IN VITRO INHIBITORY EFFECT OF LACTIC ACID BACTERIA AND THEIR SUPERNATANTS ON *FLAVOBACTERIUM COLUMNARE* BIOFILM

Mr. Farras Daffa Imtiyaz

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021

ผลทางห้องปฏิบัติการของแบคทีเรียและน้ำเลี้ยงแบคทีเรียที่สร้างกรดแลคติกในการยับยั้งไบโอฟิล์มของเชื้อฟลาโวแบคทีเรียม คอลัมแนร์

นายฟาร์รัส ดัฟฟา อิมทิยัส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	IN VITRO INHIBITORY EFFECT OF LACTIC ACID BACTERIA AND THEIR SUPERNATANTS ON *FLAVOBACTERIUM COLUMNARE* BIOFILM
By	Mr. Farras Daffa Imtiyaz
Field of Study	Veterinary Science and technology
Thesis Advisor	Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P
Thesis Co Advisor	Chumporn Soowannayan, Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor SANIPA SURADHAT, D.V.M., Ph.D., D.T.B.V.P)

THESIS COMMITTEE

..... Chairman

(Assistant Professor Ha Thanh Dong, Ph.D.)

..... Thesis Advisor

(Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P)

..... Thesis Co-Advisor

(Chumporn Soowannayan, Ph.D.)

..... Examiner

(Associate Professor NOPADON PIRARAT, D.V.M., Ph.D., D.T.B.V.P)

..... Examiner

(Associate Professor PATTRARAT CHANCHAITHONG, D.V.M., Ph.D.)

ฟาร์รัส ดัฟฟา อิมทิยัส :

ผลทางห้องปฏิบัติการของแบคทีเรียและน้ำเลี้ยงแบคทีเรียที่สร้างกรดแลคติกในการยับยั้งไบโอฟิล์ม ของเชื้อฟลาโวแบคทีเรียม คอลัมแนร์. (IN VITRO INHIBITORY EFFECT OF LACTIC ACID BACTERIA AND THEIR SUPERNATANTS ON *FLAVOBACTERIUM COLUMNARE* BIOFILM) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : ชุมพร สุวรรณยาน

Flavobacterium columnare เป็นแบคทีเรียฉวยโอกาสที่ทำให้เกิดโรคคอลัมนาริสในปลามากกว่า 37 สายพันธุ์ โดยที่แบคทีเรียชนิดนี้จะสร้างไบโอฟิล์มบนผิวหนังหรือบนพื้นผิวของปลาก่อนที่แบคทีเรียในไบโอฟิล์มจะผลิตและปล่อยเอนไซม์ที่ทำลายเนื้อเยื่อเกี่ยวพันและเนื้อเยื่ออื่นๆ ส่งผลให้เกิดแผลบนผิวปลาที่ติดเชื้อ การยับยั้งการสร้างไบโอฟิล์มของแบคทีเรีย โดยไม่รบกวนการเจริญเติบโตของเซลล์อาจเป็นวิธีการหนึ่งในการควบคุมโรคคอลัมนาริส การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของแบคทีเรียที่ผลิตกรดแลคติก ที่แยกได้จากลำไส้ของกุ้งขาว (*Penaeus vannamei*) และรากของแก่นตะวัน (*Helianthus tuberosus*) ในการยับยั้งการสร้างไบโอฟิล์มและศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างไบโอฟิล์มของเชื้อ *F. columnare* โดยการนำน้ำเลี้ยงเซลล์ของแบคทีเรียผลิตกรดแลคติกจำนวน 5 ชนิด มาทดสอบประสิทธิภาพในการยับยั้ง การสร้างไบโอฟิล์มและการเจริญเติบโตของเชื้อ *F. columnare* 3 สายพันธุ์ ได้แก่ 15, CUVET1359 และ CUVET1365 พบว่าแบคทีเรียที่ผลิตกรดแลคติก 2 สายพันธุ์ ได้แก่ WS2021 และ SC1 มีความสามารถในการยับยั้งไบโอฟิล์มของเชื้อ *F. columnare* ได้มากที่สุด จากนั้นได้ทำการศึกษาสภาวะการเลี้ยงที่เหมาะสมที่สุดของเซลล์ และน้ำเลี้ยงเซลล์ของ WS2021 และ SC1 โดยทำการเก็บเซลล์และน้ำเลี้ยงเซลล์หลังจากเลี้ยงเชื้อเป็นเวลา 0, 3, 6, 9, 12, 24, 36 และ 48 ชั่วโมง และนำไปทดสอบประสิทธิภาพในการยับยั้งไบโอฟิล์มและการเจริญเติบโตของ *F. columnare* สายพันธุ์ 15 พบว่าเซลล์และน้ำเลี้ยงเซลล์ที่เก็บที่เวลา 48 ชั่วโมง มีความสามารถในการยับยั้งไบโอฟิล์มได้ดีที่สุด จากนั้นศึกษาผลของเซลล์และน้ำเลี้ยงเซลล์ดังกล่าวต่อการแสดงออกของยีนที่เกี่ยวข้องกับสร้างไบโอฟิล์ม โดยการเลี้ยงไบโอฟิล์มของเชื้อ *F. columnare* สายพันธุ์ 15 เป็นเวลา 24, 48 และ 72 ชั่วโมง ร่วมกับเซลล์และน้ำเลี้ยงเซลล์ WS2021 และ SC1 ที่เลี้ยงเป็นเวลา 48 ชั่วโมง จากนั้นดูการแสดงออกของยีนด้วยวิธี PCR แบบเรียลไทม์เชิงปริมาณ โดยพบว่ายีนที่เกี่ยวข้องกับ iron acquisition, type IX secretion system (T9SS) และ quorum sensing (*luxR*) ของเชื้อ *F. columnare* มีการแสดงออกน้อยกว่าเมื่อมีเซลล์และน้ำเลี้ยงเซลล์ของ WS2021 และ SC1 โดยเฉพาะ *F. columnare* ไบโอฟิล์มที่เลี้ยงเป็นเวลา 48 ชั่วโมงและ 72 ชั่วโมง จากการทำ PCR โดยใช้ไพร์มเมอร์ universal bacterial primers (16S rRNA) และส่งไปหาลำดับเบสพบว่า WS2021 และ SC1 คือ *Enterococcus gallinarum* และ *Leuconostoc pseudomesenteroides* ตามลำดับ โดยสรุปทั้งน้ำเลี้ยงเซลล์และเซลล์ของแบคทีเรียที่ผลิตกรดแลคติคทั้งสองชนิด มีความสามารถในการยับยั้งไบโอฟิล์มของ *F. columnare* ซึ่งอาจเกิดจากลดการแสดงออกของการรับธาตุเหล็ก (*alcB*, *rhbC* และ *tonB*), T9SS (*porV*, *gldL* และ *sprA*) และ *luxR* ยีน

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6278502731 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY

KEYWORD: Flavobacterium columnare, biofilm, biofilm-associated genes, lactic acid bacteria, Enterococcus gallinarum, Leuconostoc pseudomesenteroides, cell-free supernatant, biofilm inhibitors, quorum sensing, Tilapia

Farras Daffa Imtiyaz : IN VITRO INHIBITORY EFFECT OF LACTIC ACID BACTERIA AND THEIR SUPERNATANTS ON *FLAVOBACTERIUM COLUMNARE* BIOFILM. Advisor: Assoc. Prof. CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P Co-advisor: Chumporn Soowannayan, Ph.D.

Flavobacterium columnare is an opportunistic bacterium causing columnaris disease to more than 37 fish species. It is believed that the bacteria form biofilm on the fish skin/surfaces before the bacteria in the biofilm produce and release enzymes that degrade connective and other tissues resulting in lesions on the infected fish. Treatments to inhibit the bacterial biofilm without interfering with its planktonic cell growth can be an alternative method to control columnaris disease. This study aimed to evaluate the efficacy of lactic acid bacteria (LAB) isolated from white leg shrimp gut (*Penaeus vannamei*) and the root of Jerusalem artichoke (*Helianthus tuberosus*) in biofilm inhibition and modulation of biofilm-associated genes of *F. columnare*. Cell-free supernatants (CFSs) of five different LAB isolates were first screened for their abilities to inhibit biofilm formation and growth in broth cultures of three highly pathogenic *F. columnare* isolates; 15, CUVET1359, and CUVET1365. Then, optimal culture conditions that resulted in the LAB cells and CFSs with the most potent *F. columnare* biofilm inhibiting activities were determined for two LAB isolates (WS2021 and SC1) with the most potent *F. columnare* biofilm inhibiting activities. The two LAB isolates were cultured for 48 h, at 0, 3, 6, 9, 12, 24, 36, and 48 h timepoints, aliquots of each culture were collected, and their cells and CFSs were separated and tested for their abilities to inhibit the biofilm and growth of *F. columnare* isolate 15. The results showed that cells and CFSs from 48 h incubating timepoints inhibited *F. columnare* biofilm strongly. To determine if LAB cells and CFSs treatment affect or modulate the expression of seven biofilm-associated genes in *F. columnare*, a quantitative real-time PCR method was used. After co-incubation at three different time points (24, 48, and 72 h) with either the CFSs or cells of WS2021 and SC1 obtained from 48 h, iron acquisition, type IX secretion system (T9SS), and quorum sensing (*luxR*) genes in *F. columnare* biofilm was predominantly downregulated, especially at 48 h and 72 h co-incubation. Based on 16S rDNA sequence, WS2021 and SC1 isolates were identified as *Enterococcus gallinarum* and *Leuconostoc pseudomesenteroides*, respectively. In summary, both CFSs or cells of these two bacteria isolates possess *F. columnare* biofilm inhibiting activity which could be due to the downregulation of iron acquisition (*alcB*, *rhbC*, and *tonB*), T9SS (*porV*, *gldL*, and *sprA*), and *luxR* genes.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

This research work was financially supported by The Agricultural Research Development Agency (ARDA, P2150487) and The 90th Anniversary of Chulalongkorn University Scholarship. The graduate scholarship program for ASEAN and NON–ASEAN countries of Chulalongkorn University is also acknowledged for master scholarship support.

In this opportunity, I would like to thank Associate Professor Dr Channarong Rodkhum and Dr Chumporn Soowannayan as my main- and co-supervisors, respectively, that have been very supportive in this journey.

Also, I would like to thank all of my thesis committee members Assistant Professor Dr Ha Thanh Dong, Associate Professor Dr Nopadon Pirarat, and Associate Professor Dr Pattrarat Chanchaithong. Their valuable advice and suggestions have formed my view on some critical points that need to be discussed further.

I would also like to thank my colleagues in CENTEX shrimp-Faculty of Science of Mahidol University; the Department of Veterinary Microbiology, Center of Excellent in Fish Infectious Disease (CEFID), Veterinary Science and Technology (VST) Program of Chulalongkorn University and all staff whose kindly helpful.

Last but not the least, I would like to thank my beloved family as my biggest motivation during this Master's degree journey.

Farras Daffa Imtiyaz

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I
INTRODUCTION

1. Importance and Rationale

Flavobacterium columnare is an important opportunistic fish pathogen causing columnaris disease in more than 37 fish species including channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), striped catfish (*Pangasianodon hypophthalmus*), and Tilapia (*Oreochromis* sp.) (Anderson and Conroy, 1969; Olivares-Fuster et al., 2011; Dong et al., 2015b; Dong et al., 2016; Evenhuis and LaFrentz, 2016; Kerddee et al., 2020). Similar to many pathogenic bacteria, *F. columnare* can produce a biofilm that is believed to be a crucial step in columnaris pathogenesis. The bacteria exploit and develop biofilm on mucus-covered fish surfaces (Olivares-Fuster et al., 2011; Declercq et al., 2015; Lange et al., 2020) before releasing chondroitin AC lyase and many proteases (Bernardet and Bowman, 2006; Suomalainen et al., 2006) that cause lesions on external surfaces of the fish. In these circumstances, fish mucus can act as a chemoattractant and stimulant for the bacteria colonization by modulating the expression of related genes such as the gliding gene (Klesius et al., 2010).

To control and treat columnaris disease, antimicrobial regimens (as metaphylaxis agents) have been commonly used (Gaunt et al., 2010; Darwish et al., 2012). However, such practice is no longer recommended partly because of the effects that they might cause to human health, such as allergic effects, adverse drug reactions (ADR) and most importantly, they might give selective advantage for antibiotic-resistant bacteria to become dominant bacteria in the human intestinal tract as well as in the environment (Buschmann et al., 2012; Tomova et al., 2015).

Since the major problem with antibiotic use is the development of antibiotic-resistant bacteria, control measures focusing on impairing the virulence properties without inhibiting the growth pattern of pathogenic bacteria would be desirable. For many pathogenic bacteria, their ability to cause diseases in animals and humans lies in/are related to their ability to form a biofilm which is the preferred mode of living for most bacteria in or on their host (Costerton et al., 1999). Thus, inhibiting the pathogenic bacterial biofilm could be a good alternative and sustainable approach to controlling the virulence of *F. columnare*. These inhibitors could come from both natural and synthetic sources such as peptides from rainbow trout and bass which have been shown to inhibit *F. columnare* biofilm without affecting its growth (Prior, 2020), lactic acid bacteria (LAB) which have been found to inhibit biofilms of many pathogenic bacteria such as oral cavity bacteria, *Porphyromonas gingivalis* (Yang et al., 2021) and *Streptococcus mutans* (Rossoni et al., 2018). In this study, the *F. columnare* biofilm inhibitors will be screened from the LAB originating from different sources and the LAB with potent biofilm inhibitors will be analysed for their abilities to modulate the expression of biofilm-related genes of the bacteria.

2. Objectives of the Study

1. To evaluate the efficacy of lactic acid bacterial cells/or their CFSs in *F. columnare* biofilm inhibition
2. To evaluate the efficacy of lactic acid bacterial cells/or their CFSs in *F. columnare* biofilm-associated genes regulation

3. Hypothesis

Lactic acid bacterial cells/or their CFSs can inhibit *F. columnare* biofilm and down-regulate the expression of some biofilm-associated genes.

