®HTB-37™) was purchased from ATCC. This epithelial cell line is an isolation from colon tissue that was used as a model to assess the adhesion ability of *S. aureus* with or without EFR3 in vitro.

The cells were routinely cultured in DMEM supplemented with 20% FBS (Gibco, NY, USA), and 1% penicillin/streptomycin (Gibco, NY, USA).

All cell lines were cultured under a 37°C and 5% CO_2 atmosphere until reaching 80% confluency, and then trypsinized using 0.25% trypsin-EDTA (Gibco, NY, USA).

3.1.2 Culture media

Tryptic soy (TS) medium (Himedia, India) contained 17 g/L of tryptone (pancreatic digest of casein), 3 g/L of soya peptone, 5 g/L of sodium chloride, 2.5 g/L of dipotassium hydrogen phosphate, and 2.5 g/L of dextrose (pH 7.3 ± 0.2). One liter of medium was supplemented with 30 g of tryptic soy medium to create the tryptic solid medium. The medium was dissolved and disinfected by at 121°C under pressure of approximately 15 pounds per square inch autoclaving for 20 minutes (min).

RPMI-1640 Medium (ATCC, USA) was modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L of glucose, and 1500 mg/L of sodium bicarbonate (pH =7.0 to 7.4).

DMEM (Himedia, India) was adjusted to include 4500 mg/L of glucose, 4 mM L-glutamine, 1500 mg/L of sodium bicarbonate, and 1 mM sodium pyruvate (pH = 7.0 to 7.4).

All culture medium for the cell lines was stored at 4°C until required.

3.1.3 phorbol 12-myristate-13-acetate (PMA)

PMA powder was purchased from Selleck Chemicals, USA and stored at -20°C until used.

3.1.4 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

MTT powder was purchased from Himedia, India and stored at -20°C until used.

3.2 Methods

3.2.1 Microorganism culture for bacterial supernatants

The bacterial strains (*S. aureus* ATCC 25923, *E. faecium* and *E. faecalis* strains R3) were initially cultured from -80°C glycerol stock on tryptic soy agar (TSA). Fresh single colonies were then inoculated in tryptic soy broth (TSB) and cultured at 37°C

with aeration, achieved by incubating in tubes/flasks with a capacity at least four times the volume of medium being used and vigorous shaking on an orbital shaker (200 rpm). Overnight cultures were diluted 1:100 in fresh TSB and cultured until reaching the post-exponential growth phase (OD_{600} = 2.5) for all experiments except the adherence assay. After this incubation, the cells were harvested by centrifugation. The filtration process involved passing through a 0.2 μm filter (MilliporeSigma, Germany) to isolate supernatants containing effector molecules into the culture media.

3.2.2 Cytotoxicity evaluation of EFR3 with THP-1 cells

The MTT assay was performed to evaluate a cytotoxic effect of EFR3 on macrophage differentiation in PMA-stimulated THP-1 cells. MTT production therefore correlates directly on the cell survival rate. THP-1 cells were seeded on 96-well plate growth medium at a concentration of 1×10^6 cells in RPMI 1640 medium at 37°C, 5% CO_2 for 24 h. Then, THP-1 cells were being differentiated into macrophages by treatment with 200 nM (123.3 ng/mL) phorbol 12-myristate-13-acetate (PMA) for 24 h. After that, 0-50% EFR3 supernatant were added into each well and the viability of the cells at 24 h after treatment were evaluated by MTT assay. MTT was added to each well at a concentration of 0.5 mg/mL and were incubated for 3 h and the medium were discarded. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO) before adding to each well of the differentiated macrophages. The reaction generated color and the absorbance at 562 nm of each well were measured using a microplate reader. The percentage of cell viability was compared with the untreated control. The values were expressed as mean ± SD, with $p < 0.05$ indicating statistical significance compared to the untreated control. The experiments were analyzed using analysis of variance (ANOVA) and Tukey's HSD (honestly significantly different) test to determine differences between the groups for statistical significance.

3.2.3 Cytotoxicity evaluation of EFR3 with OUMS-36 cells by *S. aureus* supernatant infection

The MTT assay was conducted to assess the impact of EFR3 on the survival rate of fibroblast cells in the presence of *S. aureus*-inducing injury. The viable cells

contain NAD(P)H-dependent oxidoreductase enzymes which reduce the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan. First, *S. aureus* was cultured until reaching the post-exponential growth phase (OD_{600} nm = 2.5) in TSB. After centrifugation of bacterial culture supernatants, they were combined with EFR3 solutions to final 50% concentrations. Then, fibroblast cells were cultured until they reached 80% confluence. The cells were then washed three times with phosphate-buffered saline (PBS). Next, the fibroblast cells were seeded into 96-well plates and incubated for 24 h. After that, the fibroblast cell medium was replaced. The DMEM medium of the fibroblast cells was then combined with *S. aureus* supernatants alone, *S. aureus* supernatants combined with the previously prepared EFR3, and medium without *S. aureus* supernatants using TSB as a control. Cell viability at 48 h post-treatment was assessed via the MTT assay. MTT solution was added to each well at a concentration of 0.5 mg/mL and incubated for 3 h Following this, the medium was removed, and the formazan crystals dissolved in dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 562 nm was measured using a microplate reader machine (Thermo Scientific, USA). Subsequently, the percentage of cell viability was obtained after normalizing the data with that of received from untreated control cells. Untreated cells data was served as a positive control which is 100% cell viability. The data indicate statistically significant differences between groups as determined by paired t-test.

3.2.4 Adherence assay method

To examine whether EFR3 was able to prevent *S. aureus* adherence to CaCo-2 cells. The cells were seeded in 24-well plates. Once monolayers reached 80% confluence, cells were washed three times with phosphate buffered saline (PBS). After that, *S. aureus* were grown to early exponential phase (OD_{600} = 0.5) in TSB. The suspension containing *S. aureus* was diluted with TSB at a ratio of 1:10 to achieve a *S. aureus* OD_{600} of 0.05. Then, the cells were treated with 50% of EFR3 solution in DMEM medium and co-incubated in suspension with *S. aureus* for 2 h. After incubation, the cells were washed with PBS to remove non-cell associated bacteria. Adhered

bacteria were quantified from viable counts obtained by 3 times washing and lysing the CaCo-2 cell for 30 min in water and plating serial dilutions on TSA. The colony forming units (CFU)/ml were determined to quantify the number of *S. aureus* cells adhered to CaCo-2 cells. The data indicate statistically significant differences between groups as determined by paired t-test.

3.2.5 The effect of EFR3 on *S. aureus hla* gene expression

To investigate the mechanism of EFR3 antivirulence activity, reverse transcription polymerase chain reaction (RT-PCR) was used to determine differential expression of *hla* genes.

3.2.5.1 Test of *S. aureus* culture treated with EFR3

Staphylococcus aureus ATCC 25923 was cultured in TSB with or without treatment with EFR3 for 16 h with shaking at 200 rpm. After treatment, the culture medium was removed and the cell pellets were collected before resuspended in ice cold PBS.

3.2.5.2 RNA Isolation by phenol- chloroform method

Total RNA of *S. aureus* strains was extracted from the bacterial culture using the phenol-chloroform reagent. The pellet was resuspended in 100 µl of RNase-free water. The tube was vigorously vortexed for 3 min, and then 100 µl of acid phenol was added with chloroform (1:1). It was vortexed for 1 min and incubated at 70°C for 30 min. The cell debris was centrifuged at 12,000 g for 10 min at 4°C, and the 100 µl supernatants were collected. RNA was precipitated from the upper aqueous layer with 200 µl isopropanol. The tube was vigorously vortexed for 3 min and centrifuged at 12,000×g for 10 min. Two hundred microliters of 70% ethanol were added to clean the precipitated pellet from phenol and isopropanol, then centrifuged at 8000 g for 5 min. The ethanol was removed, and the tube was left upside down to dry for 3 min before 25 µl of RNase-free water was added. The method's overview is depicted in Figure 3.1 (Atshan el at., 2012). DNA elimination was performed by incubation of the purified RNA with DNase I (PanReac AppliChem, Germany) in which one unit DNase I

recombinant, RNase-free is heat-inactivated by 10 min incubation at 75°C. Total RNA was quantified using a using NanoDrop® 100^{C} Spectrophotometer (NanoDrop, USA).

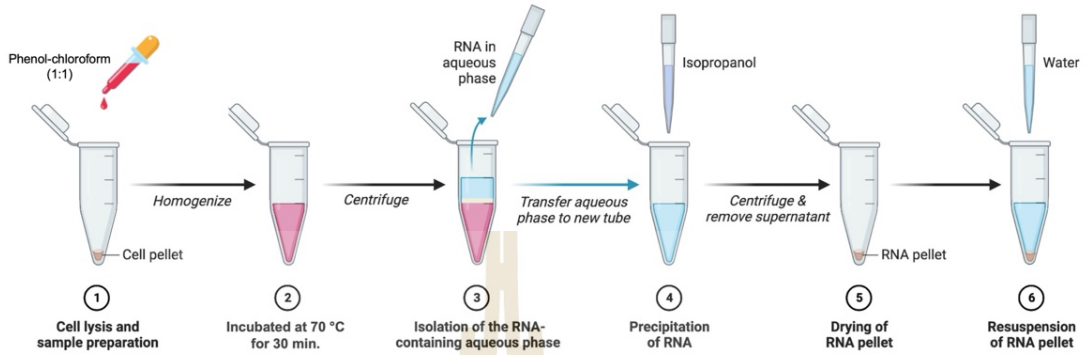

Figure 3.1 The method of phenol- chloroform for the RNA isolation from *S. aureus* (Atshan et al., 2012).

3.2.5.3 Amplification of the *hla* gene by RT-PCR

RNA molecules are converted into their complementary DNA (cDNA) sequences by reverse transcriptase (SuperScript® III First-Strand Synthesis System for RT-PCR, Invitrogen, USA). Subsequently, an assessment of optimal PCR conditions to simultaneously amplify the Hla gene has been conducted. The cDNA was used as the template for PCR amplification of the *hla* and 16S rDNA genes (Table 3.1).

Table 3.1 List of primers used in this study.

Primers	Primer's sequences (5'-3')	PCR product size	References
HlaF	GGTTTAGCCTGGCCTTC	543 bp	(Salasia et al., 2004)
HlaR	CATCACGAACTCGTTCG		(Salasia et al., 2004)
27F	AGAGTTTGATCCTGGCTCAG	1500 bp	(Lane, 1991)
1525R	AAGGAGGTGWTCCARCC		(Lane, 1991)

The PCR mixture (GoTaq® Colorless master mix, Promega, USA.) used for PCR amplification in total volume of 25 µl is shown in Table 3.2. The reaction was conducted in a thermal cycler, adhering to the following conditions: an initial denaturation step of 2 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, extension for 30 s at 72°C, and a final extension step of 7 min at 72°C. The amplification products were assessed by loading 5 µl of the total DNA onto a 1% agarose gel (Vivantis) containing 0.5× tris-borate-EDTA (TBE) buffer and subjected to electrophoresis at 100 volt for 35 min and the results were visualized using a geldoc go gel imaging system (Bio-Rad, USA).

Table 3.2 The PCR mixture condition.

Component	Volume (µl)
GoTaq® Colorless Master Mix, 2X	12.5
upstream primer, 10 µM	1
downstream primer, 10 µM	1
DNA template	1
Nuclease-Free Water to	25

3.2.6 The identification of metabolites in the EFR3 using nuclear magnetic resonance (NMR) analysis

NMR spectroscopy exploits the magnetic properties of atomic nuclei to probe and identify the quantity of metabolites present in biological samples. In this study, NMR spectra of the bacterial cultivation medium were analyzed. In this study, the metabolomics of the EFR3 were analyzed by NMR spectroscopy (Bruker, USA), comparing *Enterococcus faecium* supernatants (EFC) and culture medium (control). The NMR data were processed at Khon Kaen University International Phenome Laboratory, KKUIPL.

3.2.6.1 Preparation of bacterial cultivation medium samples

The tested microorganisms were *E. faecalis* strain R3 and *E. faecium*.

The bacterial cultivation medium is as reported in the topic 3.2.2. The cultivation medium without bacteria served as a control. In our study, five samples of supernatants received from culture medium were used. Then, the culture supernatant was stored at -20°C for further analyses.

3.2.6.2 Metabolic profiling based on ^{1}H-NMR

Each culture medium sample was passed through a 0.20 μm filter (Corning, USA). The supernatant was transferred into a 5 mm NMR tube. The analysis of ^{1}H NMR profiles of the bacterial cultivation medium was performed using the Bruker AVANCE III 400 MHz spectrometer (Bruker Corporation, USA) at 300 K. All samples were analyzed using a standard 1-dimensional pulse sequence (recycle delay-90˚-t1-90˚-tm-˚-acquisition) with t1 and t2 set to 3 ms, tm set to 10 ms, and a 90˚ pulse of 10 μs in 64 scans. Then, the raw NMR data to enhance spectral quality and remove artifacts. The phase and baseline of all NMR spectra were adjusted in MestReNova (Mestrelab Research, USA) and the TSP peak was set to 0 ppm. Data points within the range of 0.6–9.0 ppm was retained, while the water peak interval (4.5–5.0 ppm) was eliminated. The data were processed using a control spectrum following probabilistic quotient normalization (PQN). To identify the correlation between metabolites in NMR data using Pseudo-two-dimensional spectra generated from statistical total correlation spectroscopy (STOCSY). Additionally, the area under peaks was integrated and utilized for relative concentration determination. The relative concentrations of metabolites were evaluated via log2 fold-changes employing GraphPad Prism 5 (GraphPad Software, Inc., CA, US). Metabolites exhibiting log2 fold-changes underwent Spearman correlation analysis using R programming. Heatmap analysis was conducted using MetaboAnalyst (www.metaboanalyst.ca/). Access to the MBROLE2 system is available at http://csbg.cnb.csic.es/mbrole2.

3.2.6.3 Multivariate statistical analysis of metabolome data

The processed spectral data matrix was imported into SIMCA version 14.1 (Umetrics Inc., Sweden) to perform principal component analysis (PCA) employing a pareto scaling method. This facilitated visualization of metabolic similarities and

differences and identification of potential outliers. Subsequently, orthogonal partial least squares discriminant analysis (O-PLS-DA) were conducted using MATLAB R2016a (MathWorks, USA). The fitness and predictability of the models derived from OPLS-DA were assessed Q^2 values.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Previous study screening for bacterial strains with anti-hemolytic activity

All one hundred eighty-two *Enterococcus* spp. were examined for its anti-hemolytic activity by using reverse CAMP tests. The results exhibited that seven isolates of *E. faecalis* can interfere hemolytic activity *of S. aureus*. The representative *Enterococcus* spp. demonstrating a positive result in reverse-CAMP test is shown in Figure 4.1. However, *E. faecium* was found to be the most prevalent enterococcal species (172 isolates), followed by *E. raffinosus* (1 isolates), and *E. avium* (1 isolates) which illustrated negative results in reverse-CAMP tests.

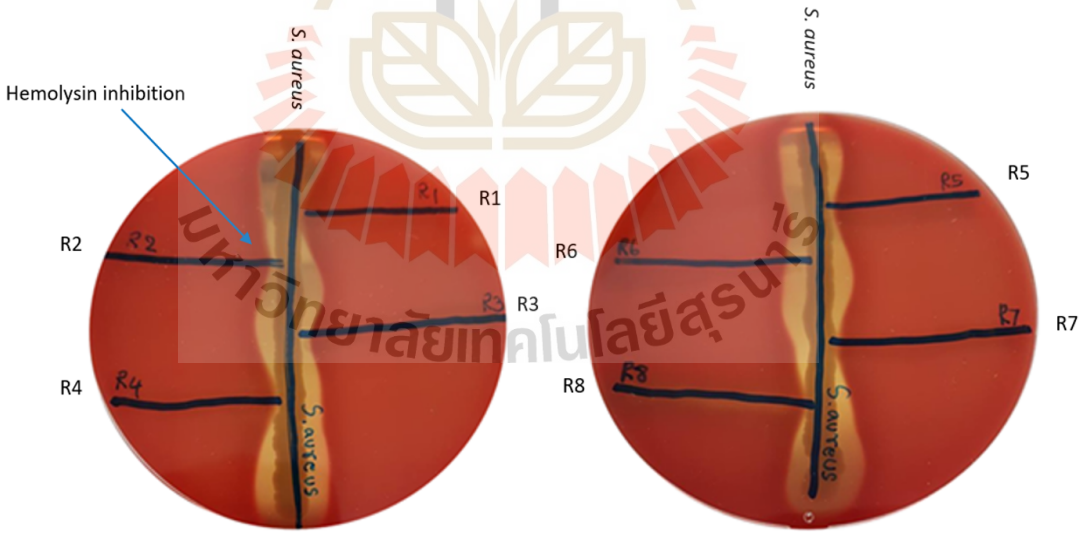

Figure 4.1 Seven strains of *E. faecalis* (R1-R5, R7-R8) demonstrated the attenuation of *S. aureus* hemolysis.

Here, we demonstrate that only *E. faecalis* are species that have hemolysis attenuation ability in *S. aureus*.

Moreover, our previous studies using a co-culture method confirmed that the *E. faecalis* strain R3 (EFR3) was the most efficient strain in hemolytic activity attenuation. The red blood cell lysis was remarkably attenuated when the *E. faecalis* R3 were added with equal volumes of *S. aureus* supernatant and red blood cell incubation compared to the condition of red blood cells incubated with *S. aureus* alone (Figure 4.2). Therefore, in this study, we aimed to study the anti-virulence properties of EFR3 in the *in vitro* model of *S. aureus* infections.

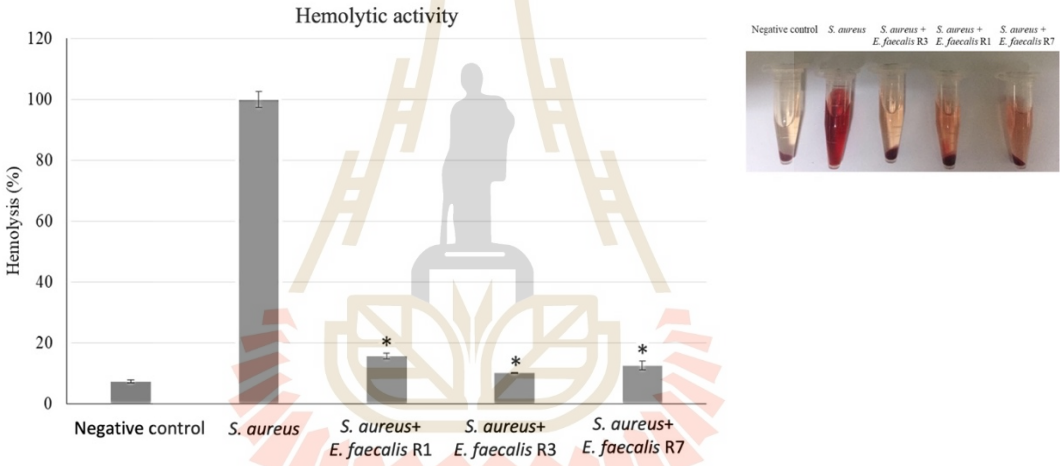

Figure 4.2 *E. faecalis* R3 was the most effective at inhibiting the hemolytic activity of *S. aureus*.

4.2 The Cytotoxic effect of EFR3 on PMA-differentiated THP-1 cells

To investigate the cytotoxicity of EFR3 on PMA-differentiated THP-1 cells, we determined the concentration of EFR3 that does not cause cell death, ensuring that any observed effects are due to EFR3 itself and not cytotoxicity. Firstly, an optimal concentration of EFR3 was optimized. A cytotoxicity assay was performed to examine the effects of EFR3 and *S. aureus* supernatant on THP-1 with different concentrations of EFR3 on THP-1 cells, using MTT assay. The THP-1 cells were first grown by incubating

for 48 h to allow the monocytes to differentiate into macrophages by adding PMA to the culture medium at a final concentration of 200 nM (123.3 ng/mL). The various concentrations of PMA are shown in appendix B. As assessed by light microscopy, changes in cell morphology were appeared more macrophage-like phenotype (Figure 4.3). Then, differentiated THP-1 cells were treated with different concentrations of EFR3 (0-50% of EFR3). The MTT assay analysis of differentiated THP-1 cells revealed that EFR3 at a concentration of 3.31 - 50% EFR3 concentration significantly reduced the cell viability compared to the untreated control group ($p < 0.05$). The highest non-toxicity concentration of EFR3 on THP-1 cells was 1.56% EFR3 concentration. (Figure 4.4). In this experiment, we used PMA-differentiated THP-1 cells serve as a model for macrophages, which are essential for studying cytotoxicity. The result suggests that treatment with EFR3 leads to a dose-dependent decrease in the viability of PMA-differentiated THP-1 cells, indicating cytotoxic effects. Therefore, the non-toxicity concentrations of 1.56% EFR3 concentration were selected for further investigation of the ability of EFR3 to inflammatory responses and their interaction with other immune cells or pathogens.