CHAPTER II
LITERATURE REVIEW

1. *Flavobacterium columnare*

Flavobacterium columnare was first isolated in 1922 by Davis (1922) from diseased fish and called *Bacillus columnaris* due to its column-like form of the cells. The bacterium was later classified as *Chondrococcus columnaris* because of the microcyst (spheroplast) and fruiting body that the bacteria produced (column-like cells on the tissue-infected wet mount) (Ordal and Rucker, 1944). However, in 1945, Garnjobst (1945) observed that microcyst is absent from this bacterium and reclassified it into *Cytophaga columnaris*. The name of bacteria changed again for the second time to *Flexibacter columnaris* (Bernardet and Grimont, 1989) based on DNA relatedness analysis before the same group of authors Bernardet et al. (1996) reclassified it to *Flavobacterium columnare* after studying its DNA-rRNA hybridization and protein and fatty acid profiles. Through restriction fragment length polymorphism (RFLP) assay based on 16S rRNA gene, Triyanto and Wakabayashi (1999), Olivares-Fuster et al. (2007) and LaFrentz et al. (2014) subclustered *F. columnare* into five distinct genetic groups described as genomovar I, I/II, II, II-B and III. In 2018, through multi-locus phylogenetic analysis (MLPA) of 50 isolates, LaFrentz et al. (2018) demonstrated that 16S-RFLP does not accurately reflect this genetic diversity and proposed that isolates be assigned to four genetic groups rather than genomovars, with group 4 is assigned for isolates recovered from tilapia in South America, Central America, and Asia. In 2021, LaFrentz and colleagues (2022) re-characterized all four genetic groups (five isolates each) and found out that biochemical and physiological characteristics were similar between the groups. However, fatty acid profile MALDI-TOF characterization, and the average nucleotide identity (ANI)/ digital DNA-DNA

hybridization (GGDC) calculations were found to be able to distinguish between different genetic groups, so they suggested the placement of each group into distinct species namely, *F. columnare*, *F. covae* sp. nov., *F. davisii* sp. nov., and *F. oreochromis* sp. nov. for genetic groups 1, 2, 3, and 4, respectively.

F. columnare is a Gram-negative, column-like and gliding bacterium commonly isolated from the external surface of columnaris disease-affected fish inhabiting the freshwater environment. This bacterium presents identical phenotypic features for gross recognition. The bacteria exhibit flat, dry, rhizoid or non-rhizoid, shiny, mucoid colonies on Anacker and Ordal's (AO) and tryptone yeast extract salts (TYES) agar with tight or non-tight adherence. The colony absorbs Congo red, produces flexirubin pigment which gives a yellow appearance and produces cytochrome oxidase (Bernardet and Bowman, 2006). *F. columnare* grows well on the temperature ranging from 28°C to 30°C correlating with the incidence of columnaris disease in warm periods of the year (Triyanto and Wakabayashi, 1999). The disease affects a wide range of economically important cultured fish species such as channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), striped catfish (*Pangasianodon hypophthalmus*), and other fishes (Olivares-Fuster et al., 2011; Dong et al., 2015b; Dong et al., 2016; Evenhuis and LaFrentz, 2016; Kerddee et al., 2020).

2. Biofilm and columnaris disease

Naturally, most microorganisms live in communities together with other microbes called biofilm. These biofilms generally attach to suitable surfaces and distribute in various areas such as in/on the soil, animal and human surfaces, plants, and aquatic environment. Bacterial cells in the biofilm are embedded into a matrix called extracellular polymeric substances (EPS) which help maintain the biofilm architecture and protect the bacteria living in it against harmful substances. In other words, biofilm provides the most favourable state of living for the bacteria. The EPS

is made of polysaccharides which could be homopolysaccharides (sucrose-derived glucans) or heteropolysaccharides (β-1, 6-linked N-acetylglucosamine) (Branda et al., 2005), extracellular proteins which can be both functional enzymes and structural proteins. Some of these enzymes help degrade the complex biopolymer needed by the bacterial cells inside the biofilm. Extracellular DNA is also a crucial component of EPS as has been shown in *Listeria monocytogenes,* that extracellular DNAs are required for initial attachment to the substrate (Harmsen et al., 2010). Lipid is also required for EPS to be hydrophobic on the area of cell-inert surface interaction whereas water which accounts for 98% of the biofilm is required as a carrier for nutrients as well as other components (Evans, 2000).

Generally, biofilm formation consists of five main steps: planktonic cell attachment, formation of microcolonies, biofilm maturation, and dispersion. Planktonic bacteria cells initiate attachment on suitable substrates by forming the first reversible bonds between cells and substrates. The temporarily attached cells generally claw toward one another to form micro-colonies and permanent/stronger bonding are formed. The cells within these microcolonies multiply and larger colonies are formed. After the bacterial cell concentrations in these colonies reach a certain threshold or quorum, the bacterial cells in the colonies will produce extracellular matrices (EPS) to cover the bacterial cells and the bacterial biofilms are formed. As the bacterial cells in the biofilm grow and biofilm will become more complex and mature under the three-dimensional structure provided by their EPS. This maturation process is mainly controlled by a quorum-sensing regulatory system carried out by chemical signals among bacterial cells to monitor and respond to the bacterial density on biofilm. When the cell density is high, the chemical signal produced is also high and this condition will regulate the expression of certain genes controlling virulence, competence, motility, biofilm formation, and other regulation

(Miller and Bassler, 2001). When the biofilm is fully matured or in the case when the conditions are no longer suitable for bacterial growth, cells from the biofilm will produce enzymes that degrade the EPS and bacterial embedded in the biofilms will be released and dispersed into the surrounding and free-living bacterial cells can now move to another suitable environment to colonize and form biofilm again (Costerton et al., 1999; An et al., 2000; Branda et al., 2005).

For *F. columnare,* biofilm formation is believed to be a crucial stage of columnaris disease pathogenesis. To form a biofilm, *F. columnare* planktonic cells use the fish mucus as a chemoattractant to adhere and glide toward other *F. columnare* cells to form microcolonies and biofilms in later stages (Olivares-Fuster et al., 2011; Declercq et al., 2015; Lange et al., 2020). In the biofilm, iron acquisition genes including *tonB*, *rhbC*, and *alcB* were found to be upregulated when compared to the same in planktonic cultures. These genes may be responsible for iron acquisition for the bacterial cells in both the biofilm and in the surrounding environment (Lange et al., 2018; Lange et al., 2020). Quorum sensing gene in *F. columnare* has not been well-characterized yet. So far, only the LuxR family transcriptional regulator is upregulated in planktonic culture in a calcium-enriched medium (Cai et al., 2019). Several genes that are important for bacterial survival such as the type IX secretion system (T9SS) which includes components such as *sprA* and *porV* are upregulated in biofilm culture (Declercq et al., 2019). These components of T9SS are responsible for secreting mobile surface adhesins (*sprB* and *remA*) that are involved in gliding motility and adhesion to the surface of the bacterial cells (McBride and Zhu, 2013; Shrivastava et al., 2013). After adhesion and colonization on the fish surface, *F. columnare* cells in the biofilm release chondroitin AC lyase (ChonAC) which degrades acidic polysaccharide such as chondroitin sulphate and hyaluronic acid of the connective tissue. At the same time, cells in the biofilms also release

proteases that degrade gelatine, elastin, collagen, fibrinogen, laminin, myosin and actin that resulted in wounding on the fish skins or surface (Bernardet and Bowman, 2006; Suomalainen et al., 2006). These two types of enzymes result in gross lesions affecting external surfaces such as dorsal fin, tail, gill and mouth but these manifestations after infection are vary depending on the virulence of the strain, age of the fish, and the infection model. In fish infected with less virulent *F. columnare* strains, the induction of ChonAC production is slowed or much delayed, so the slowed progression of the disease (Suomalainen et al., 2006).

3. Current control and treatments of Columnaris disease

Management practices such as stocking density and water quality play a crucial role in controlling columnaris disease in several farmed fish species. Suomalainen et al. (2005) revealed that the reduction of fish density to a quarter capacity on high temperature can decrease the mortality of rainbow trout experimentally challenged with *F. columnare*. By lowering the density on the pond together with reduced feeding, the level of organic matter and nitrate would decrease which eventually will impair the adhesion capability of *F. columnare* (Decostere et al., 1999) and also will give unfavourable conditions to ectoparasite. Altinok and Grizzle (2001) reported that artificial salt at 1, 3, and 9‰ can decrease the growth and adhesion pattern of *F. columnare* which also prevented columnaris disease-associated mortality on channel catfish, goldfish and striped bass.

Vaccinations through immersion, oral administration and intraperitoneal injection have offered a successful rate in preventing fish from mortality caused by columnaris disease. Bath vaccination with recombinant *F. columnare* DnaK protein and mucoadhesive formalin-killed *F. columnare* has been shown to promote immune responses and protect against columnaris disease in channel catfish and red tilapia, respectively (Lange et al., 2019; Kitiyodom et al., 2021). Heat-killed *F.*

columnare administered orally for 2 months through feeding has been shown to protect coho salmon (*Oncorhynchus kisutch*) against columnaris disease throughout 17 weeks culture period (Fujihara and Nakatani, 1971). Modified live *F. columnare* (live attenuated vaccine) has also been shown to reduce mortality of largemouth bass (*Micropterus salmoides floridanus*) fry by 43% when apply orally Bebak et al. (2009). Additionally, intraperitoneal injection of formalin-killed vaccine (lyophilized *F. columnare* G4cpN22 ghost) has also been found to protect grass carp from columnaris infection with 70.9% relative percentage survival (RPS) when compared to the formalin-killed *F. columnare* injected group with 41.9% RPS (Zhu et al., 2012).

Probiotic bacteria are another commonly used products to prevent or to reduce the effects of many diseases. Many probiotic bacteria products administered through either oral supplementation or immersion are effective in preventing many bacterial diseases in aquaculture in Nile tilapia (Hai, 2015; Saputra et al., 2016; Tan et al., 2019). These microorganisms are regarded as safe replacements for antibiotics. Seven bacterial strains from the indigenous skin of brook charr (*Salvelinus fontinalis*) has been found to have an antagonistic effect against *F. columnare* and *F. psychrophilum* (Boutin et al., 2012). These antagonistic bacteria were found to be protective against *F. columnare* infection when the mixture of all strains was mixed all together and added to the pond water. *Pseudomonas fluorescens* from the healthy skin of Walleye (*Sander vitreus*) has been shown to inhibit *F. columnare* and protect experimentally infected Walleye from columnaris disease (Seghouani et al., 2017).

Antimicrobial compounds are among the most commonly used products to mitigate columnaris disease. Darwish et al. (2012) reported that oral administration of florfenicol can decrease the mortality of sunshine bass (*Morone chrysops*) from concurrent infections of *Aeromonas hydrophila* and *F. columnare*. Florfenicol in the

feed is effective in treating columnaris disease in channel catfish (8.7% mortality) when compared to fish fed with unmedicated feed (54.2% mortality) (Gaunt et al., 2010). However, antimicrobial regimens are no longer recommended partly because of their ineffectiveness and partly because of public and environmental health concerns. *F. columnare* originated from 17 fish species worldwide is resistant to enrofloxacin, flumequine and oxolinic acid oxytetracycline (Declercq et al., 2013). Moreover, pathogenic bacteria producing biofilm may be less susceptible to antimicrobial drugs than their planktonic cell counterpart, partly because the EPS that encase the biofilm are not easily penetrated by the antibiotics (Evans et al., 1991; Ishida et al., 1998). After prolonged and overuse medication, the tissues of aquaculture animals can contain antibiotic residue and the consumption of such products can harm human health by causing allergies, adverse drug reactions (ADR) and antibiotic resistance of human intestinal bacteria (Tomova et al., 2015). As a result, importing countries have rejected and restricted aquaculture products contaminated with a high residual level of antimicrobial compounds (Lulijwa et al., 2020). Unmetabolized antimicrobial compounds (75%) formulated to the feeds would be excreted in the surrounding environment as residues which can affect phytoplankton, zooplankton (Ferreira et al., 2007), and wild fish of non-target species (Björklund et al., 1990); and increasing the incidence of antimicrobial-resistant bacteria (Buschmann et al., 2012).

4. Bacterial biofilm inhibitors

Since bacterial biofilm are known to be associated with pathology and virulence of the infection, interference with pathogenic bacterial biofilm instead of using antimicrobial compounds is an interesting strategy. The biofilm inhibitors can control biofilm through displacement, exclusion, and/or competition which eventually block the bacterial invasion. The displacement strategy consists of the degradation of the

pre-formed biofilm after the addition of substances or cells; exclusion approach comprises of the pre-coated area with substances or cells to inhibit the adhesion of biofilm; and competition strategy consists of the co-culture of substances or cells and bacterial biofilm (Carvalho et al., 2020). These biofilm inhibitors could be originated from both natural and synthetic sources. So far, there is only one study conducted to search for *F. columnare* biofilm inhibitor (Prior, 2020) which, peptides from Rainbow trout and bass (concentrations ranging from 10 μM to 100 μM) have been found to have varying biofilm inhibiting activities against *F. columnare* biofilm while not showing any growth inhibition towards the *F. columnare* cells. Several lactic acid bacteria have also been found to have biofilm inhibitory activity against pathogenic bacteria. Cell-free supernatant of *Lactobacillus reuteri* has been found to inhibit biofilm formation and regulate biofilm-associated gene expressions of *Porphyromonas gingivalis* (Yang et al., 2021). Rossoni et al. (2018) and Wasfi et al. (2018) have also found that *Lactobacillus spp.* has a significant biofilm inhibitory effect against clinical isolates of *Streptococcus mutans*. Melo et al. (2016) have also found that *Lactobacillus* isolated from fine cocoa have inhibited biofilm formation and modulated the expression level of *ica* operon of *Staphylococcus aureus*.