Figure 4.3 Morphologies of THP-1 cells in different stages under light microscopy. (A) Undifferentiated THP-1 cells are free floating round cells. (B). Differentiated THP-1 cells are adherent cell and elongated macrophage-like morphology.

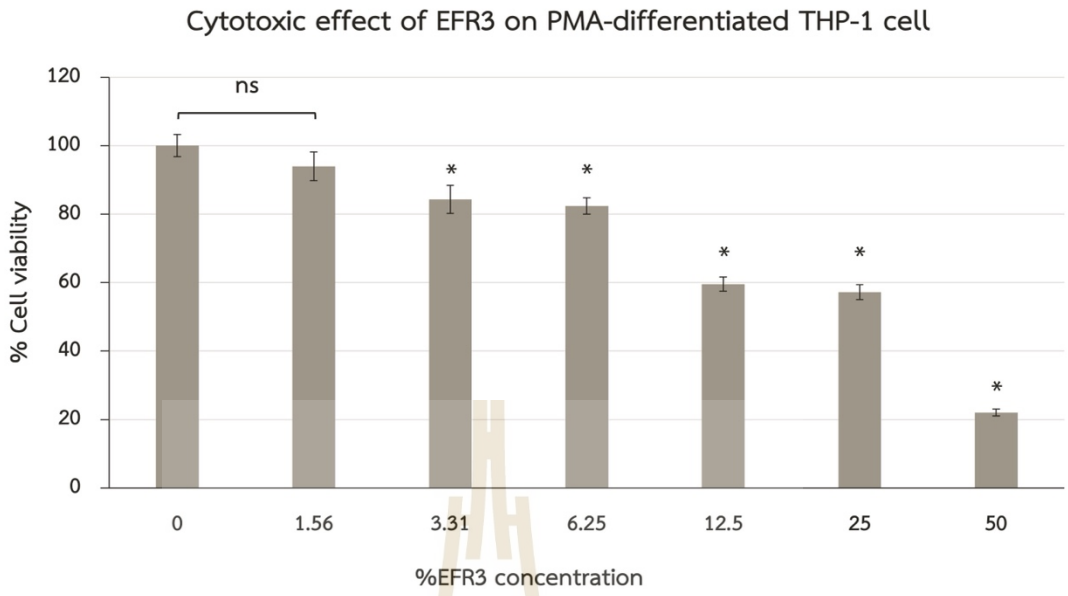

Figure 4.4 The cytotoxicity of EFR3 on differentiated THP-1 cells. The cells were treated with various concentrations of EFR3 (0-50 % EFR3 concentration). The untreated cells were used as controls. All values are represented as the mean ± SD, with "ns" indicating not significant ($p > 0.05$). An asterisk (*) indicates significant differences in the group treated with various concentrations of EFR3 ($p < 0.05$).

4.3 Cytotoxic activity of EFR3 in OUMS-36 cell by *S. aureus* supernatant infection

The bacterial exoproducts released by *S. aureus* mainly include a large amount of toxins and enzymes, such as Toxic Shock Syndrome Toxin (TSST), which acts as a superantigens to trigger a complex molecular pathway, enterotoxins that pose a high risk for food-borne diseases, and hemolysin, which has the ability to lyse RBCs causing hemolysis (Otto, 2014). Our previous studies using a reverse CAMP test and a co-culture test have found that EFR3, a supernatant received from the culture of *E. faecalis* strain R3, has the ability to inhibit the hemolytic activity of *S. aureus*. To determine the EFR3's capability to reduce that effect of the *S. aureus* supernatant on normal cells, cytotoxicity test was used to examine this capability. In our study, we used OUMS-36

which is a fibroblast cell lines as a normal cell control.

In order to investigate the effects of *S. aureus* supernatant received from the *S. aureus* culture, on OUMS-36 cell viability after treatment with EFR3 for 48 hours at 37°C, the cytotoxicities were analyzed using the absorption at 562 nm in the MTT assay (Figure 4.5). The results showed that there was no significant difference ($p > 0.05$) in the cell viability after EFR3 treatment compared to the untreated cells. The cell viability of OUMS-36 tested in *S. aureus* supernatant and *S. aureus* supernatant treated with EFR3 were 77.59 ± 3.3% and 81.27 ± 2.2%, respectively. These results indicate that the inhibition of EFR3 in the hemolytic activity from *S. aureus* was not a result of OUMS-36 cell damage at a concentration of 50% of EFR3. There are some studies elsewhere have reported a similar data in which exosubstance produced from *E. faecalis* was able to inhibit the hemolysis caused from *S. aureus* beta-hemolysin in vitro using of Blood Agar Base (Vítkova and Votava, 2005). In addition, *E. faecalis* EF478 supernatants were demonstrated the highest antibacterial activity against several multidrug-resistant enterococci and vancomycin-resistant enterococci strains (Phumisantiphong et al., 2017). However, the cell viability was significantly reduced in the presence of *S. aureus* supernatant in infected OUMS-36 ($p > 0.05$). Therefore, this finding suggests that *S. aureus* supernatant inhibits the cell viability of OUMS-36.

Zhu Cheng (2013) reported the effects of methicillin-resistant-*S. aureus* (MRSA) supernatant (SAS) on the release of mouse β-defensin-14 (MBD-14) in mouse osteoblasts (OBs) were examined. OBs were treated with SAS at various concentrations, including dilutions based on the number of OBs versus the concentration of bacterial exoproducts from a number of MRSA. The report showed that in the experimental groups treated with higher concentrations of SAS, there was consistently a lower number of cells compared to the groups treated with concentrations equivalent to or lower than 1:10 and 1:20. This observation was statistically significant, indicating a dose-dependent effect of the SAS on cell numbers that induced MBD-14 expression. This suggests that lower concentrations of *S. aureus* may have an effect on cell viability compared to EFR3 supernatant.

In addition, a recent study found that *E. faecalis* MN1 supernatant concentrated 480× relative to the original culture plays a role in IL-8 inhibition (Brosnahan et al.,

2013). This suggests that higher concentrations of EFR3 may have an effect on cell viability compared to *S. aureus* supernatant. Therefore, further study on the variation of the concentrations of EFR3 or *S. aureus* supernatant might show different effects on the cell viability of OUMS-36 cells.

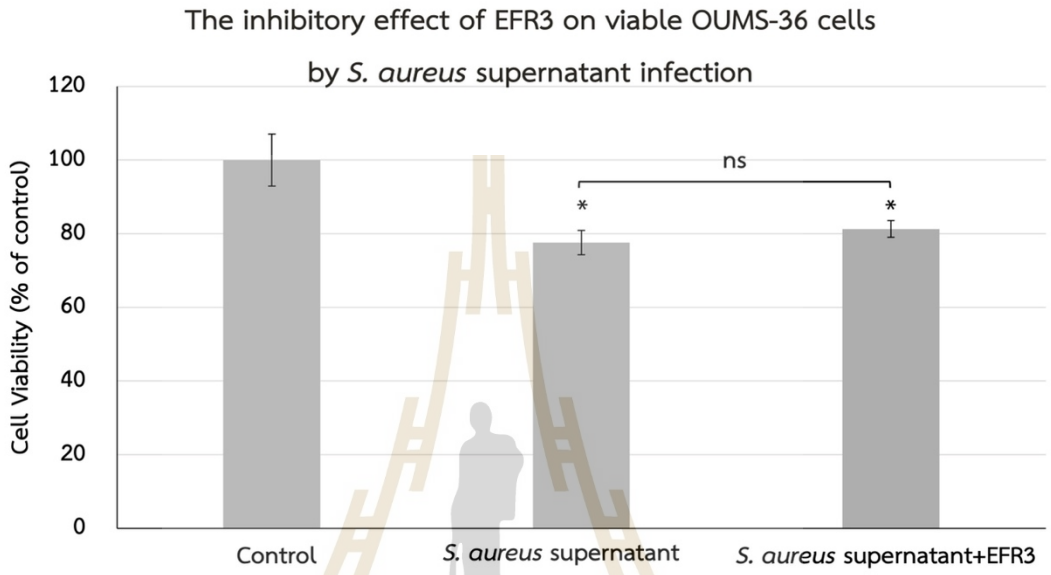

Figure 4.5 The effect of EFR3 on enhancing the viability of OUMS-36 cells infected with *S. aureus* supernatant is comparable to the effect observed with treatment using *S. aureus* supernatant alone. Data are presented as means ± SD, with "ns" indicating not significant ($p > 0.05$), whereas an asterisk (*) indicates statistically significant differences compared to the control (untreated cells) ($p < 0.05$).

4.4 Adhesion of *S. aureus* and EFR3 to Caco-2 cells

Adherence of *S. aureus* to host intestinal epithelial cells can lead to various adverse effects. Many adhesins and other surface proteins of *S. aureus* serve as virulence determinants involved infection. In this study, the Caco-2 cells were used as a model to study adherence ability of *S. aureus*. The Caco-2 cells were co-incubated with 50% EFR3 and *S. aureus* for 2 h with an OD_{600} = 0.05. The colony-forming units (CFUs) were quantified following overnight incubation at 37°C to ascertain the quantity of adherent bacteria on Caco-2 cells by plating method. These results indicate that, the potential of EFR3 to reduce attachment of *S. aureus* to Caco-2 cells was determined by comparing the adhesion of *S. aureus* in the presence of EFR3 to that of *S. aureus* alone is shown in Figure 4.6. The number of *S. aureus* was significantly decreased to 4.3×10^6 CFUs/ml in the Caco-2 cells treated with 50% concentrations of EFR3, while the adhesion cell number of *S. aureus* untreated with EFR3 was 6.6×10^6 CFU/ml. Previous studies revealed the ability of certain strains of *E. faecalis* to adhere to Caco-2 cells (Wang et al., 2020). The higher ability of *E. faecalis* to adhere to epithelial cells suggests a greater potential to colonize the host intestine, making them suitable candidates for probiotic screening. This ability allows them to compete with pathogenic bacteria such as *S. aureus* at a colonization sites.

Moreover, a report from Tinrat and co-workers in 2018 demonstrated an inhibition of pathogenic adhesion by *E. faecalis* MTC 1032 in Caco-2 cells. The ability of *E. faecalis* MTC 1032 could adhere to host epithelial cells and reduce the cell count of *E. coli* ATCC 25922 and *S.* Typhimurium ATCC 13311 (Tinrat et al., 2018).

In conclusion, our results demonstrated similar effects observed with EFR3 significantly inhibiting adhesion on Caco-2 cells compared to treatment with *S. aureus* alone. This suggests that *E. faecalis* strain R3 secretes exoproduct molecules, which are EFR3, with the ability to interfere *S. aureus* infection. Interestingly, this study also provided results investigating molecules from EFR3 (as detailed in section 4.6).

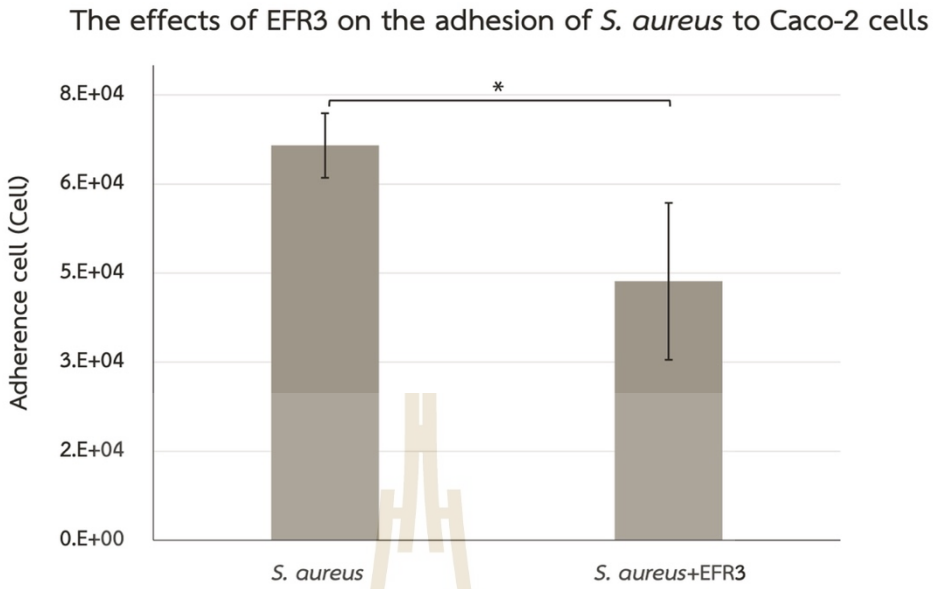

Figure 4.6 The effects of EFR3 on the adhesion of *S. aureus* to Caco-2 cells were assessed. The inhibition of *S. aureus* was calculated by estimating the number of CFUs/ml attached to the Caco-2 cells. The data as represented as the mean ± SD of three repeats (*P < 0.05).

4.5 The effect of EFR3 on staphylococcal *hla* gene and α-hemolysin expression

Alpha hemolysin is produced by *S. aureus* and is a potent pore-forming cytotoxin. This toxin plays a significant role in the pathogenesis of *S. aureus* infections. Several previous studies have reported the dangerous effects of α-toxin from *S. aureus*. The previous results from McElroy and co-workers in 1999 indicated that a mutant strain lacking α-toxin showed decreased toxicity in animal models on the air-blood barrier. Additionally, previous studies have reported that resveratrol (Tang et al., 2019) and flavone (Lee et al., 2012) exhibited antihemolytic activity against *S. aureus*. The expression of virulence genes, such as *hla* gene that encodes alpha hemolysin, was significantly downregulated when exposed to polyphenols.

This down-regulation is likely due to polyphenols binding to the toxin, preventing it from attaching to and damaging host cells. To investigate the effect of

EFR3 on the Staphylococcal *hla* gene and α-hemolysin expression, RT-PCR was utilized to determine the differential expression of *hla* (alpha hemolysin) genes in *S. aureus* cells both with and without EFR3.

The results of culturing *S. aureus* strains under EFR3 conditions were assessed. As a first step, the quality of the extracted RNA from bacteria was evaluated using a NanoDrop® 100^{C} Spectrophotometer. In Table 4.1, RNA concentrations are shown as 703.1 ng/μl for *S. aureus* and 629 ng/μl for *S. aureus* treated with EFR3, respectively. The ratio of absorbance at 260 to 280 ratio (A260/A280) of a pure sample of RNA is ideally 2.0±0.15. Excess salt, DNA, and contaminating proteins can affect the absorbance of RNA, leading to altered values. To remove any contaminating DNA, 5 μg of each RNA was treated with DNase I and then used as a template for converting into complementary DNA (cDNA) molecules, which could subsequently be amplified by PCR.

Table 4.1 List of RNA samples of *S. aureus* the ratios measured via Nanodrop.

Sample	RNA concentration (ng/ μl)	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio
S. aureus	703.1	2.41	2.35
S. aureus treat with EFR3	629.0	2.23	2.20

Next, our results confirmed the presence of *hla* gene. After amplification of genes by optimized PCR conditions and expression of these genes was studied by RT-PCR. The 16s rRNA have been frequently used for reference gene (internal control) analysis in *S. aureus*. The *hla* gene in *S. aureus* encodes for alpha hemolysin, which is a significant virulence factor. Alpha hemolysin disrupts cell membranes by forming pores, leading to cell damage and potentially cell death. This mechanism facilitates the spread of *S. aureus* within the host and contributes to the severity of infections caused by this bacterium.

As depicted, the gel electrophoresis results likely demonstrate the successful amplification of *hla* genes alongside the 16s rRNA gene, which serves as a control. Both S. *aureus* and *S. aureus* treated with EFR3 were positive for the *hla* gene. The results are shown in Figure 4.7. According to previous studies, a PCR investigation of genetic determinants revealed that the *hla* genes were found in all strains investigated, suggesting an important role of these elements in the pathogenicity of *S. aureus* (Salasia et al., 2004).

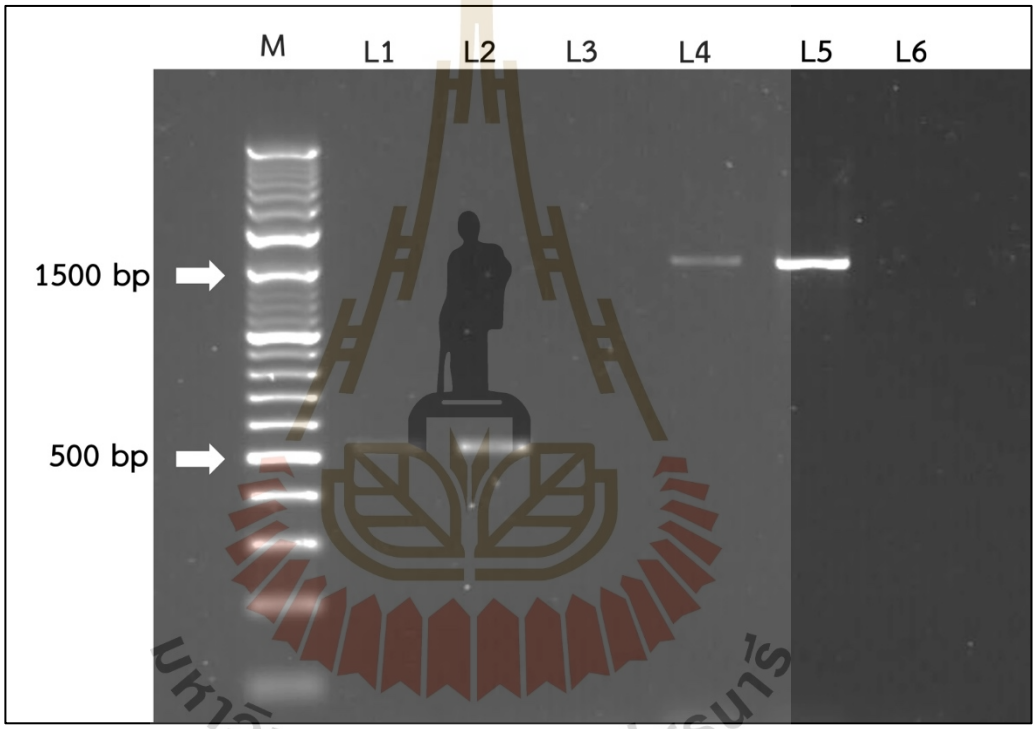

Figure 4.7 The results of gel electrophoresis displayed in the agarose gel show the RT-PCR amplification of *hla* (543 bp) and 16S rRNA (1500 bp) in *S. aureus* strains. The lanes are labeled as follows: M= DNA ladder marker, L1 and L4 = *S. aureus*, L2 and L5= *S. aureus* treated with EFR3, L3 and L6 = negative control.

However, further studies are necessary to confirm the effect of EFR3 on staphylococcal *hla* gene and α-hemolysin expression. These studies should utilize a combination of random hexamers and oligo(dT) primers to ensure the entire length of mRNA molecules is reverse transcribed. Additionally, designing primers so that the

amplification product is shorter will allow for more efficient and accurate quantification, as longer products can lead to variability and reduced sensitivity. Furthermore, investigating quantification in qRT-PCR involves determining the amount of *hla* gene expression in samples.

4.6 The identification of metabolites in EFR3 using Nuclear magnetic resonance (NMR) analysis

The production of bacterial culture supernatants was analyzed using nuclear magnetic resonance (NMR) spectroscopy. The tested isolates that released hemolysis inhibiting molecules, EFR3 were selected for metabolite characterization using NMR spectroscopic data. The EFR3 were analyzed by proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics, comparing with the data received from EFC and culture medium (control). Our previous study found that *E. faecium* is the most prevalent *Enterococcus* species in the gastrointestinal tract, followed by *E. faecalis*, which is frequently found in human stool samples. However, *E. faecium* did not show attenuation of hemolysis. Thus, we are using *E. faecium* as a control to better understand the unique characteristics of *E. faecalis*, leading to informative experimental results.

The chemical shift refers to the displacement of a resonance signal from its position in a reference standard. Protons (^{1}H NMR) are commonly used as the reference standard, with a chemical shift of 0 ppm. In this study, all 27 metabolites produced from bacterial culture supernatants were identified and detected in the NMR spectra. The figure shows the full spectrum followed by extended spectral regions (Figure 4.8) and metabolite list presented in Table 4.2.