CHAPTER III

MATERIAL AND METHODS

1. Conceptual Framework

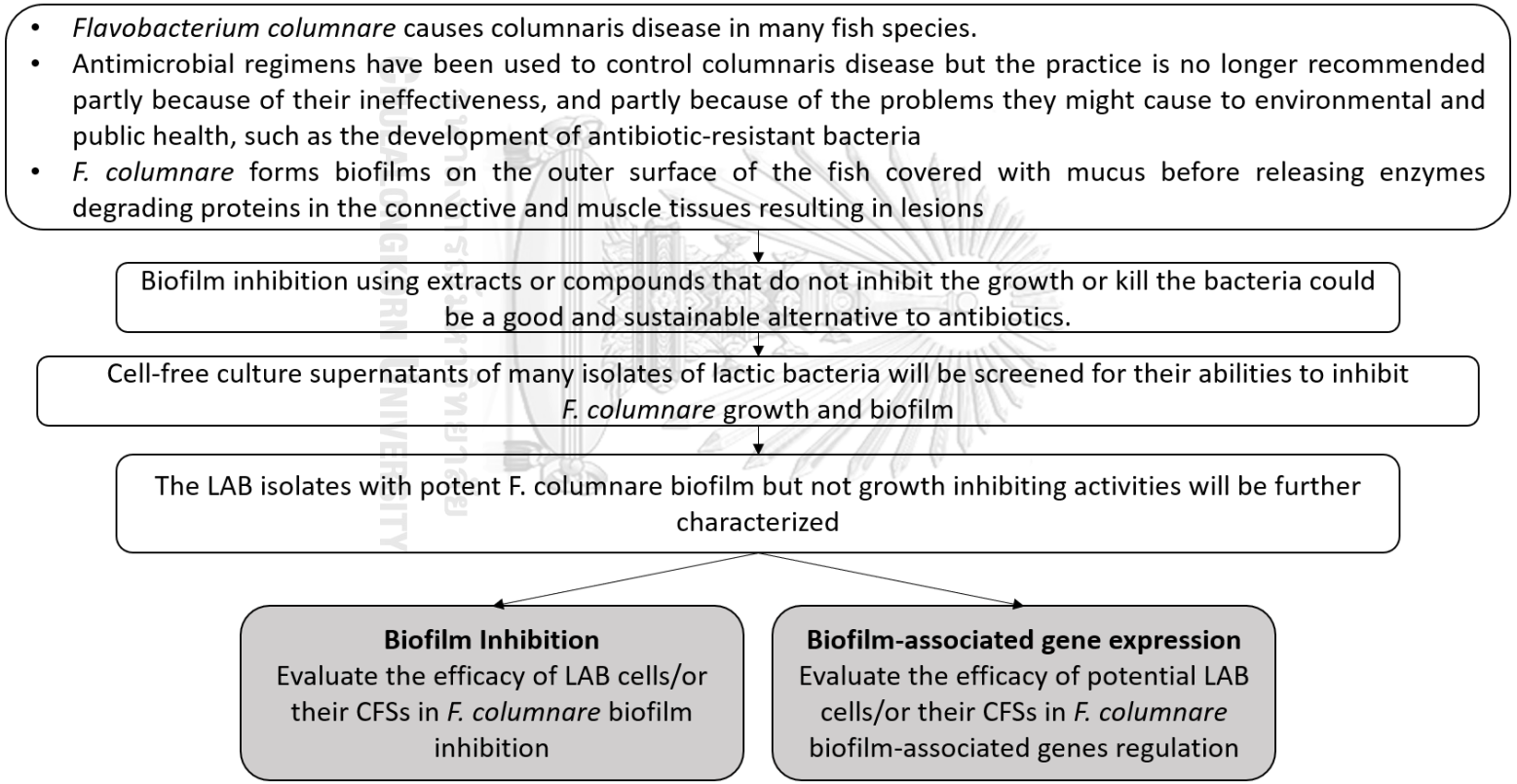

Figure 1. Conceptual framework of the study.

2. *Flavobacterium columnare* isolates and Lactic acid bacteria (LAB)

Three isolates of *Flavobacterium columnare* (*F. columnare* isolates; 15, CUVET1359, and CUVET1365) used for growth and biofilm inhibition assays were isolated from the gill of red tilapia (*Oreochromis sp.*) experiencing columnaris disease in Kanchanaburi province, Thailand (Dong et al., 2015a). To prepare the bacteria starter cultures, glycerol stock culture maintained in a refrigerator at -80°C was streaked onto Tryptone Yeast Extract Salts (TYES) agar and incubated at 28°C for 24 h. A loopful of the bacterial colony was inoculated into a 15 ml sterile centrifuge tube (Corning, USA) containing 5 ml of TYES broth and incubated for 18 h with 250 rpm agitation. The optical density (O.D.) at 600 nm of the cultures were determined and were diluted to get an OD of 0.5 and the bacterial culture were diluted 10 times further.

Six lactic acid bacteria (LAB) isolates originated from the gut of white leg shrimp (*Penaeus vannamei*) and the root of Jerusalem artichoke (*Helianthus tuberosus*) were used in this study. These LAB isolates were revived from a culture stock maintained at -80°C into De Man, Rogosa and Sharpe (MRS; Difco™, USA) agar and incubated at 30°C for 24 h.

Table 1. The code and sources of bacterial isolates used in this study for growth and biofilm inhibition screening against *Flavobacterium columnare*.

Bacterial Code	Sources
MRS-WS2020	The gut of white leg shrimp
MRS-WS2021	
MRS-WS2022	
MRS-SC1	The root of Jerusalem artichoke
MRS-SC2	

3. Preparation of cells and cell-free culture supernatants of LAB isolates for *Flavobacterium columnare* growth and biofilm inhibition assays

A single colony of each LAB isolate was inoculated into a 50 ml sterile centrifuge tube (Corning, USA) containing 25 ml of MRS broth and incubated at 30°C with 250 rpm agitation for 36 h. After incubation, each tube of the LAB culture was centrifuged using a refrigerated centrifuge machine (Kubota 6200, Japan) at 10,000 × ***g*** for 5 min at 4°C. The culture supernatants were collected into new tubes and their pH was adjusted to 7 using 2 M of NH_4HCO_3 (Sigma-Aldrich, USA) and filtered through a 0.2 µm filter to obtain the bacterial cell-free supernatant (CFS) before storing at -20°C until used in the biofilm and growth inhibition assay against *Flavobacterium columnare*. The bacterial cell pellets were resuspended in 2 ml of new MRS medium before storing at -20 °C.

4. Identification of LAB isolates

To identify the two LAB isolates with the most potent *F. columnare* biofilm inhibiting activities, 16S rRNA sequencing of the bacteria was used. The genomic DNA of both LAB isolates were extracted from overnight bacterial cultures (2 ml) of each

isolate using the phenol-chloroform extraction method (Sambrook et al., 1989). Briefly, the cells were spun down by centrifugation at 5000 × g for 5 min at 4°C, lysed by lysis buffer, and extracted using phenol-chloroform-isopropanol (25:24:1). The extracted DNA samples were used as templates for PCR amplification using primers targeting 16S rRNA region [primer 40F (5'-GCCTAACACATGCAAGTCGA-3') and 802R (5'-GACTACCAGGGTATCTAATCC-3')]. Each 25 µl of PCR reaction comprised of 100 ng genomic DNA, 0.5 µM each primer, 0.25 µM $MgCl_2$, 0.2 mM dNTPs, 1-unit Taq polymerase (Promega, USA), and 1 × reaction buffer. The PCR condition for amplification of primers used in this identification section was set as follows, 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were then ligated into pGEM-T Easy vector (Promega, USA) and transformed into competent cell *Escherichia coli* DH5**α** (Thermo Fisher, USA). White colonies grew at 37°C on Luria-Bertani (LB) medium supplemented with 100 mM X-gal + 100 mM IPTG + 100 mg/l ampicillin were then selected for colony PCR using M13R and T7 primers with the same PCR protocol as previous. Colony showing the expected size of base-pair was then grown overnight at 37°C with 250 rpm agitation on LB broth supplemented with ampicillin and subjected to plasmid extraction using Exprep™ Plasmid SV mini (Geneall, South Korea). The recombinant plasmids were amplified using the same pair of primers to check the expected product before the plasmids were sent for sequencing by Macrogen (Macrogen, Inc., South Korea) targeting M13R and T7 primers. Sequences were assembled and aligned from both forward and reverse direction using BioEdit 7.0.3 (Hall, 1999) and Clustal W (Thompson et al., 1994). The sequences were subjected to BLAST analysis on the NCBI website (http://www.ncbi.nlm.nih.gov), followed by a phylogenetic tree construction using MEGA X software with neighbour-joining method (Kumar et al., 2018).

5. *Flavobacterium columnare* growth inhibition assay

To determine the effect of CFS or cells of LAB isolates on the growth of *F. columnare* microtiter plate growth inhibition assay as described by Yatip et al. (2018) was used. Briefly, 180 µl *F. columnare* starter culture prepared as described in section 1. was seeded into each well of 96-well flat-bottomed microplate (Corning, USA) before 20 µl of CFS or cells from LAB isolates prepared as described in section 2 was added into each well of the seeded wells (done in eight replicates). For the negative control wells, 20 µl of MRS broth was added into each well instead of the CFS or cells of LAB isolates (also done in 8 replicates). All the plates were incubated for 24 h with 250 rpm agitation at 28°C and the OD_{600} was measured using an ELISA microplate reader (SpectraMax ID3, USA). Growth inhibition of CFS or cells of LAB isolates toward *F. columnare* was expressed as a mean percentage of relative inhibition of eight replicates from OD_{600} readings as the following formula,

$$\text{Mean relative inhibition (\%)} = \left[1 - \left(\frac{\text{Mean OD}_{600}\text{ of test}}{\text{Mean OD}_{600}\text{ of Negative Control}}\right)\right] \times 100\%$$

6. *Flavobacterium columnare* biofilm inhibition assay

A similar protocol as described in section *F. columnare* growth assay was applied to measure the effect of CFS or cells from LAB isolates to the biofilm of *F. columnare* as described by O'Toole (2011) with a slight modification (Yatip et al., 2018). Briefly, in the starter culture preparation step, TYES supplemented with 1% glycerol (Sigma-Aldrich, USA) was used for diluting the overnight bacterial cultures instead of TYES alone and the flat-bottomed 96-well microplate used were pre-coated with 1% gelatine (isolated from fish skin). The plates inoculated with the bacteria were incubated for 72 h at 28°C in a static condition.

To determine the bacterial biofilm inhibition, the bacterial culture plates were gently overturned to remove the culture medium and washed two times with distilled water. After letting the plate dry, 200 µl of 0.3% crystal violet (Sigma Aldrich,

USA) was added into each well for 15 minutes to stain the bacterial biofilms, excess stain was removed by overturning the plates and gently washing three times with tap water. After that, the stained biofilms were allowed to dry at room temperature (RT), before 200 µl of 33% acetic acid (Sigma-Aldrich, USA) was added to each well and plates were allowed to stand for 15 minutes to dissolve the crystal violet-stained biofilms. Optical densities at OD_{600} of dissolved stained biofilms which is an indicator of biofilm thickness was measured using an ELISA microtiter plate reader. Mean OD_{600} of biofilm thickness was transformed into the mean percentage of the relative inhibition value using the same formula as for the growth inhibition assay.

7. Determination of optimal conditions for growth and biofilm inhibitions by the LAB isolates

Optimal conditions for growth and biofilm inhibitions by the most potent LAB isolates from the screening process were further determined by testing both their cells and cell-free culture supernatants collecting at different growth/culture stages for their abilities to inhibit biofilm and/or growth of *F. columnare.* To do this, the LAB isolates were cultured (20 ml) for 48 h at 30 °C and at the following time points: 0, 3, 6, 9, 12, 24, 36 and 48 h, 2 ml of the culture broth was collected and their cells and cell-free culture supernatants were separated by centrifugation at 3,000 × ***g*** for 5 min at 4°C and stored at -20°C for use in *F. columnare* isolates 15 growth and biofilm inhibition assays. Before being used, cells were diluted with 2 ml of MRS broth.

8. Gene expression analysis by quantitative real-time PCR

F. columnare isolate 15 stater culture (900 µl) was seeded into a flat-bottomed 12-well microplate (Corning, USA). Subsequently, 100 µl of LAB cells and CFSs (cultured for 48 h) or MRS broth (control) was seeded into the well in three replicates. *F. columnare* cells within the biofilm were collected at 3 different time points (24, 48, and 72 h after co-incubation) by rinsing the well with sterile distilled

water and scrapping using pipette tips. The scrapped biofilms were suspended and their total RNA was extracted using TRIzol (Invitrogen, USA) reagent using the manufacturer's protocol. The total RNA samples obtained were quantified using a Nanodrop machine at 260 nm. The total RNA (100 ng) was reverse-transcribed into complementary DNA (cDNA) using random hexamer and ImProm-II™ Reverse Transcriptase (Promega, USA) according to the manufacturer's protocol. To determine the expression levels of biofilm and virulent related genes of *F. columnare*, quantitative real-time PCR (qPCR) using published primers as described in Table 2. Primers targeting adenylate kinase (*AK*) were served as the reference gene. Each qPCR reaction (20 µl) comprised of 1 µl of cDNA (5 ng of cDNA), 0.4 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer, 0.4 µl of low ROX (carboxyrhodamine), 10 µl of SYBR premix (Kapa Biosystem, Inc), and 7.8 µl of molecular-grade water. The qPCR conditions were as follow, pre-denaturation (95°C for 3 min) followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The qPCR level of transcription was normalized to adenylate kinase (*AK*) and analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) before being expressed as log2 fold change of relative expression.

Table 2. Real-time quantitative PCR primer for analysis of biofilm-associated genes expression of *Flavobacterium columnare*.

Genes	Gene function	Oligo sequence (5′ ⟶ 3′)	References
gldL	Gliding motility	F: GCTTCTGTACCTAAACCAGCA R: TTGGTGCGGCAGTAGTAATC	(Declercq et al., 2019)
sprA	Secretion of mobile surface adhesin	F: AGGCGATGGTATTTCGTTAGG R: GTACGCGTCCTGCTTGATAA	(Declercq et al., 2019)

tonB	iron-siderophore uptake	F: TGCAATGCAAGCCTATCTTATTC R: TGCTGAACCCTTCCGTTATATT	(Lange et al., 2018)
rhbC	Siderophore biosynthesis	F: GAATTGGGAGACGAAACCATTG R: CCAAGGATGTGTTGGCATAAAG	(Lange et al., 2020)
alcB	Siderophore biosynthesis	F: TCGTGTAGTTGTAGAACCTGATG R: GAGCATTGTAGTATTGTTGTCTTGT	(Lange et al., 2020)
PorV	Secretion of mobile surface adhesin	F: GTGCCAACTCCTAAAACAGCC R: AAACCTCCTGGAGCATCACC	(Penttinen et al., 2018)
luxR	Quorum sensing regulator	F: ACCTTTCGCACACACAGAAA R: GCAATGTCGTTCTTTAGGCTGT	(Cai et al., 2019)
AK	Housekeeping gene	F: TTCTTTTCAACTTCAGATTCCAACA R: GGAAAACCTGGAGCGGGAA	(Cai et al., 2019)

9. Statistical analysis

The differences in mean OD_{600} of growth and biofilm formation and gene expression of *F. columnare* between negative control and the mixture of bacterial CFS or cell were analysed using student t-test in the SPSS version number 22 software (IBM, USA). The level of significance at all comparisons was $p < 0.05$ considered as statistically significant.

CHAPTER IV
RESULTS

1. Cell-free supernatants (CFS) of all lactic acid bacteria (LAB) isolates inhibited biofilms and some inhibited growth of *Flavobacterium columnare*

The biofilm inhibition assay revealed that CFSs from all five LAB isolates significantly ($p < 0.05$) inhibited biofilms of all three *F. columnare* isolates with relative percent inhibitions ranging from 58.4% to 93.3%, 67.2% to 93.0%, and 28.2% to 89.2% for *F. columnare* isolates 15 (**Figure 2A**), CUVET1359 (**Figure 2C**) and CUVET1365 (**Figure 2E**), respectively. As for the planktonic cell growth inhibition assays, except for the CFS of WS2020 which consistently inhibited the planktonic cells of all three *F. columnare* isolates, the results for other isolate CFSs were found to be varied (**Figure 2B**, **Figure 2D**, and **Figure 2F**). Based on these data, the LAB isolates SC1 and WS2021 that inhibited biofilms of all three *F. columnare* isolates without affecting their planktonic cell growth were chosen for further analyses.

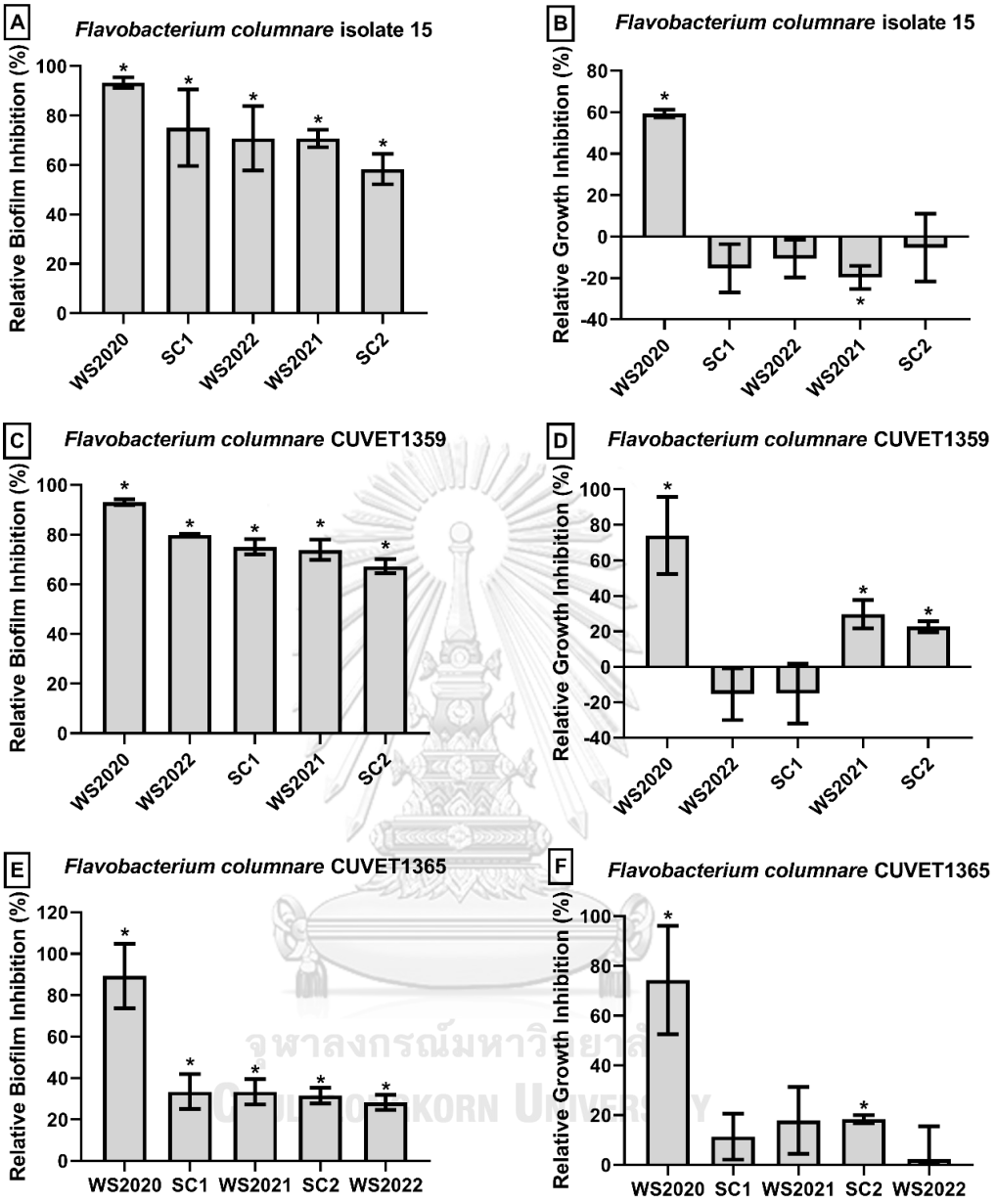

Figure 2. Relative biofilm and growth inhibitions of *Flavobacterium columnare* isolate 15 (**A** and **B**), CUVET1359 (**C** and **D**), and CUVET1365 (**E** and **F**) by cell-free supernatants (CFSs) of five different lactic acid bacteria (LAB) isolates after 72 h (biofilm) and 24 h (growth) co-culture respectively. The CFSs of all five LAB bacterial isolates were significantly ($p < 0.05$) inhibited biofilms of *F. columnare* isolates 15 (**A**), CUVET1359 (**C**) and CUVET1365(**E**). However, only the CFS of WS2020 inhibited planktonic cell growth of all three *F. columnare* isolates (**B, D and F**). The CFSs of SC2 were found to marginally but significantly inhibit the growths of *F. columnare* isolates CUVET1359 and CUVET1365 but not affected the growth of isolate 15. The CFS of

WS2021 was found to marginally but significantly inhibit planktonic cell growth of *F. columnare* isolates CUVET1359 (**D**) but promote *F. columnare* isolates 15 (**B**). Bars represent the percentage of mean relative growth inhibition ± standard deviation of eight replicates and asterisks (*) indicate significant difference at $p < 0.05$ when compared to vehicle control (student t-test; $p < 0.05$).

2. CFSs and cells of SC1 and WS2021 inhibited *F. columnare* biofilm but not its growth

Cells and CFSs of SC1 and WS2021 isolates were obtained from cultures at 8 different time points (0, 3, 6, 9, 12, 24, 36, and 48 h) and evaluated for their abilities to inhibit biofilm and growth of *F. columnare* isolate 15. As shown in **Figures 3A** and **4A**, SC1 and WS2021 cells from all incubation time points were found to inhibit *F. columnare* biofilm with relative percent inhibitions between 54.4% and 91.9% and 93.0% and 94.3% whereas the CFSs obtained from only 36 h and 48 h cultures were found to inhibit *F. columnare* biofilm formation with relative percent inhibitions between 64.9% and 95.9% and 53.5% and 96.6%, respectively. The CFSs of both isolates collected at other time points did not affect the *F. columnare* biofilm formation. In the *F. columnare* growth inhibition assays, none of these CFSs and cells were found to inhibit the planktonic cell growth of *F. columnare*, instead they stimulated it (**Figures 3B and 4B**).

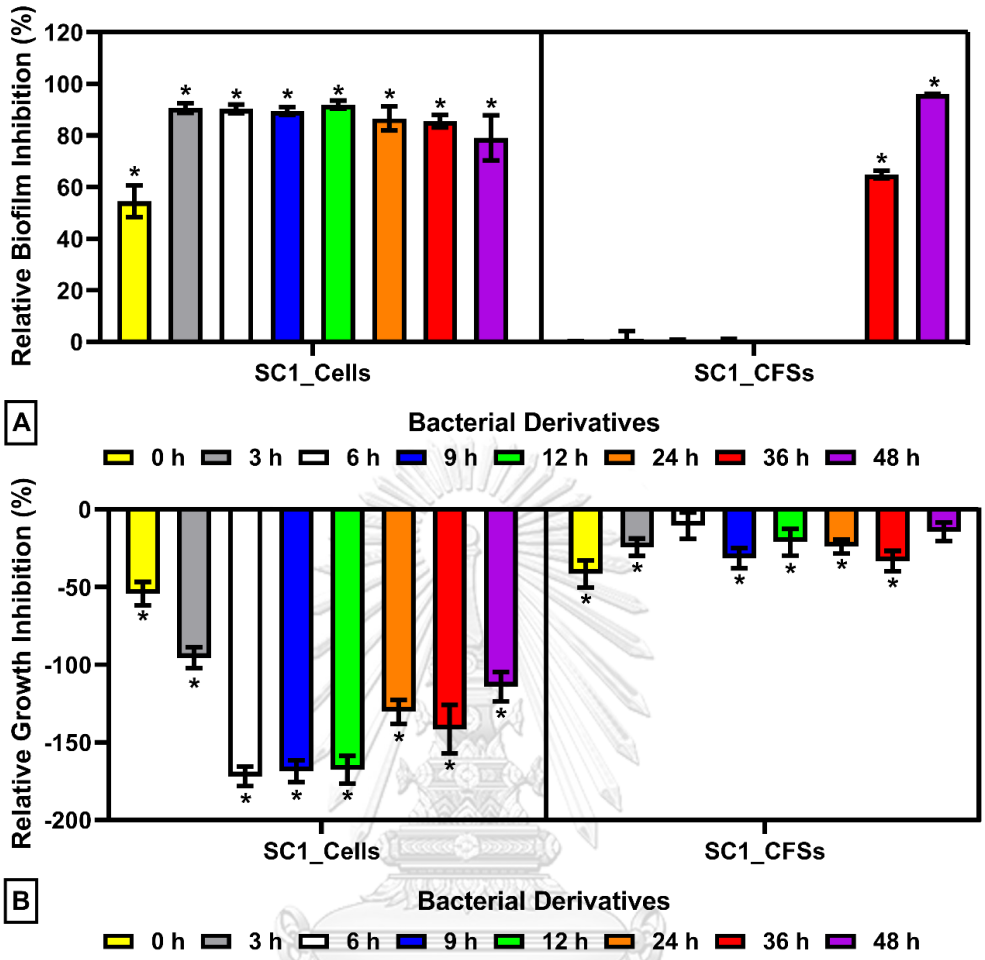

Figure 3. Relative biofilm (**A**) and growth (**B**) inhibitions of *Flavobacterium columnare* (isolate 15) by the cells and cell-free supernatant (CFS) of *Leuconostoc pseudomesenteroides* isolate SC1 obtained from eight different time points. As shown in panel **A**, cells of *L. pseudomesenteroides* SC1 isolated from eight different time points significantly inhibited biofilm formation of *F. columnare* ($p < 0.05$) by 54.4% to 91.9% inhibition, meanwhile only CFS isolated from 36 h (64.9%) and 48 h (95.9) significantly inhibited *F. columnare* biofilm formation. As shown in panel **B**, both cells and CFSs of *L. pseudomesenteroides* SC1 obtained from all time points did not inhibit the planktonic cell growth of *F. columnare*, but many stimulated cell growths ranging from 21.1% to 171.9% stimulation. Bars represent the percentage of mean relative growth inhibition ± standard deviation of eight replicates and asterisks (*) indicate significant difference at $p < 0.05$ when compared to vehicle control (student t-test; $p < 0.05$).

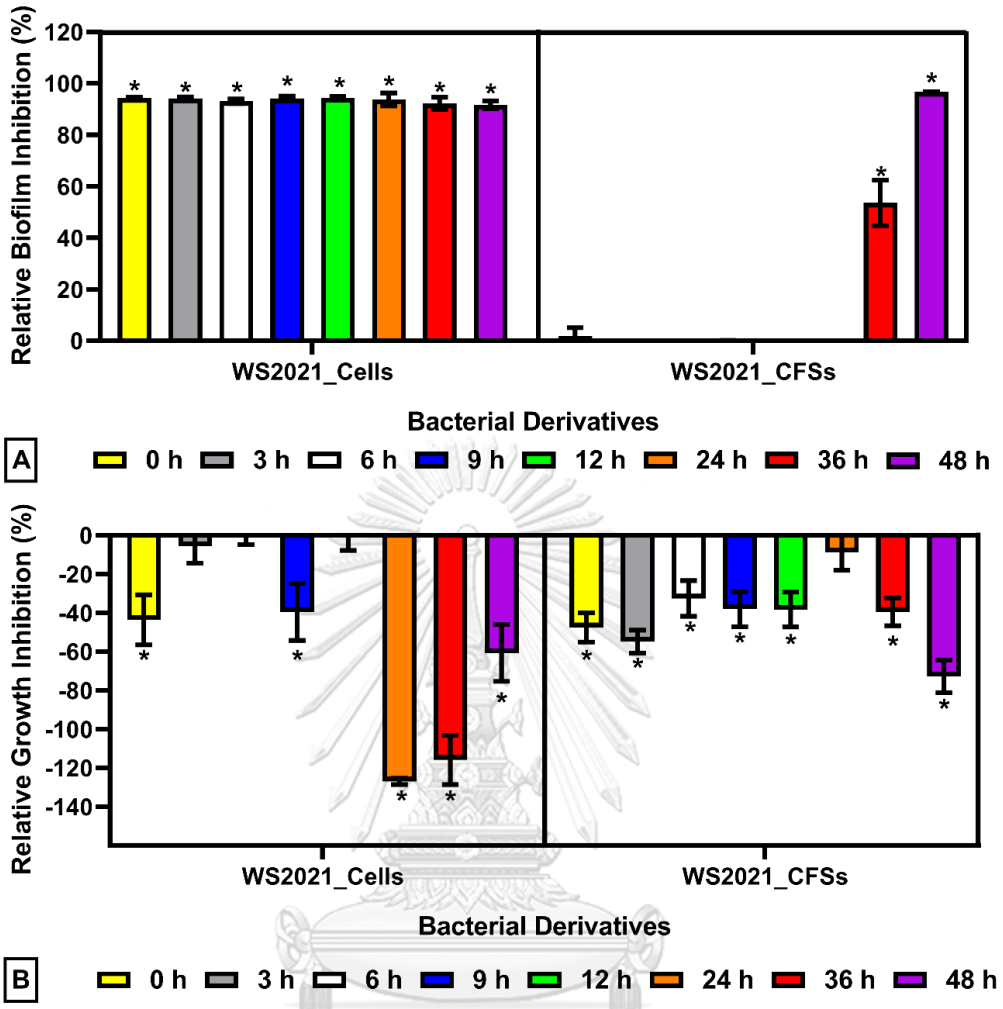

Figure 4. Relative biofilm (**A**) and growth (**B**) inhibitions of *Flavobacterium columnare* (isolate 15) by the cells and cell-free supernatant (CFS) of *Enterococcus gallinarum* WS2021 obtained from eight different time points. As shown in panel **A**, cells of *E. gallinarum* isolate WS2021 isolated from eight different time points significantly inhibited biofilm formation of *F. columnare* ($p < 0.05$) with relative inhibition between 93.0% and 94.3%, meanwhile only CFS isolated from 36 h (53.5%) and 48 h (96.6%) significantly inhibited *F. columnare* biofilm formation. As shown in panel **B**, both cells and CFSs of *E. gallinarum* WS2021 obtained from all time points did not inhibit the planktonic cell growth of *F. columnare*, but many stimulated cell growths ranging from 32.5% to 126.9% stimulation. Bars represent the percentage of mean relative growth inhibition ± standard deviation of eight replicates and asterisks (*) indicate significant difference at $p < 0.05$ when compared to vehicle control (student t-test; $p < 0.05$).