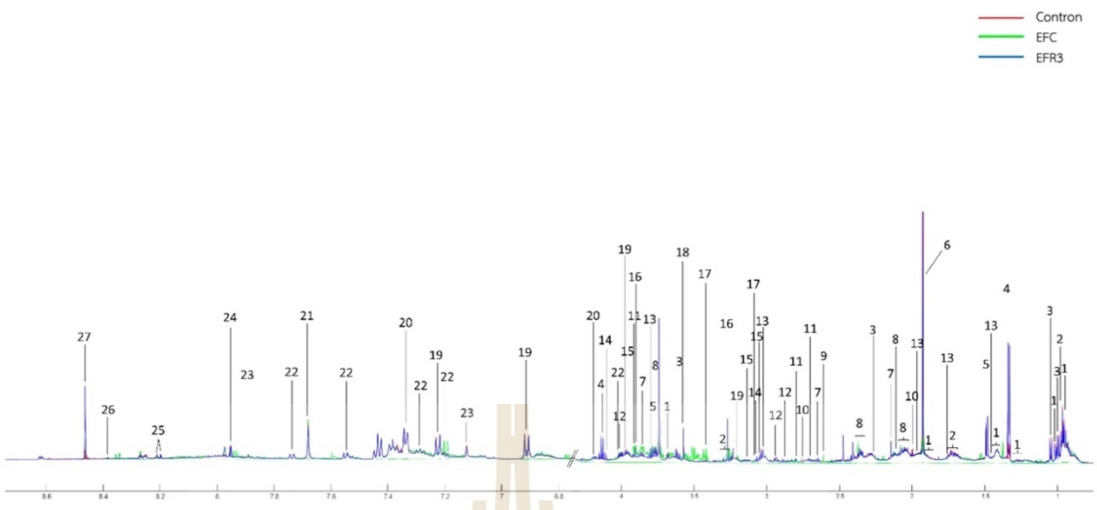

Figure 4.8 Median standard one-dimensional ^{1}H NMR spectrum of all of bacterial culture supernatants were identified. The blue line indicates metabolites found in EFR3, the green line indicates metabolites found in EFC, whereas the red line indicates metabolites found in the control.

Table 4.2 List of all metabolites that found in NMR spectra of bacterial culture supernatants.

NO.	Metabolite	Chemical shift
1	Isoleucine	0.929 (t); 1.007 (d); 1.254 (m); 1.449 (m); 1.895 (m); 3.678 (d)
2	Lucine	0.955 (t); 1.7 (m); 3.732 (m)
3	Valine	0.9897 (d); 1.04 (d); 2.262 (d); 3.597 (d)
4	Lactic acid	1.331 (d); 4.116 (q)
5	Alanine	1.481 (d); 3.754 (q)
6	Acetic acid	1.925 (s)

Table 4.2 List of all metabolites that found in NMR spectra of bacterial culture supernatants (Continued).

NO.	Metabolite	Chemical shift
7	Methionine	2.145 (s); 2.63 (t); 3.848 (dd)
8	Glatamic acid	2.029 (m); 2.114 (m); 2.342 (m); 3.754 (dd)
9	Methylamine	2.611 (s)
10	Unknown1	1.997 (s); 2.76 (s)
11	Aspartic acid	2.67 (dd); 2.798 (dd); 3.891 (dd)
12	Asparagine	2.855 (dd); 2.92 (dd); 4.001 (dd)
13	Lysine	1.46 (m); 1.721 (q); 1.903 (m); 3.011(t); 3.778 (t)
14	Creatinine	3.065 (s); 4.009 (s)
15	Cysteine	3.03 (dd); 3.118 (dd); 3.98 (dd)
16	Betaine	3.269 (s); 3.89 (s)
17	UnKnown2	3.018 (dd); 3.404 (dd)
18	Glycine	3.571 (s)
19	Tyrosine	3.024 (dd); 3.187 (dd); 3.93 (dd); 6.905 (m); 7.218 (m)
20	Phenylalanine	7.331 (m); 4.17 (m); 3.28 (m); 3.118 (m)
21	Unknown3	7.68 (s) 3.285 (dd); 3.470 (dd); 4.057 (dd); 7.19 (m); 7.274 (m);7.524 (d);
22	Tryptophan	7.73 (d)
23	Histidine	3.10 (dd); 3.233 (dd); 3.985 (dd); 7.123 (d);7.89 (d)

Table 4.2 List of all metabolites that found in NMR spectra of bacterial culture supernatants (Continued).

NO.	Metabolite	Chemical shift
24	Unknown4	7.952 (s)
25	Adenine	8.198 (s); 8.211 (s)
26	Unknown5	8.383 (s)
27	Formic acid	8.464 (s)

Abbreviations: S: Singlet, d: Doublet, dd: Doublet of doublet, t: Triplet, q: Quartet, m: Multiplet

Principal Component Analysis (PCA) was conducted to visualize the metabolic similarities and differences of EFR3, EFC, and control metabolome datasets. According to the PCA, the bacterial culture supernatants (Blue and Green) clustered separately from the different cultivation medium (Red) (Q^2 =0.813) (Figure 4.9A). Moreover, slight metabolomic differences were found between the bacterial culture supernatants, from EFR3 (bule) and EFC (green) (Q^2 =0.611) (Figure 4.9B). The Q^2 value can range from negative infinity to 1. A value close to 1 indicates good predictive ability, meaning the model can accurately predict the data it was trained on.

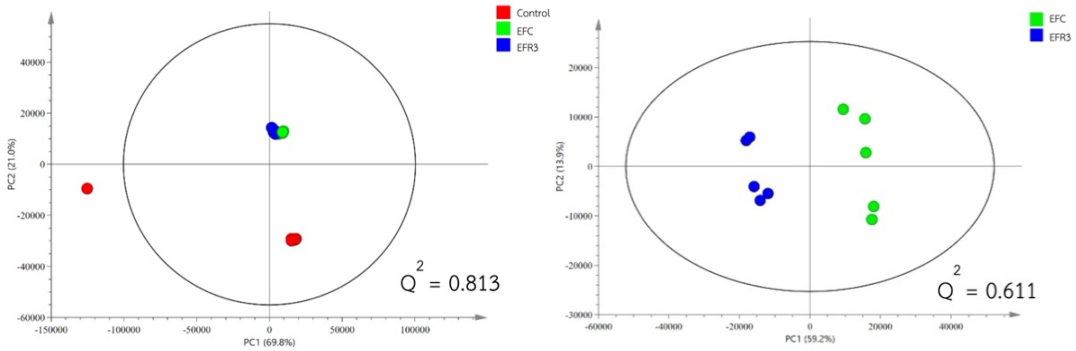

Figure 4.9 Principal Component Analysis (PCA) score plot of ^{1}H NMR spectral data obtained from EFR3 (bule) and EFC (green) of bacterial culture supernatants and control of cultivation medium (Red). (A) PCA score plot of all groups. (B) PCA score plot between medium EFC and EFR3.

The Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model is employed to identify spectral regions or metabolites that exhibit strong supervised clustering, thereby separating the different metabolic profiles based on the sample groups. In this study, ^{1}H NMR EFR3 metabolomes were statistically correlated with EFC and control using OPLS-DA regression analysis. The score plot of the OPLS-DA regression model between EFR3 metabolome and control (R^2X = 0.97 Q^2Y = 0.999, CV-ANOVA $p < 0.01$) revealed prominent class clustering (Figure 4.10A). Similarly, the score plot between EFR3 metabolome and EFC (R^2X = 0.998 Q^2Y = 0.984, CV-ANOVA $p < 0.01$) also demonstrated notable class clustering (Figure 4.10B). The statistical analysis revealed significant differences, and the trend was similar to the PCA analysis. This result indicates that the EFR3 were separated into distinct groups. Previous work by Chen, Y. M. in 2022 studied how probiotics can improve gastrointestinal sensation and demonstrated the different potential active ingredients from the supernatants of *E. faecium* and *E. faecalis* on sensation and movement by regulating the serotonin transporter (SERT) expression in intestinal epithelial cells. Therefore, the supernatants from *E. faecium* and *E. faecalis* were found to be distinct for each species, containing reported bioactive compounds.