3. WS2021 and SC1 isolates were identified as *Enterococcus gallinarum* and *Leuconostoc pseudomesenteroides*, respectively

Two LAB isolates (WS2021 and SC1 isolates) with the most potent *F. columnare* biofilm inhibiting activities were chosen for identification using 16sRNA sequence analysis. Both are Gram-positive bacteria with milky white raised colonies on MRS agar. From 16S rRNA sequence analysis, that the sequence of 16S sRNA gene of isolate WS2021 was found to have 99% identity to that of *Enterococcus gallinarum* while the same of the SC1 isolate was found to have 99% identity to that of *Leuconostoc pseudomesenteroides*. The phylogenetic tree was constructed with MEGA X software (**Figure 5**).

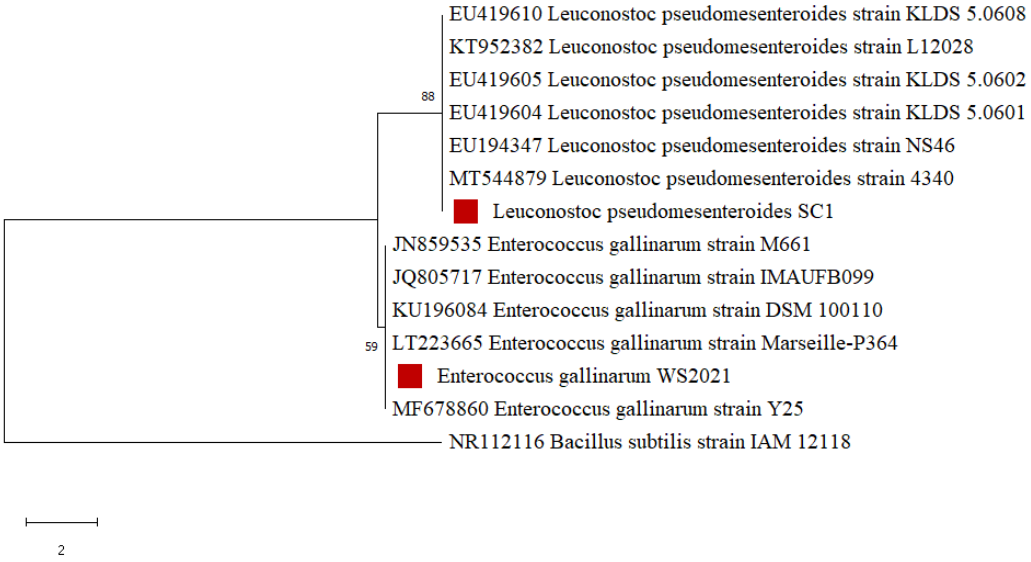

Figure 5. Phylogenetic tree constructed using MEGA X software and based on 16S rRNA sequences using neighbor-joining methods. WS2021 was assigned as *Enterococcus gallinarum* and SC1 was assigned as *Leuconostoc pseudomesenteroides*. The phylogenetic tree was statistically evaluated using 1000 bootstrap replicates.

4. Modulations of *F. columnare* iron acquisition genes by LAB CFS and cells

To determine the effect of the cells and CFSs of SC1 and WS2021 on the expressions of iron acquisition genes that are involved in biofilm formation and

virulence of *F. columnare*, the cells and CFSs obtained from 48 h cultures of each isolate were incubated with the *F. columnare* isolate 15 biofilm cultures for 72 h. At 24 h, 48 h, and 72 h after incubations, biofilm samples from each culture were collected and their RNA were extracted and used as templates for quantitative real-time RT-PCRs using primers targeting three iron acquisition genes, namely, *alcB*, *rhbC*, and *tonB*. For this qRT-PCR analysis, the adenylate kinase (*AK*) gene was used as the housekeeping gene to normalize the data. From the analyses, *alcB* gene was found to be up regulated at early time point (at 24 h). But as the biofilms grew older (48 and 72 hours), the *alcB* of all the *F. columnare* biofilm cultures were found to be downregulated except for that of the WS2021 CFS treated *F. columnare* biofilm at 48 h which was not affected (**Figure 6A**). It is interesting to note that the degree of *alcB* inhibitions increase with the culture times of the *F. columnare* biofilms.

The *rhbC* is the gene that is responsible for rhizobactin 1021 siderophore synthesis, down regulation of the gene could mean that there will be less this type of siderophore to trap iron (Fe^{3+}). In this study, using quantitative real-time RT-PCR, the *rhbC* gene expression of *F. columnare* biofilms in the present and absent of cells and CFSs of the two LAB isolates were determined. From the results obtained, all cells and CFSs of the two LAB bacterial isolates were found to significantly supress the *rhbC* expressions, except the cells of WS2021 at 72 h and SC1 cells that were incubated with *F. columnare* biofilms for 48h that were found to have no effect on the *rhbC* gene expression (**Figure 6B**).

For the *tonB* gene expression analysis, the CFSs and cells of the two LAB isolates were found to have no effect on the *tonB* gene expression in early time point (24 h) except for the CFSs from SC1 isolate which was found to stimulate the gene expression. However, all the cells and CFS collected from both of the LAB isolates that have been co-incubated with *F. columare* biofilms for 48 and 72 hours,

were all found to inhibit the *tonB* gene expressions except for the CFS from SC1 isolate which was found to have no effect on the gene expression (**Figure 6C**).

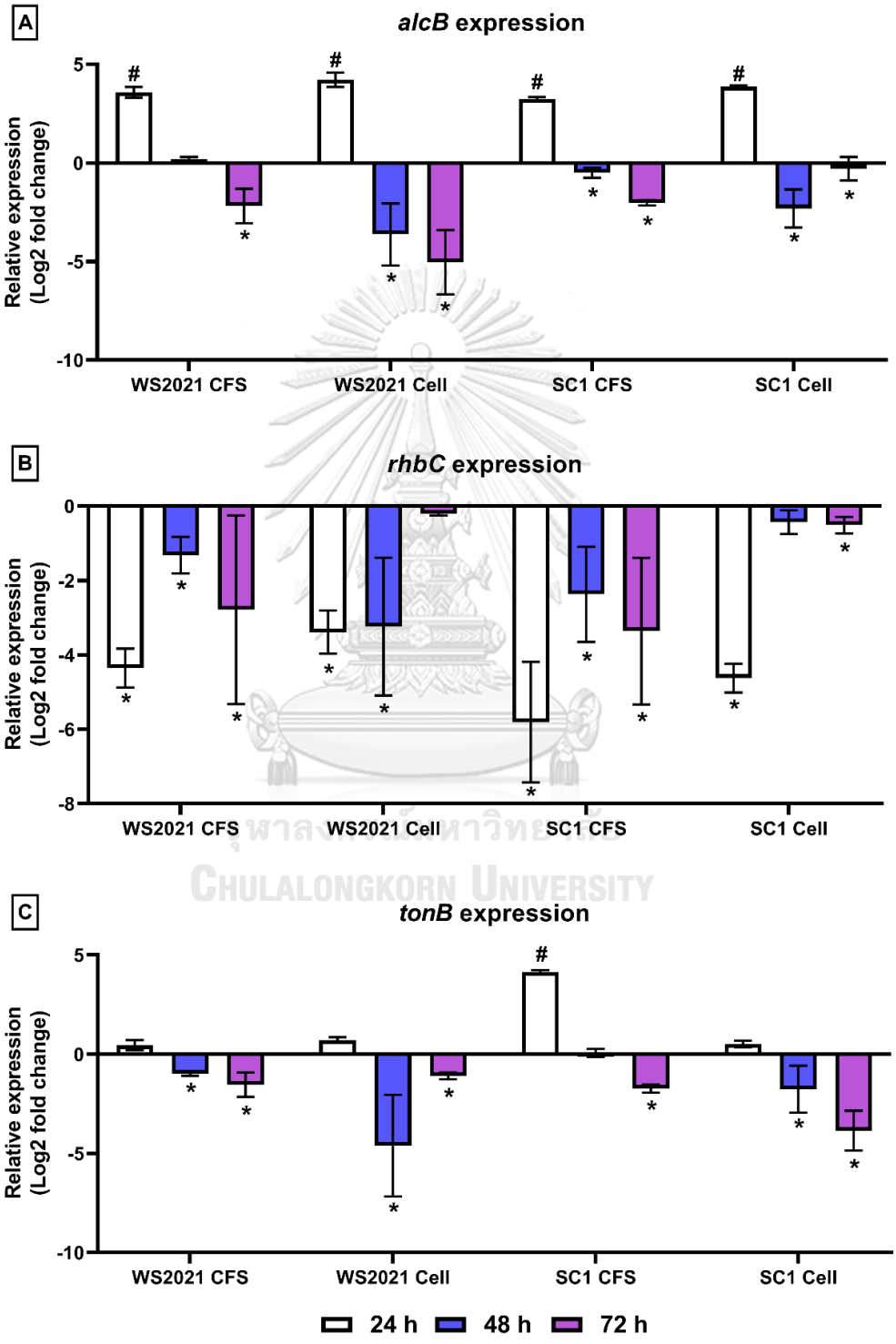

Figure 6. Comparative analysis of the expression of three iron acquisition genes in *Flavobacterium columnare* isolate 15 at three different time points (24 h, 48 h, and 72 h) after being incubated with either cells or CFSs of SC1 and WS2021 isolates. On panel **A**, *alcB* expression at 24 h co-incubation was enhanced in all treatment groups but the expression was mostly downregulated at 48 h and 72 h co-incubation. On panel **B**, the *rhbC* expression was mostly downregulated at all co-incubation timepoints. On panel **C**, *tonB* expression at *F. columnare* biofilm co-incubated with all treatment was no differences at 24 h timepoint, except for upregulation to that of treated with SC1 CFS. The expression was gradually downregulated at the two remaining timepoints (48 h and 72 h). Bars represent the mean relative expression (Log2 fold change) ± standard deviation. An asterisk (*) or octothorpe (#) on top of some bars represents significant downregulation or upregulation, respectively, of gene expression in each treatment when compared to that of vehicle control biofilm at each timepoints (student t-test; $p < 0.05$).

5. CFS and cells modulated type IX secretion system genes of *F. columnare* biofilm

To determine the effect of the cells and CFSs of SC1 and WS2021 on the expressions of the type IX secretion system (T9SS) genes that are involved in biofilm formation and virulence of *F. columnare*, the cells and CFSs obtained from 48 h cultures of each isolate were incubated with the *F. columnare* isolate 15 biofilm cultures for 72 h. At 24 h, 48 h, and 72 h after incubations, biofilm samples from each culture were collected and their RNA were extracted and used as templates for quantitative real-time RT-PCRs using primers targeting three T9SS genes, namely, *porV*, *gldL*, and *sprA*. For this qRT-PCR analysis, the adenylate kinase (*AK*) gene was used as the housekeeping gene to normalize the data. From the analyses, *porV* expression at 24 h co-incubation was upregulated in *F. columnare* biofilm treated with CFSs of WS2021 and SC1, but the expression *porV* was mostly downregulated at 48 h and 72 h co-incubation (**Figure 7A**). On **Figure 7B**, the *gldL* expression was upregulated in *F. columnare* biofilm treated with WS2021 CFS, but the other three treatments were mostly downregulated at 24 h co-incubation timepoint. Similar pattern of *gldL* gene modulation at 24 h was also observed at 48 h with only

WS2021 CFS was unaffected. All the treatments downregulated the expression of *gldL* at 72 h timepoint. On **Figure 7C**, *sprA* expression at *F. columnare* biofilm co-incubated with all treatment was mostly upregulated at 24 h timepoint, except that treated with SC1 cell presenting slight downregulation. At 48 h and 72 h timepoints, the *sprA* expression in *F. columnare* biofilm was gradually downregulated.

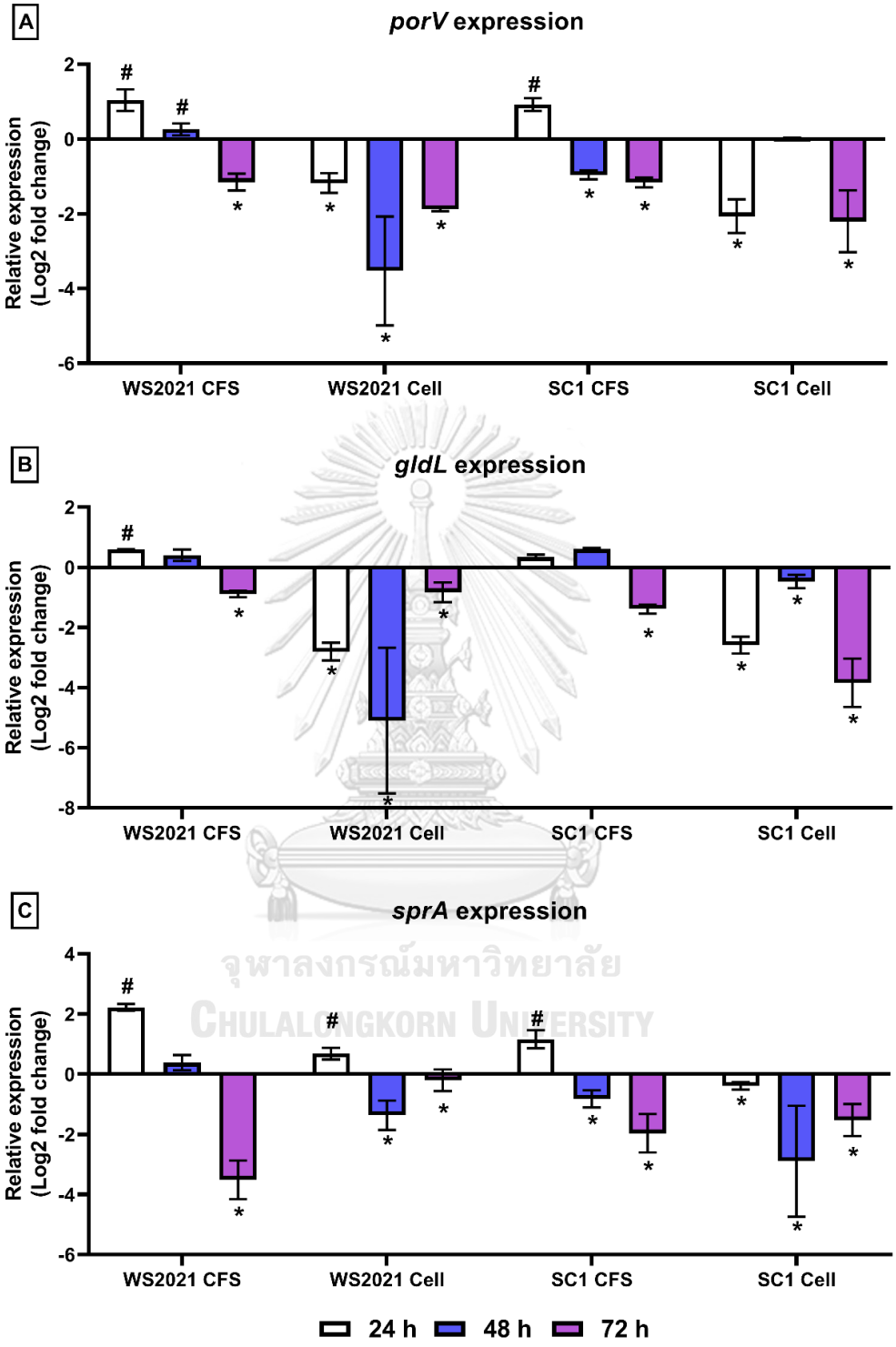

Figure 7. Comparative analysis of the expression of three genes of the type IX secretion system (T9SS) in *Flavobacterium columnare* isolate 15 at three different time points (24 h, 48 h, and 72 h) after being incubated with either cells or CFSs of SC1 and WS2021 isolates. On panel **A**, *porV* expression at 24 h co-incubation was enhanced in CFSs of WS2021 and SC1, but the expression

was mostly downregulated at 48 h and 72 h co-incubation. On panel **B**, the *gldL* expression was upregulated in *F. columnare* biofilm treated with WS2021 CFS, but the other three treatments were mostly downregulated at 24 h co-incubation timepoint. Similar pattern of 24 h gene modulation was observed at 48 h, but all the treatments downregulated the expression of *gldL* at 72 h timepoint. On panel **C**, *sprA* expression at *F. columnare* biofilm co-incubated with all treatment was mostly upregulated at 24 h timepoint, except that treated with SC1 cell presenting slight downregulation. The expression was gradually downregulated at the two remaining timepoints (48 h and 72 h). Bars represent the mean relative expression (Log2 fold change) ± standard deviation. An asterisk (*) or octothorpe (#) on top of some bars represents significant downregulation or upregulation, respectively, of gene expression in each treatment when compared to that of vehicle control biofilm at each timepoints (student t-test; $p < 0.05$).

6. CFS and cells modulated *luxR* gene (a master transcriptional regulator of quorum sensing gene) of *F. columnare*

To determine the effect of the cells and CFSs of SC1 and WS2021 on the expressions of *luxR* that are involved in biofilm formation and virulence of *F. columnare*, the cells and CFSs obtained from 48 h cultures of each isolate were incubated with the *F. columnare* isolate 15 biofilm cultures for 72 h. At 24 h, 48 h, and 72 h after incubations, biofilm samples from each culture were collected and their RNA were extracted and used as templates for quantitative real-time RT-PCRs using primers targeting a master regulatory gene, *luxR*. For this qRT-PCR analysis, the adenylate kinase (*AK*) gene was used as the housekeeping gene to normalize the data. From the analyses, *luxR* gene was found to be up regulated at two time point (at 24 h and 48 h) in the *F. columnare* biofilm treated with SC1 CFS. The other treatments at all three time points predominantly downregulated the expression of *luxR* in *F. columnare* biofilm (**Figure 8**).

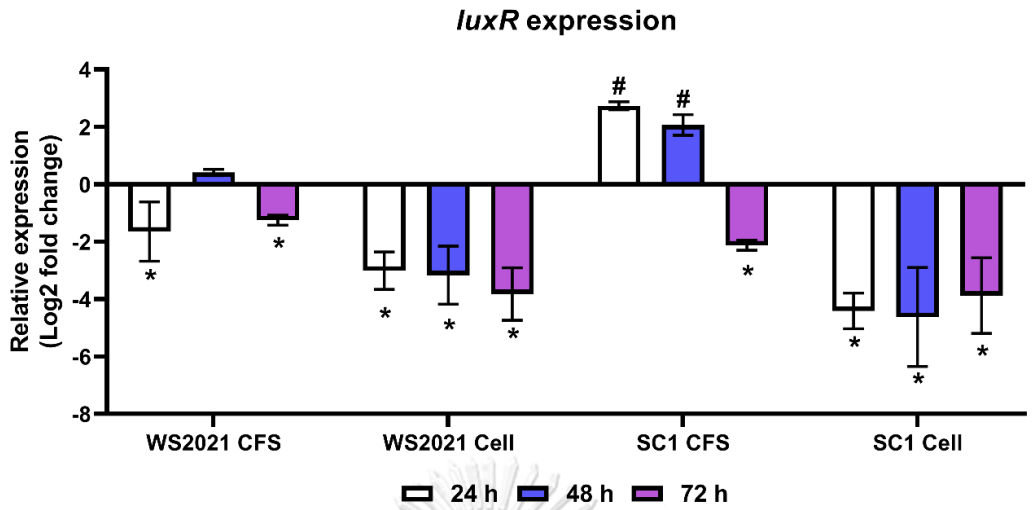

Figure 8. Comparative analysis of the expression of *luxR* gene in *Flavobacterium columnare* isolate 15 at three different time points (24 h, 48 h, and 72 h) after being incubated with either cells or CFSs of SC1 and WS2021 isolates. WS2021 CFS, WS2021 cell, and SC1 cell downregulated the expression of *luxR*, but upregulation was observed in SC1 CFS in 24 h co-incubation. Similar pattern of *luxR* expression downregulation in 48 h co-incubation was also observed in *F. columnare* biofilm treated with WS2021 cell and SC1 cell, while SC1 CFS still upregulated the *luxR* expression. At 72 h co-incubation, all of treatments downregulated the expression of *luxR*. Bars represent the mean relative expression (Log2 fold change) ± standard deviation. An asterisk (*) or octothorpe (#) on top of some bars represents significant downregulation or upregulation, respectively, of gene expression in each treatment when compared to that of vehicle control biofilm at each timepoints (student t-test; $p < 0.05$).

CHAPTER V
DISCUSSION AND CONCLUSION

1. Discussion

Biofilm lifestyle is the preferred mode of living for many bacteria including pathogenic bacteria because the bacterial cells are protected from many adversities such as sunlight, host immunity and antibiotics. To eradicate cells in biofilm generally requires higher concentration of antimicrobial compounds than the planktonic cells, partly because the extracellular polymeric substances (EPS) that encase the biofilm are not easily penetrated by the antibiotics (Evans et al., 1991; Ishida et al., 1998). The use of antibiotics at high concentrations could lead to the development of antibiotic resistant bacteria or antibiotic resistant bacteria could become dominant bacteria in the animals and in the environment. Therefore, biofilm inhibitors from natural and/or synthetic sources that do not possess antibiotic properties could be a good alternative tool to prevent or control bacterial infection. In this study, cell-free supernatant (CFS) of 4 lactic acid bacteria (WS2021, WS2022, SC1, and SC2) isolates originated from the gut of white leg shrimp and the root of Jerusalem artichoke were tested and found to inhibit biofilms of three tilapia pathogenic *F. columnare* strains. The cells and CFSs of the two LAB isolates; WS2021 and SC1, which were identified as *Enterococcus gallinarum* and *Leuconostoc pseudomesenteroides* respectively, were found to be the most potent *F. columnare* biofilm inhibitors. The abilities of these two LAB isolates were further characterized in *F. columnare* isolate 15. These two LAB isolates were found to downregulate the expression of iron acquisition genes (*alcB*, *rhbC*, and *tonB*), type 9 secretion system genes (T9SS; *porV*, *gldL*, and *sprA*), and the quorum sensing master transcriptional regulator gene, *luxR* genes of the *F. columnare* isolate 15.

We found that the cells at all growth phases of the most potent isolate (WS2021, identified as *Enterococcus gallinarum*) were shown to strongly inhibit biofilm formation of pathogenic *F. columnare*. *E. gallinarum* WS2021 cells may compete with *F. columnare* cells through the competition of exclusion for sources such as nutrients and surfaces (**Figure 9**). By that, *F. columnare* will not have enough sources for its fitness as well as to form biofilm. Addition of the cells of LAB has been found to inhibit biofilm formation of human's pathogenic bacteria in culture broth. For example, co-culture of *Listeria monocytogenes* and *L. paracasei* or *L. rhamnosus* at a rate of 10^7 CFU/mL each reduces the counts of *L. monocytogenes* biofilm cells by ~4 $\log_{10}$ CFU/mL (Woo and Ahn, 2013). Also, we found that CFSs of *E. gallinarum* WS2021 at only the stationary phases (36 and 48 h incubation time) inhibited the *F. columnare* biofilm strongly, compared to other earlier phases (0 to 24 h). This inhibition may be attributed by the presence of antagonistic products in CFSs (**Figure 9**) which may be produced more at the later/late growth phase of LAB as studied in *L. plantarum* which produces maximal biosurfactants at 48 h under stationary conditions (Montoya Vallejo et al., 2021). Some products from LAB have been found to inhibit the biofilm formation of pathogenic bacteria. Examples are biosurfactants produced by *P. acidilactici* 27167 and *L. plantarum* 27172 inhibiting biofilm formation of *Staphylococcus aureus* CMCC26003 (Yan et al., 2019).

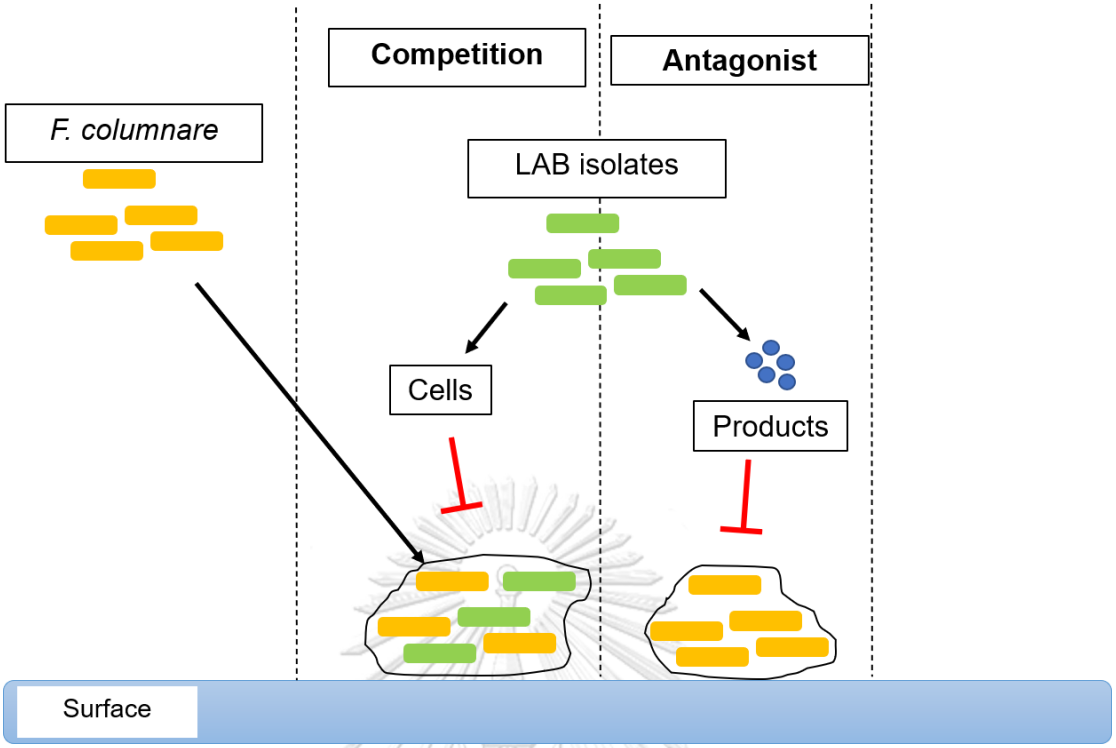

Figure 9. The possible mode of action of lactic acid bacteria (LAB) used in this study to inhibit biofilm formation of *Flavobacterium columnare*. Since we apply cells and cell-free supernatant (CFS) of LAB isolates to *F. columnare* culture, competition and antagonistic mechanisms can be the possible mechanisms.