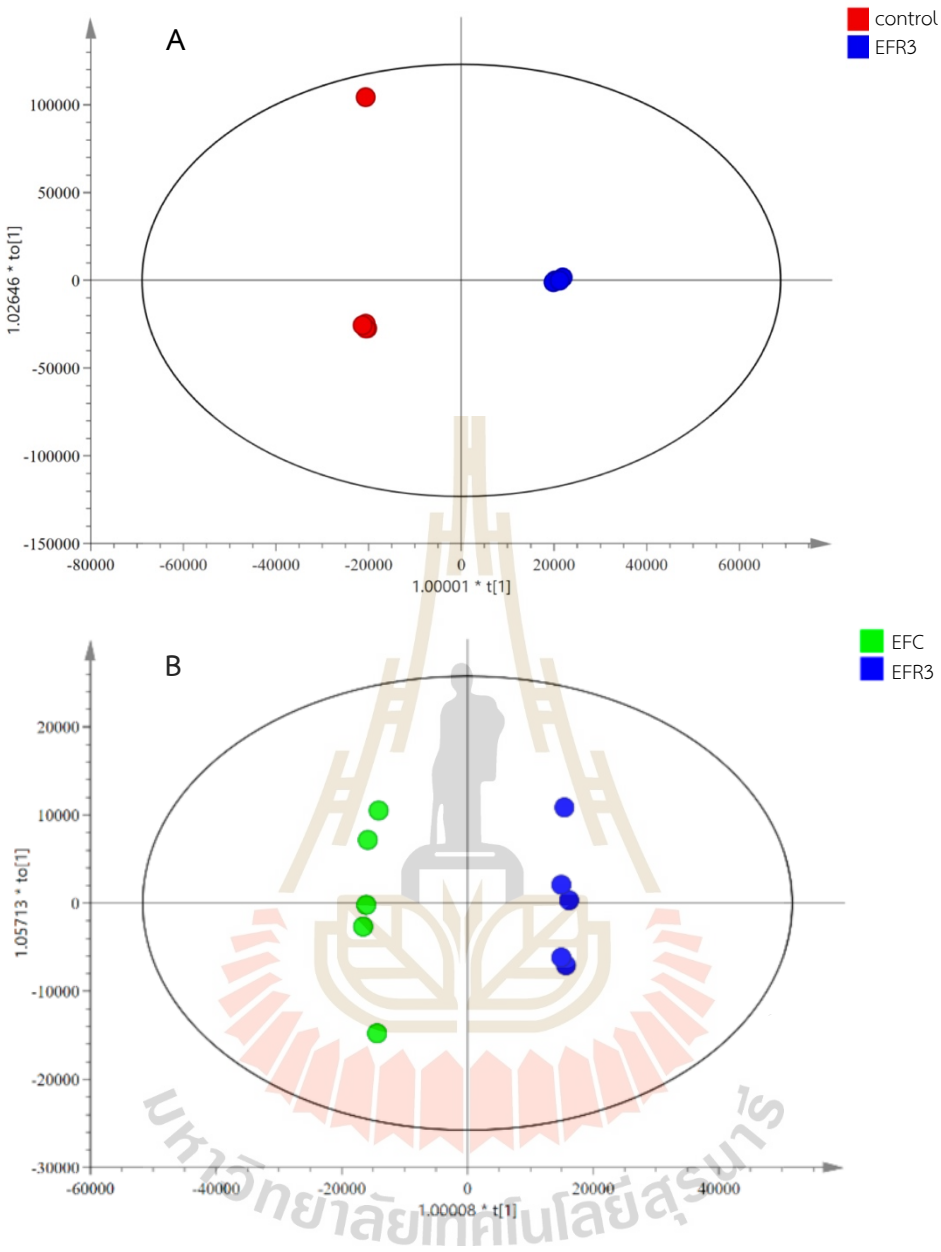

Figure 4.10 OPLS-DA score plot with significant p-values for EFR3 between the control (A) and EFC (B), indicating that each bacterial cultivation medium can be differentiated into a distinct group.

In addition, OPLS-DA color-coded correlation coefficient loading plots, as shown in Figure 4.11, included a label representing the importance of different metabolites, demonstrating discrimination of metabolite levels between EFR3 and EFC.

The key metabolites included lactate, formate, and isovalerate. The color gradient showed a higher correlation (red) with lactate and formate and a lower correlation (blue) with isovalerate.

Moreover, Maximum intensity of all significant metabolites between group of EFC and EFR3 was used for quantification of relative concentrations by comparing integration values in arbitrary units (AU) of different peaks in the spectrum. The EFR3 group showed a significant decrease in relative concentration of glycine, isoleucine, and unknown 1 compared to the EFC group, while lactic acid and formic acid significantly increased in the EFC group (Figure 4.12) The overall metabolite assignment from ^{1}H NMR analysis results are shown appendix C for all the studied groups.

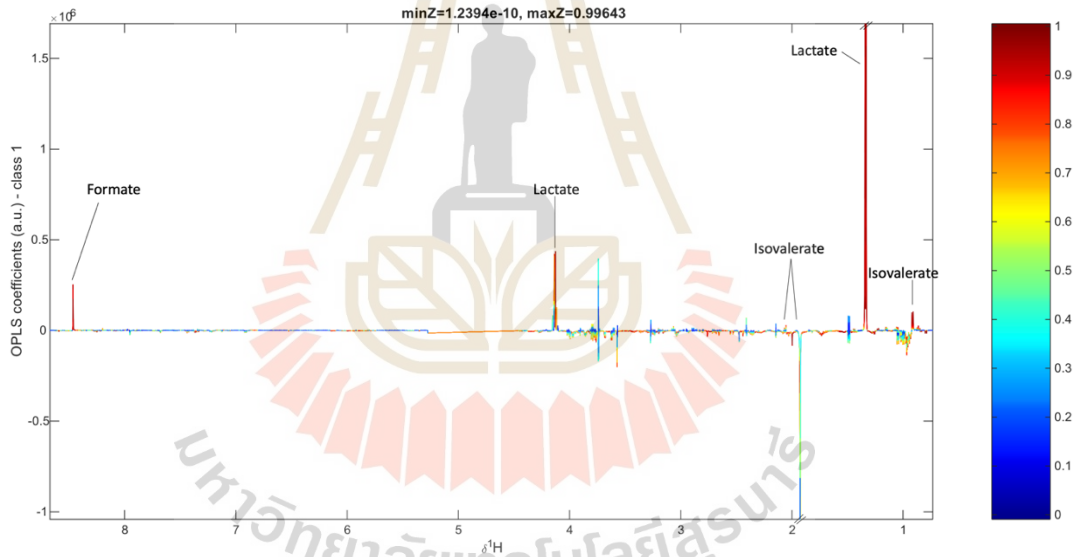

Figure 4.11 OPLS-DA color-coded correlation coefficient loading plots analyses of EFR3 and EFC metabolome data.

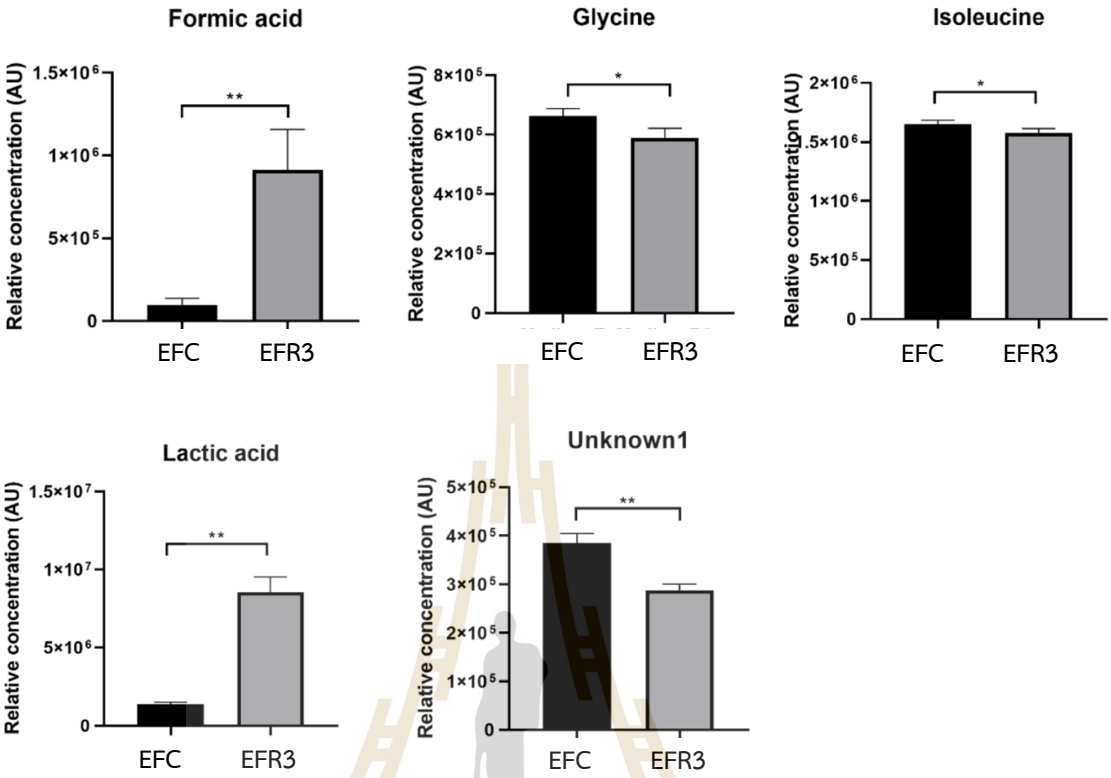

Figure 4.12 The significantly metabolites between group of EFC and EFR3.

Thus, the summary of OPLS-DA metabolic changes profiles between the EFR3 group and the direction of its correlation in the EFR3 group are consistent with additional metabolite data provided in Table 4.3. The OPLS-DA prediction model results revealed that lactic acid and formic acid were significantly increased in key metabolites of EFR3 in relation to both EFC and controls. The correlation of lactic acid in the EFR3 was 0.984 and 0.9854 relative to control and EFC, respectively. Additionally, the correlation of formic acid was 0.961 and 0.9606 relative to control and EFC, respectively. Both metabolites indicate a higher correlation in EFR3. The results from the student's t-test ($p < 0.01$) indicate significant differences between groups.

Table 4.3 Summary of OPLS-DA metabolic changes between EFR3 group ^{1}H NMR spectra.

NO.	Metabolite	O-PLS-DA model		
		control VS EFR3 (p-value = 0.01)	**EFC VS EFR3 (p-value = 0.01)**	**control VS EFR3 (p-value = 0.01)**
1	Acetate	0.9725**	-	-
2	Arginine	0.7845**	-	-
3	Tyrosine	0.7931**	-	-
4	Unknown1	-	-0.9592**	-0.9592**
5	Unknown4	-	-	-
6	Lactic acid	0.984**	0.9854**	0.9854**
7	Formic acid	0.961**	0.9606**	0.9606**
8	Isoleusine	-	0.9859*	0.9859*
9	Glycine	-	-0.8842*	-0.8842*
10	Leucine	-	-0.931	-0.931

"+" indicates higher correlation in medium EFR3, whereas "-" indicates higher correlation in medium EFR3. "*" indicates $p < 0.05$, "**" indicates $p < 0.01$. The p values were obtained from student's t-test.

Rautureau and co-workers, 2019 used metabolomic profiling to compare the family Enterobacteriaceae for discrimination between *Escherichia coli* (*E. coli*) and *Shigella* spp. in culture media. The bacterial culture media have demonstrated the identification of metabolites in NMR-based metabolomics, enabling the distinction of

even very closely related species Moreover, our results are consistent with previous studies. This model demonstrates remarkable segregation of bacterial culture supernatants, including the discrimination of EFR3 and EFC. The strain *Enterococcus* spp. exhibited metabolomic markers in the medium, enabling the differentiation of species belonging to the genus *Enterococcus* and most recently, by Psotta and co-workers in 2023 found that ^{1}H-NMR results suggested *E. faecalis* can be differentiated from *E. coli* and *Klebsiella pneumoniae*. *E. faecalis* utilizes citric acid as a substrate, releasing metabolites such as acetic acid and ethanol, whereas the two Gram-negative (*E. coli* and *K. pneumoniae*) utilize lactic acid as a primary substrate for growth. These differences are due to changes in the artificial urine medium's (AUM) composition caused by bacterial metabolic activity (Psotta et al., 2023). Our results are correspondent with previous studies that lactic acid and formic acid are found at higher levels after the growth of *E. faecalis* and *E. faecium*. This finding potentially paving the way to identify differences between species for more precise diagnostics.