Iron is one of the key components required by all life forms to carry out numerous cellular processes including vertebrates which employ several iron-binding proteins such as lactoferrin and transferrin. These proteins limit the amount of free ferric iron ions available to pathogens. However, Gram-negative bacteria have unique responses to this iron restriction by employing siderophore-based and/or enzyme-based iron acquisition systems to retain and acquire iron from vertebrate hosts. In *F. columnare*, several iron acquisition genes have been identified and shown to be related to biofilm formation (Beck et al., 2016; Lange et al., 2018; Lange et al., 2020). In this study, we performed gene expression analysis using quantitative real-time PCR on three iron acquisition genes namely: *alcB*, *rhbC*, and *tonB* in *F. columnare* biofilm

co-incubated for 24 h, 48 h, and 72 h with either CFSs or cells of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 isolates. The *alcB* and *rhbC* are the genes responsible for different siderophore biosynthesis pathways called alcaligin and rhizobactin 1021, respectively (Giardina et al., 1997; Lynch et al., 2001). Whereas, *tonB* gene product spans the periplasmic membrane of the bacterial cell anchoring the inner membrane which associates with ExbB and ExbD proteins. The tonB protein is responsible for active transport of iron-chelated siderophore (in this case; rhizobactin 1021 and alcaligin) into bacterial cells (Noinaj et al., 2010).

We found that, at 24 h after the *F. columnare* cultures were co-incubation with either cells or CFSs of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 isolates, the *alcB* gene expression in the biofilm was predominantly upregulated along with *tonB* gene, but *rhbC* was downregulated in biofilm at 24 h timepoint. Generally, iron availability affects the production of siderophore and its transport system into the bacterial cells. Indeed, 60 strains in *Enterococcus* genus including *E. gallinarum* secrete siderophore with hydroxamate type to their culture supernatant fluid (Lisiecki et al., 2000). On the other hand, no report reveals the presence of siderophore in *Leuconostoc* genus so far, but other bacterium in Lactobacillales order, *L. casei* ATCC334 secretes ferrichrome siderophore into its culture supernatant fluid (Konishi et al., 2016). Therefore, CFS and cell of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 may compete with *F. columnare* to acquire iron from TYES medium (yeast extract as the iron sources) which results in upregulation of *alcB* and *tonB* in *F. columnare*. This type of competition is also observed in *Pseudomonas aeruginosa* PAO985 when being cultured with *Staphylococcus aureus* FZ21 under iron restricted condition found that the presence of *S. aureus* in microcosm upregulates the siderophore production (per capita) (Harrison et al., 2008). CFS or cell of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 may cause disturbances to

iron regulation in *F. columnare* biofilm, so upregulation of *alcB* and *tonB*, and downregulation of *rhbC* might be part of regulatory network of *F. columnare* to reach a balance in iron homeostasis.

In contrast, consistent and predominant downregulation in *alcB*, *rhbC*, and *tonB* was observed at 48 h and 72 h timepoints in *F. columnare* biofilm treated with cells or CFSs of WS2021 and SC1 isolates. The downregulation in *alcB* and *rhbC* expression blocks the synthesis of alcaligin and rhizobactin 1021 siderophores, respectively (Giardina et al., 1997; Lynch et al., 2001), while downregulation of *tonB* may affect the transport of iron-chelated siderophore into bacterial cells (**Figure 10**). As a result, *F. columnare* will not have enough physiological iron for its fitness and survivability which will be detrimental for biofilm development. This statement is supported by some studies conducted in different bacteria. Examples are a study conducted by Banin et al. (2005) revealing that *P. aeruginosa* PAO1 mutant with no functional pyoverdine siderophore grew on iron-sufficient medium develop thin biofilm similar to the wildtype under low iron condition. In addition, *Staphylococcus epidermidis* 1457 mutant with no staphyloferrins siderophore is defective in biofilm formation at medium under iron-restricted condition which is more or less similar to human host condition (Oliveira et al., 2021). In *P. aeruginosa* PAO6609, Abbas et al. (2007) has revealed that mutant of this bacterium with *tonB1* iron transport defect is unable to form biofilm in glass even though the mutant has similar growth pattern as the wildtype. Therefore, downregulation of *alcB*, *rhbC*, and *tonB* after treatment with cells or CFSs of WS2021 and SC1 isolates may contribute to the failure of *F. columnare* in developing mature and thick biofilm.

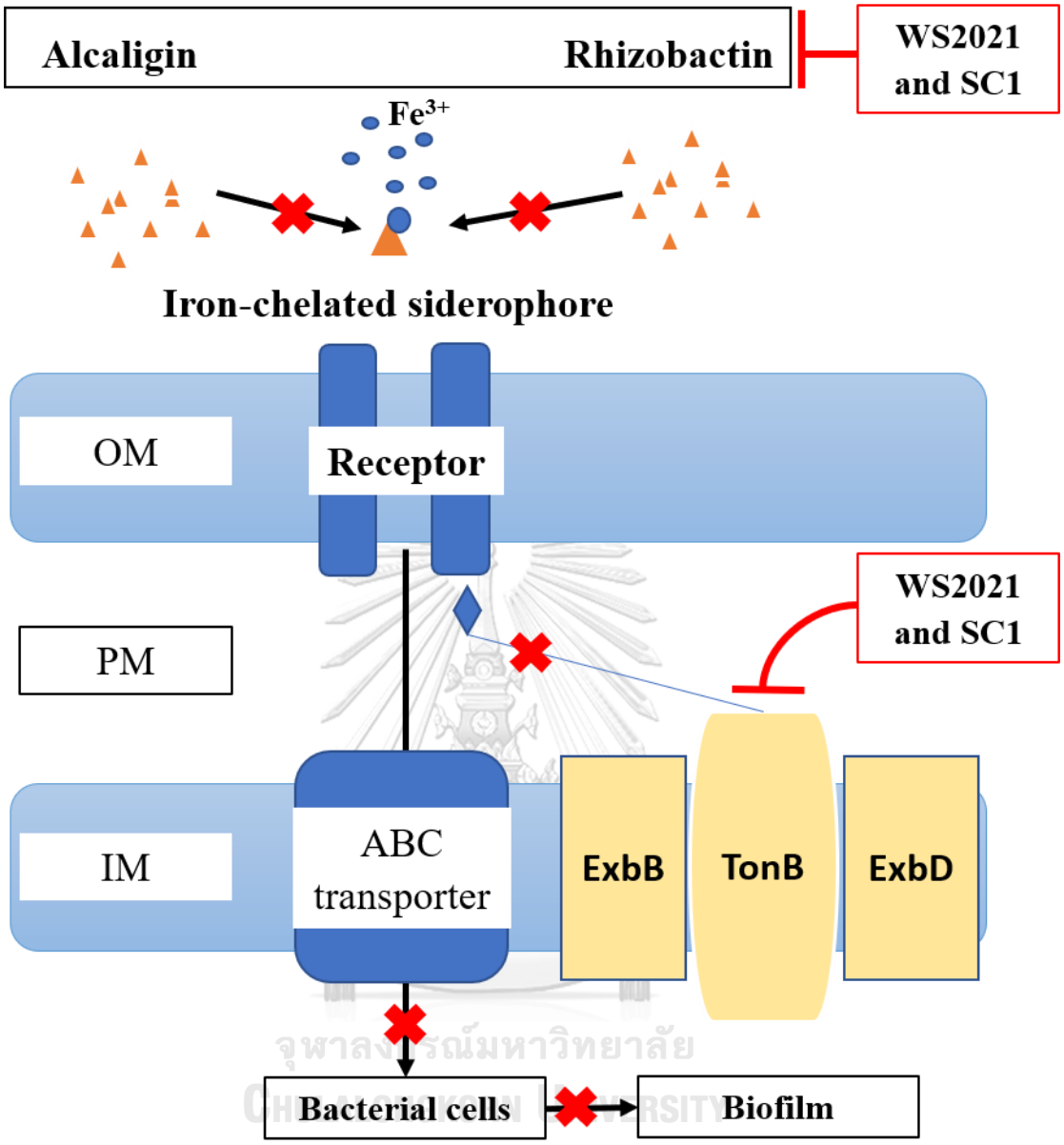

Figure 10. The schematic diagram showing the interplay of pathway involved in the transportation of iron-chelated siderophores into bacterial cells with the possible mechanisms of action from CFS and cell of WS2021 and SC1 isolates by downregulating the expression of *alcB*, *rhbC*, and *tonB*. The downregulation will affect the synthesis of the proteins required for iron homeostasis in *Flavobacterium columnare* which may affect its fitness, including its ability to produce thick biofilm.

In Gram-negative bacteria, there are at least nine types of secretion systems (T1SS to T9SS) transporting proteins across membranes. The types IX secretion system (T9SS)

is common on Bacteroidetes phylum contributing to the secretion of some virulent proteins as studied in *F. columnare* by Li et al. (2017). This group revealed that *F. columnare* mutant with some T9SS-defected components has lower proteolytic and chondroitin sulphate activities which eventually fails to cause mortality in rainbow trout, channel catfish, and zebrafish experimental models compared to the wild type *F. columnare*. T9SS components also have critical roles in the alteration of behavioural characters of bacteria in the community level such as the bacterial attachment (Shrivastava et al., 2013). In our study, we found that the expression of some studied T9SS component genes of *F. columnare* biofilm was predominantly downregulated in the course of three co-incubating timepoints with either cells or CFSs of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 isolates. The *gldL*, *porV*, and *sprA* and along with other genes contribute to some mechanisms in the T9SS machinery including the secretion of mobile surface adhesins called SprB and RemA involved in gliding motility and attachment to the glass (**Figure 11**). It is possible that the downregulation of the studied genes on *F. columnare* biofilm following co-incubation with either cells or CFSs of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021 isolates contributed to the failure of *F. columnare* in developing mature and thick biofilm. These results are in agreement with the study conducted by Shrivastava et al. (2013). They reveal that *F. johnsoniae* mutants with the deletion of either *gldK*, *gldL*, *gldM*, or *sprA* still produce the SprB and RemA but these mutant cells have lower attachment ability to the cover slip than the wildtype. This suggests that defective on one of these genes does not affect the production of mobile surface adhesins but it will affect the secretion of these adhesins to the cell surface which eventually affect the bacterial attachment (biofilm) to the surface.

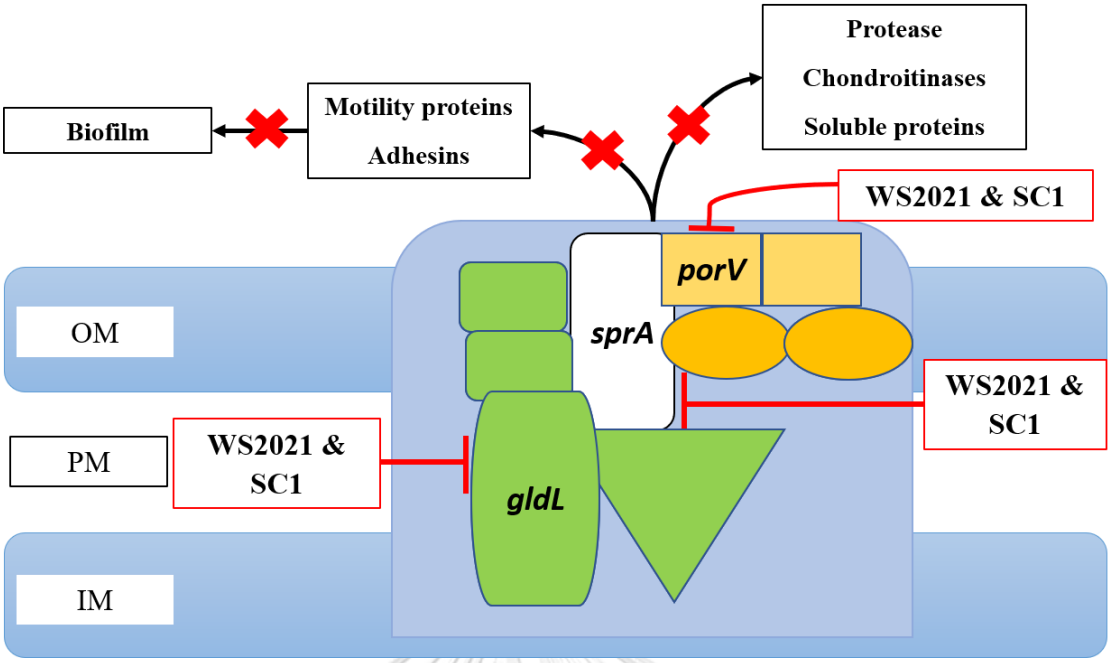

Figure 11. The schematic diagram showing the interplay of the type IX secretion system (T9SS) machinery in *Flavobacterium columnare* with the possible mechanisms of action from CFS and cell of WS2021 and SC1 isolates to the downregulation of *gldL*, *sprA*, and *porV* genes. The downregulation will affect the functionality of T9SS machinery required for synthesis of many proteins for the survival of *Flavobacterium columnare* including its ability to produce thick biofilm.

Quorum sensing is a cell-to-cell communication of bacteria mediated by signal molecules called autoinducers to coordinate the expression of certain genes for group behaviour such as biofilm formation. In *Vibrio* bacteria, there are three autoinducer types regulating three different pathways called harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2), and cholerae autoinducer 1 (CAI-1) synthesized by *luxM*, *luxS*, and *cqsA* (**Figure 12**), respectively (Cao and Meighen, 1989; Chen et al., 2002; Higgins et al., 2007). However, there are no report characterizing the quorum sensing regulation in *F. columnare* yet, with only two reports identify the presence of *luxR* response regulator gene (Lange et al., 2018; Cai et al., 2019). In the present study, the *luxR* in *F. columnare* biofilm was predominantly downregulated in all three

timepoints after co-incubation with either cells or CFSs of *L. pseudomesenteroides* SC1 or *E. gallinarum* WS2021. The *luxR* gene along with *aphA* is responsible in the downstream regulation that individually and jointly control more than 700 quorum sensing-regulated genes in *V. harveyi* (Kessel et al., 2013). Indeed, *luxR* is shown to be associated with the biofilm formation as presented in *V. anguillarum*, where *luxR* mutant produces much less biofilm compared to that of the wildtype (Croxatto et al., 2002). Also, *luxR* is also believed to be correlated with the production of several virulence genes such as *vhp* metalloprotease, the *srp* serine protease, and the regulatory gene *toxR* in Vibrio bacteria (Ruwandeepika et al., 2011). The brine shrimp (*Artemia franciscana*) challenged with *luxR*-producing Vibrio present higher mortality (77% and 51%) than non-producing *luxR* (1%). Thus, it is possible that the downregulation of *luxR* gene may contribute to the failure of *F. columnare* in developing mature and thick biofilm, and may also affect the production of *F. columnare* virulence factors.