In our study, out of the 27 quantified metabolites, lactic acid and formic acid appeared to contribute to this separation. This indicates that they are present at higher levels after the growth of microbes such as *E. faecalis* and *E. faecium*, as shown by their significance in the OPLS-DA model. However, the *E. faecalis* culture media exhibited the highest levels of lactic acid and formic acid. It has been previously reported that pyruvate plays a pivotal role as a catabolic compound in *E. faecalis*, serving as the end product of glycolysis and other catabolic pathways. Pyruvate can undergo metabolism through various enzymes or pathways, contingent on the specific enzymes and environmental conditions present (Leblanc, 2006). The production of lactate and formate results from the activity of the lactic dehydrogenase (LDH) or pyruvate-formate lyase (PFL), respectively (Figure 4.13).

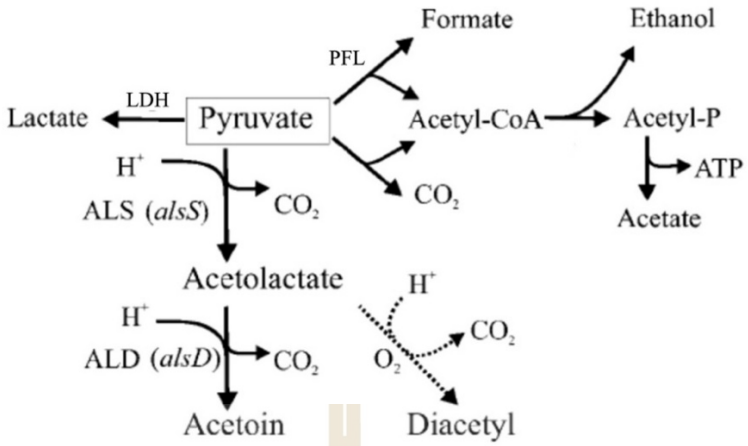

Figure 4.13 Pyruvate metabolism in *E. faecalis.*

The lactic acid is a major metabolic by product of lactic acid bacteria (LAB), including *Lactobacillus plantarum*, *Lactococcus lactis*, and *E. faecalis*. LAB produce various antibacterial substances, including organic acids such as lactic acid and acetic acid, hydrogen peroxide, and bacteriocins. Many lactic acid bacteria have been used in applications in fermentation processes, but also used for potential health benefits. Lactic acid was found to be elevated, consistent with previous studies on the fermentation of carbohydrates, such as glucose, in higher levels of anaerobic conditions of *E. faecalis* (Portela et al., 2014) and were discovered recently from Díaz and collaborators in 2024 was shown 2,5-diketopiperazines (2,5-DKPs), which are cyclic forms of peptides. Present at high concentrations in the cell-free extracts of lactic acid bacterial supernatants, have been found inhibit the activation of regulatory proteins involved in the expression of the *hla* gene (Díaz et al., 2024).

In addition, it was revealed that the increased lactic acid production by *E. faecalis* antagonizes the growth of *P. aeruginosa*. Growth inhibition arises as a result of elevated l-lactate production by *E. faecalis*, facilitated by LDH converting pyruvate. *E. faecalis* modulates the microenvironment over time, the acidity of the environment subsequently exceeds a pH threshold at which *P. aeruginosa* can no longer grow (Tan et al., 2022). Similar to the findings in formic acid, *E. faecalis* R3 carries out mixed-acid fermentation. The formation of formic acid was previously implicated in the

fermentation of *Lactobacillus* plantarum LAT3 and *E. faecalis* KE06, observed in single-strain cultures after 24 hours of fermentation (Chaves-López et al., 2017). The result indicates that *E. faecalis* R3, bacteria in general can produce a variety of organic acids through metabolic pathways. These acids can serve various purposes, including energy production, pH regulation, and interactions with their environment.

Furthermore, investigating lactic acid or formic acid as silage additives is a proven strategy to improve fermentation quality and extend storage time. A previous study by Lei and collaborators in 2024 shows that lactic acid treatment appears to enhance silage fermentation quality by increasing the population of beneficial bacteria like lactic acid bacteria, while formic acid treatment improves fermentation quality by reducing the activities related to "membrane transport" and "signal transduction" of harmful bacteria. Therefore, further studies hypothesize that pure lactic acid or formic acid may have anti-hemolytic activity against *S. aureus* supernatants (Lei et al., 2024).

CHAPTER V

CONCLUSION

Staphylococcus aureus (*S. aureus*), a commensal bacterium and human pathogen, is a leading cause of numerous diseases, ranging from minor skin infections to potentially fatal conditions. One of the characteristic features of *S. aureus* is the ability to lyse red blood cells (RBCs) causing hemolysis by producing α-hemolysin that directly damages the host cell plasma membrane. Our previous studies have revealed that *Enterococcus faecalis* can interfere a hemolytic activity of hemolysins secreted from *S. aureus*. Antivirulence strategies that target alpha-hemolysin (Hla) represent a promising approach to combatting *S. aureus* infections.

In this study, the cell-free supernatant produced by *Enterococcus faecalis* strains R3 (EFR3) demonstrated capacity to inhibit *S. aureus* infection. The results suggest that EFR3 concentration at 1.56% is associated with minimal toxicity to differentiated THP-1 cells. However, when assessing the cytotoxic activity of *S. aureus* supernatant in OUMS-36 cells after treatment with EFR3, there was no significant difference in cell damage observed at a concentration of 50% EFR3 concentration. The inhibitory effect of EFR3 on *S. aureus* adhesion to Caco-2 cells could be attributed to the bioactive compounds produced by *E. faecalis* during its growth. Specifically, the presence of EFR3 at a concentration of 50% has been shown to effectively inhibit *S. aureus* adhesion. Furthermore, the acidification caused by the production of organic acids, particularly lactic acid and formic acid, by *E. faecalis* could also contribute to this inhibitory effect. Further investigation showed the the presence of the virulence genes, *hla* which encodes for alpha hemolysin. In this study *S. aureus* and *S. aureus* treated with EFR3 were positive for the *hla* gene.

In conclusion, EFR3 shows promise as a natural anti-adhesive agent against *S. aureus* infections, as evidenced by its inhibitory effect on *S. aureus* adhesion to Caco-2 cells. However, our investigation did not reveal a significant association between EFR3 and the expression of the *hla* gene in *S. aureus*. Therefore, in future studies, exploring gene expression patterns using quantitative real-time PCR (qPCR) could provide further insights into the effects of EFR3 on *S. aureus* infections.

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APPENDICES

APPENDIX A

EQUIPMENTS AND INSTRUMEN

Name	Source
Autoclave	Hirayama, Japa
Autopipettes (10, 100, 1000 ul.)	Satorius, Finland
Balance	Denver instrument, Germany
Bruker AVANCE III 400 MHz spectrometer	Bruker Corporation, USA.
Cell culture flask (25 cm^2)	Thermo scientific Nunc, China
Cell culture flask (75 cm^2)	Thermo scientific Nunc, China
Centrifuge himac CF12RX	Hitachi, Japan
Class II Biohazard Safety Cabinet	Esco, Singapore
Conical tube (15, 50 ml)	Thermo scientific, USA
Cryovial	Thermo scientific, USA
Cuvettes	Hellma, Germany
Pipet filter	Thermo scientific, USA
Forma Series II Water Jacked CO_2	Thermo scientific, USA
Haemocytometer	Hausser scientific, USA
Geldoc go gel imaging system	Bio-Rad, USA
Hot Air Oven	Binder, Germany
Hotplate stirrer C-MAG HS 7	IKA, USA
Incubator shaker	New Brunswick Innova, USA
Light microscope Olympus DP20	Olympus, Japan
pH meter	Mettler toledo, China
24-well cell culture plate	Thermo scientific Nunc, Denmark
96-well cell culture plate	Thermo scientific Nunc, Denmark
Serological pipette (5ml,10ml)	Thermo scientific Nunc, Korea

EQUIPMENTS AND INSTRUMENTS (Continued)

Name	Source
Serological pipette (5ml,10ml)	Thermo scientific Nunc, Korea
Sterile syringe filter (0.2 μm)	MilliporeSigma, Germany
Sterile syringe filter (0.2 μm)	Corning, USA
Syringe without needle (10, 50 ml)	Nipro, Thailand
Tips (10, 100, 1000ul)	Thermo scientific, Mexico
T80+ UV/VIS spectrophotometer	PG Instruments, UK

APPENDIX B

PMA CONCENTRATION

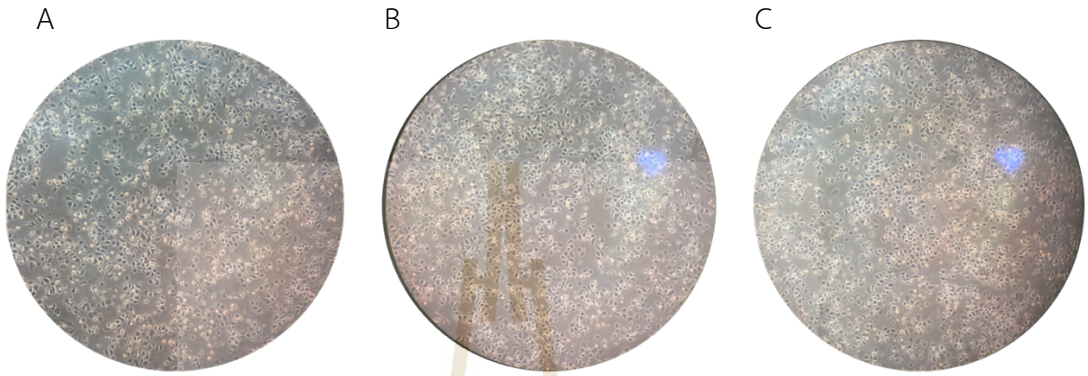

Figure B1 The conditions used to differentiate THP-1 cells are variable, with PMA concentrations ranging from 100 nM (61.65 ng/mL) (A), 200 nM (123.3 ng/mL) (B), to 300 nM (184.95 ng/mL) (C), respectively and treatment for 48 h.

APPENDIX C

THE METABOLITE ASSIGNMENT FROM ^{1}H NMR ANALYSIS

The production of bacterial culture supernatants was analyzed using nuclear magnetic resonance (NMR) spectroscopy. In this study, culture medium (control) is red, *Enterococcus feacium* supernatants (EFC) is green, and *Enterococcus faecalis* strains R3 supernatants (EFR3) is blue.

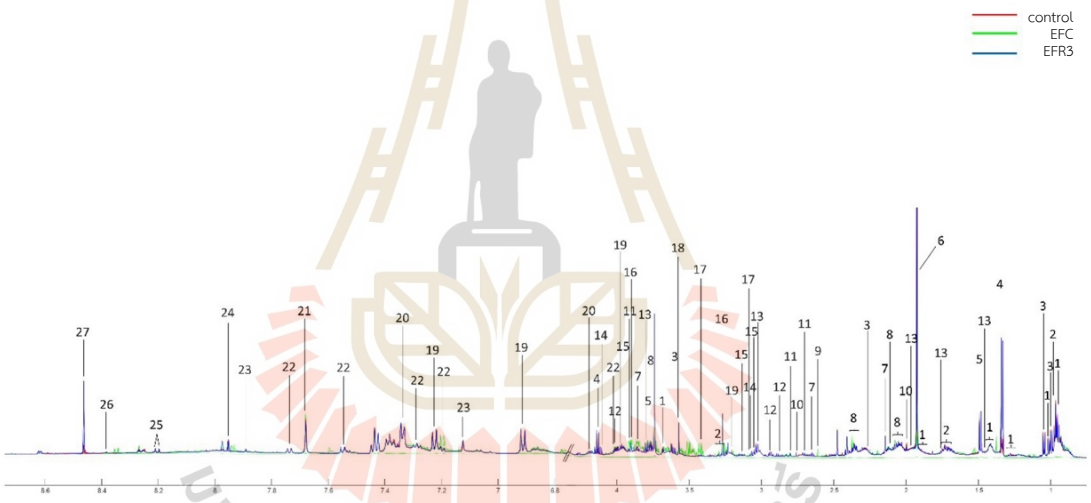

Figure C1 ^{1}H NMR spectrum of all samples. 1= Isoleucine, 2=Leucine, 3=Valine, 4=Lactic acid, 5=Alanine, 6=Acetate, 7=Methionine, 8=Glutamate, 9=Methylamine, 10=Unknown1, 11=Aspartic acid, 12=Asparagine, 13=Lysine, 14=Creatinine, 15=Cysteine, 16=Betaine, 17=UnKnown2, 18=Glycine, 19=Tyrosine, 20=Phenylalanine, 21=Unknown3, 22=Tryptophan, 23=Histidine, 24=Unknown4, 25=Adenine, 26=Unknown5, 27=Formic acid.

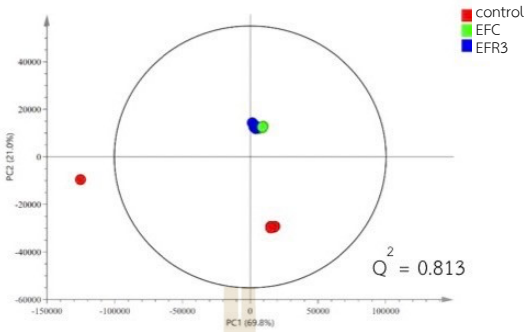

Figure C2 PCA score plot of all groups.

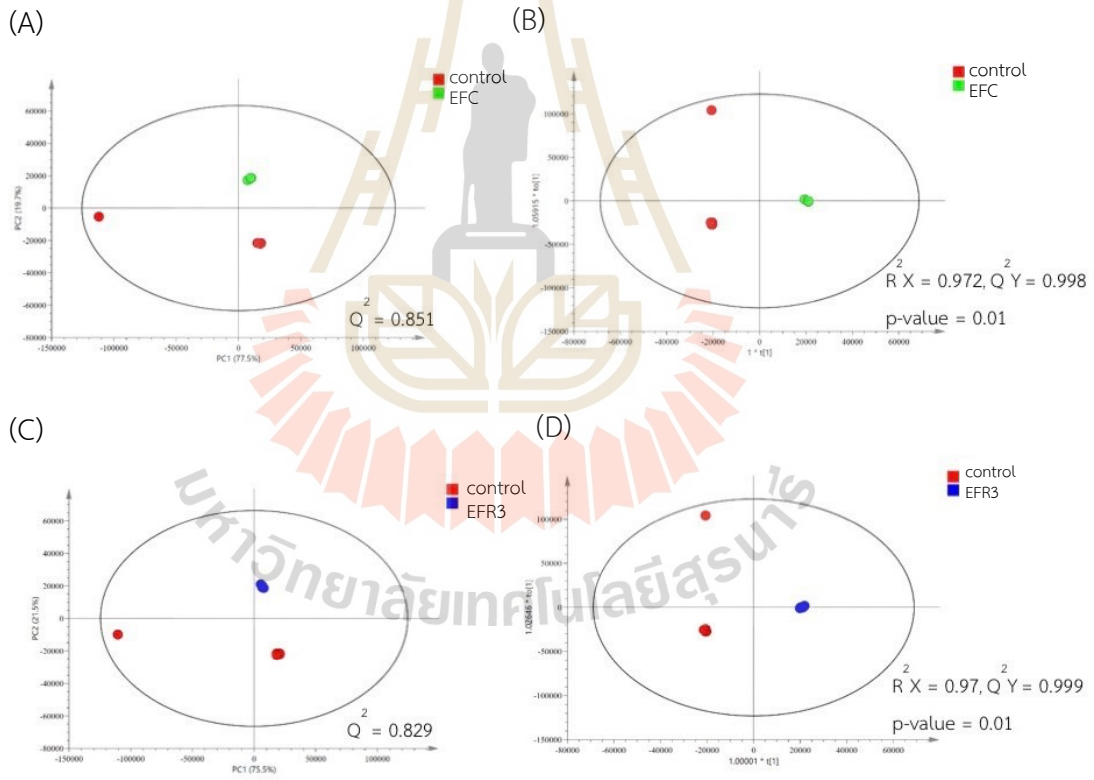

Figure C3 (A) PCA score plot between control and EFC. (B) The OPLS-DA score plot between control and EFC. (C) PCA score plot between control and EFR3. (D) OPLS-DA score plot between control and R3.

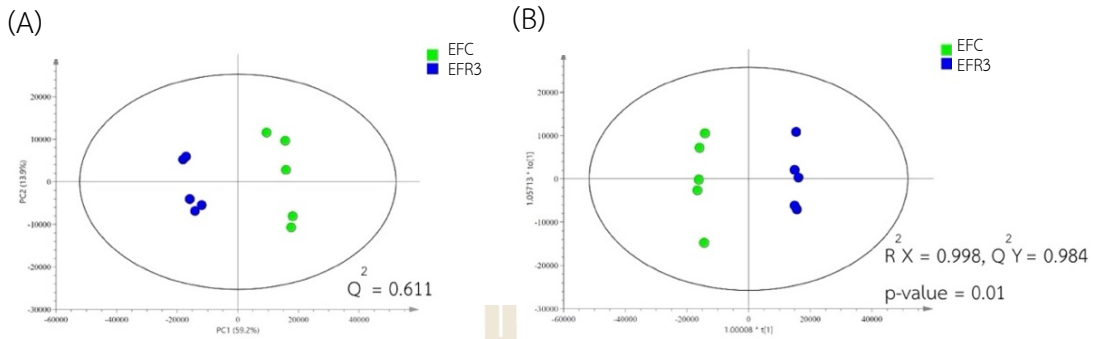

Figure C4 (A) PCA score plot between EFC and EFR3. (B) The OPLS-DA score plot between EFC and EFR3. The data were Par scaling.

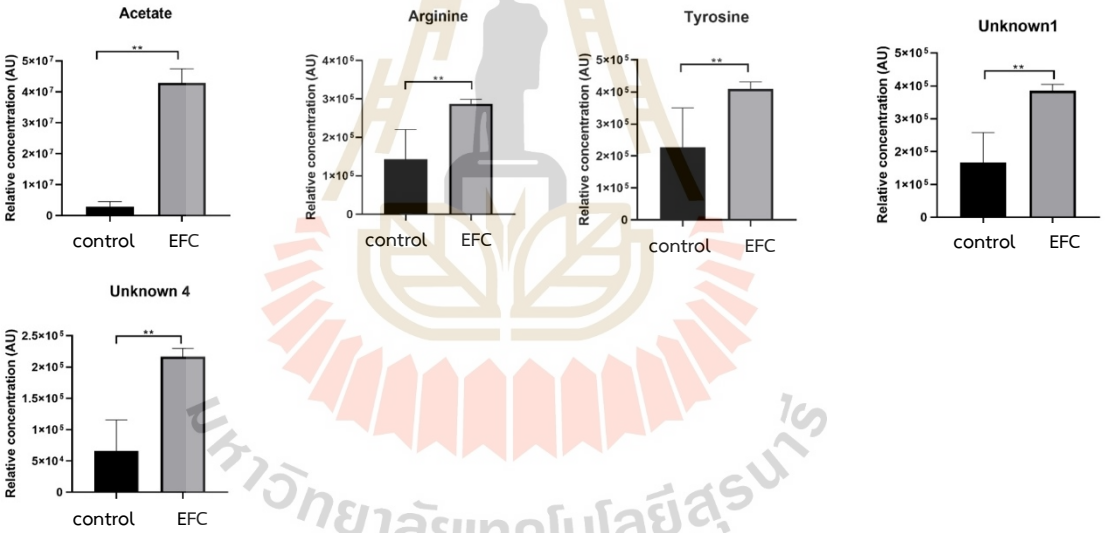

Figure C5 The significantly metabolites between group of control and EFC.

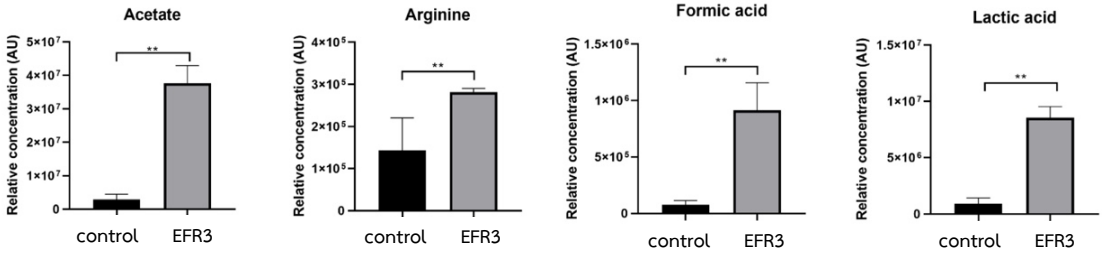

Figure C6 The significantly metabolites between group of control and EFR3.

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