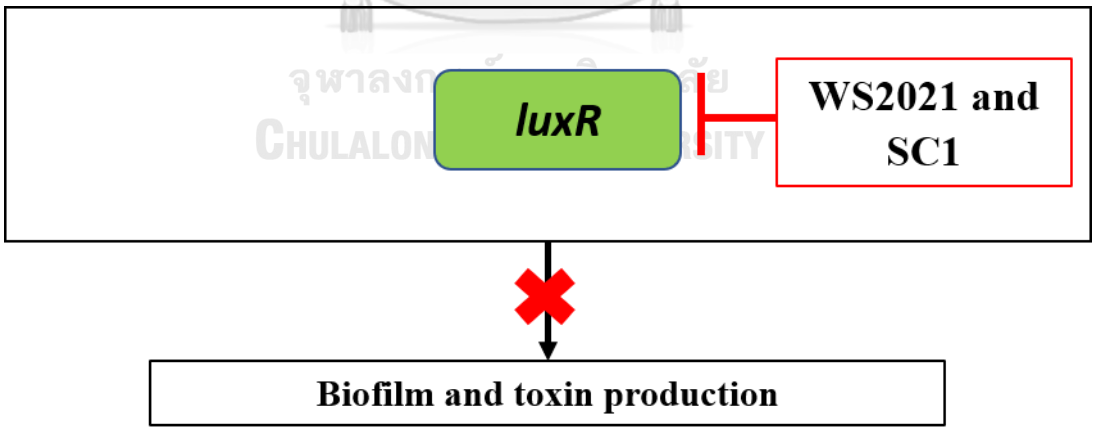

Figure 12. The schematic diagram showing the interplay of the *luxR* gene with the possible mechanisms of action from CFS and cell of WS2021 and SC1 isolates.

2. Conclusion and suggestion

Overall, this study suggests that co-incubation of *F. columnare* with either cells or CFSs of WS2021 or SC1 has detrimental effect on its biofilm formation. This inhibition might be partly associated with the iron competition between LAB isolates and *F. columnare* cells, since the LAB species is previously reported to secrete siderophore to acquire iron. In addition, the downregulation of three iron acquisition genes (*alcB*, *rhbC*, and *tonB*), the three T9SS components (*porV*, *gldL*, and *sprA*), and the quorum sensing gene master regulator (*luxR*) may also contribute to the thin *F. columnare* biofilm formed after being incubated with the LAB cells and cell products.

3. Advantages of the study

This study will suggest the efficacy of lactic acid bacteria (LAB) cells/or their CFSs in *F. columnare* biofilm inhibition and biofilm-associated genes regulation. The knowledge obtained from this study may be applied to control *F. columnare* infection in fish in the future.

REFERENCES

Abbas A, Adams C, Scully N, Glennon J and O'Gara F 2007. A role for TonB1 in biofilm formation and quorum sensing in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 274(2): 269-278.

Altinok I and Grizzle JM 2001. Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and freshwater stenohaline fish. J. Fish Dis. 24(6): 361-367.

An YH, Dickinson RB and Doyle RJ 2000. Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections. In: Handbook of bacterial adhesion. Springer. 1-27.

Anderson JIW and Conroy DA 1969. The Pathogenic Myxobacteria with Special Reference to Fish Diseases. J. Appl. Bacteriol. 32(1): 30-39.

Banin E, Vasil ML and Greenberg EP 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A. 102(31): 11076-11081.

Bebak J, Matthews M and Shoemaker C 2009. Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*. Vaccine. 27(32): 4297-4301.

Beck BH, Li C, Farmer BD, Barnett LM, Lange MD and Peatman E 2016. A comparison of high- and low-virulence *Flavobacterium columnare* strains reveals differences in iron acquisition components and responses to iron restriction. J. Fish Dis. 39(3): 259-268.

Bernardet J-F and Bowman JP 2006. The genus *Flavobacterium*. Prokaryotes. 7: 481-531.

Bernardet J-F and Grimont PAD 1989. Deoxyribonucleic Acid Relatedness and Phenotypic Characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986. Int. J. Syst. Evol. Microbiol. 39(3): 346-354.

Bernardet J-F, Segers P, Vancanneyt M, Berthe F, Kersters K and Vandamme P 1996.

Cutting a Gordian Knot: Emended Classification and Description of the Genus *Flavobacterium*, Emended Description of the Family Flavobacteriaceae, and Proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int. J. Syst. Evol. Microbiol. 46(1): 128-148.

Björklund H, Bondestam J and Bylund G 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture. 86(4): 359-367.

Boutin S, Bernatchez L, Audet C and Derôme N 2012. Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*. Vet. Microbiol. 155(2): 355-361.

Branda SS, Vik S, Friedman L and Kolter R 2005. Biofilms: the matrix revisited. Trends Microbiol. 13(1): 20-26.

Buschmann AH, Tomova A, López A, Maldonado MA, Henríquez LA, Ivanova L, Moy F, Godfrey HP and Cabello FC 2012. Salmon Aquaculture and Antimicrobial Resistance in the Marine Environment. PLOS ONE. 7(8): e42724.

Cai W, De La Fuente L and Arias CR 2019. Transcriptome analysis of the fish pathogen *Flavobacterium columnare* in biofilm suggests calcium role in pathogenesis. BMC Microbiol. 19(1): 151.

Cao JG and Meighen EA 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J. Biol. Chem. 264(36): 21670-21676.

Carvalho FM, Teixeira-Santos R, Mergulhão FJM and Gomes LC 2020. The Use of Probiotics to Fight Biofilms in Medical Devices: A Systematic Review and Meta-Analysis. Microorganisms. 9(1).

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL and Hughson FM 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature. 415(6871): 545-549.

Costerton JW, Stewart PS and Greenberg EP 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science. 284(5418): 1318.

Croxatto A, Chalker VJ, Lauritz J, Jass J, Hardman A, Williams P, Cámara M and Milton DL 2002. VanT, a Homologue of Vibrio harveyi LuxR, Regulates Serine, Metalloprotease, Pigment, and Biofilm Production in Vibrio anguillarum.

J. Bacteriol. 184(6): 1617-1629.

Darwish AM, Bebak JA and Schrader KK 2012. Assessment of Aquaflor®, copper sulphate and potassium permanganate for control of *Aeromonas hydrophila* and *Flavobacterium columnare* infection in sunshine bass, *Morone chrysops* female × *Morone saxatilis* male. J. Fish Dis. 35(9): 637-647.

Davis H 1922. A New Bacterial Disease of Fresh-water Fishes. In: Bulletin of the US Bureau of Fisheries. 261-280.

Declercq AM, Boyen F, Van den Broeck W, Bossier P, Karsi A, Haesebrouck F and Decostere A 2013. Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. J. Fish Dis. 36(1): 45-55.

Declercq AM, Cai W, Naranjo E, Thongda W, Eeckhaut V, Bauwens E, Arias C, De La Fuente L, Beck BH, Lange MD, Peatman E, Haesebrouck F, Aerts J and Decostere A 2019. Evidence that the stress hormone cortisol regulates biofilm formation differently among *Flavobacterium columnare* isolates. Vet. Res. 50(1): 24.

Declercq AM, Chiers K, Haesebrouck F, Van den Broeck W, Dewulf J, Cornelissen M and Decostere A 2015. Gill infection model for columnaris disease in common carp and rainbow trout. J. Aquat. Anim. Health. 27(1): 1-11.

Decostere A, Haesebrouck F, Turnbull J and Charlier G 1999. Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. J. Fish Dis. 22(1): 1-11.

Dong HT, LaFrentz B, Pirarat N and Rodkhum C 2015a. Phenotypic characterization and genetic diversity of Flavobacterium columnare isolated from red tilapia, Oreochromis sp., in Thailand. J. Fish Dis. 38(10): 901-913.

Dong HT, Nguyen VV, Phiwsaiya K, Gangnonngiw W, Withyachumnarnkul B, Rodkhum C and Senapin S 2015b. Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand. Aquaculture. 448: 142-150.

Dong HT, Senapin S, LaFrentz B and Rodkhum C 2016. Virulence assay of rhizoid and non-rhizoid morphotypes of *Flavobacterium columnare* in red tilapia, *Oreochromis* sp., fry. J. Fish Dis. 39(6): 649-655.

Evans DJ, Allison DG, Brown MR and Gilbert P 1991. Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. J. Antimicrob. Chemother. 27(2): 177-184.

Evans LV 2000. Biofilms: recent advances in their study and control. In: CRC press.

Evenhuis JP and LaFrentz BR 2016. Virulence of *Flavobacterium columnare* genomovars in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ. 120(3): 217-224.

Ferreira CS, Nunes BA, Henriques-Almeida JM and Guilhermino L 2007. Acute toxicity of oxytetracycline and florfenicol to the microalgae *Tetraselmis chuii* and to the crustacean *Artemia parthenogenetica*. Ecotoxicol. Environ. Saf. 67(3): 452-458.

Fujihara MP and Nakatani RE 1971. Antibody Production and Immune Responses of Rainbow Trout and Coho Salmon to *Chondrococcus columnaris*. Journal of the Fisheries Research Board of Canada. 28(9): 1253-1258.

Garnjobst L 1945. *Cytophaga columnaris* (Davis) in pure culture: a myxobacterium pathogenic to fish. J. Bacteriol. 49(2): 113.

Gaunt PS, Gao D, Sun F and Endris R 2010. Efficacy of florfenicol for control of mortality caused by *Flavobacterium columnare* infection in channel catfish. J. Aquat. Anim. Health. 22(2): 115-122.

Giardina PC, Foster LA, Toth SI, Roe BA and Dyer DW 1997. Analysis of the alcABC operon encoding alcaligin biosynthesis enzymes in *Bordetella bronchiseptica*. Gene. 194(1): 19-24.

Hai NV 2015. Research findings from the use of probiotics in tilapia aquaculture: A review. Fish Shellfish Immunol. 45(2): 592-597.

Hall T 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser.:95-98.

Harmsen M, Lappann M, Knøchel S and Molin S 2010. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl. Environ. Microbiol. 76(7): 2271-2279.

Harrison F, Paul J, Massey RC and Buckling A 2008. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. The ISME Journal. 2(1): 49-55.

Higgins DA, Pomianek ME, Kraml CM, Taylor RK, Semmelhack MF and Bassler BL 2007. The major *Vibrio cholerae* autoinducer and its role in virulence factor production. Nature. 450(7171): 883-886.

Ishida H, Ishida Y, Kurosaka Y, Otani T, Sato K and Kobayashi H 1998. In vitro and in vivo activities of levofloxacin against biofilm-producing *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 42(7): 1641-1645.

Kerddee P, Dong HT, Chokmangmeepisarn P, Rodkhum C, Srisapoome P, Areechon N, Del-Pozo J and Kayansamruaj P 2020. Simultaneous detection of scale drop disease virus and *Flavobacterium columnare* from diseased freshwater-reared barramundi *Lates calcarifer*. Dis Aquat Organ. 140: 119-128.

Kessel JCv, Rutherford ST, Shao Y, Utria AF and Bassler BL 2013. Individual and Combined Roles of the Master Regulators AphA and LuxR in Control of the Vibrio harveyi Quorum-Sensing Regulon. J. Bacteriol. 195(3): 436-443.

Kitiyodom S, Trullàs C, Rodkhum C, Thompson KD, Katagiri T, Temisak S, Namdee K, Yata T and Pirarat N 2021. Modulation of the mucosal immune response of red tilapia (*Oreochromis* sp.) against columnaris disease using a biomimetic-mucoadhesive nanovaccine. Fish Shellfish Immunol. 112: 81-91.

Klesius PH, Pridgeon JW and Aksoy M 2010. Chemotactic factors of *Flavobacterium columnare* to skin mucus of healthy channel catfish (*Ictalurus punctatus*). FEMS Microbiol. Lett. 310(2): 145-151.

Konishi H, Fujiya M, Tanaka H, Ueno N, Moriichi K, Sasajima J, Ikuta K, Akutsu H, Tanabe H and Kohgo Y 2016. Probiotic-derived ferrichrome inhibits colon cancer progression via JNK-mediated apoptosis. Nature Communications. 7(1): 12365.

Kumar S, Stecher G, Li M, Knyaz C and Tamura K 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution. 35(6): 1547.

LaFrentz BR, García JC, Waldbieser GC, Evenhuis JP, Loch TP, Liles MR, Wong FS and Chang SF 2018. Identification of Four Distinct Phylogenetic Groups in Flavobacterium columnare With Fish Host Associations. Front. Microbiol. 9.

LaFrentz BR, Králová S, Burbick CR, Alexander TL, Phillips CW, Griffin MJ, Waldbieser GC, García JC, de Alexandre Sebastião F, Soto E, Loch TP, Liles MR and Snekvik KR

2022. The fish pathogen *Flavobacterium columnare* represents four distinct species: *Flavobacterium columnare*, *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov. and *Flavobacterium oreochromis* sp. nov., and emended description of *Flavobacterium columnare*. Syst. Appl. Microbiol. 45(2): 126293.

LaFrentz BR, Waldbieser GC, Welch TJ and Shoemaker CA 2014. Intragenomic heterogeneity in the 16S rRNA genes of *Flavobacterium columnare* and standard protocol for genomovar assignment. J. Fish Dis. 37(7): 657-669.

Lange MD, Abernathy J and Farmer BD 2019. Evaluation of a Recombinant *Flavobacterium columnare* DnaK Protein Vaccine as a Means of Protection Against Columnaris Disease in Channel Catfish (*Ictalurus punctatus*). Front. Immunol. 10(1175).

Lange MD, Farmer BD and Abernathy J 2018. Catfish mucus alters the *Flavobacterium columnare* transcriptome. FEMS Microbiol. Lett. 365(22).

Lange MD, Farmer BD and Abernathy J 2020. Vertebrate mucus stimulates biofilm development and upregulates iron acquisition genes in *Flavobacterium columnare*. J. Fish Dis. 43(1): 101-110.

Li N, Zhu Y, LaFrentz BR, Evenhuis JP, Hunnicutt DW, Conrad RA, Barbier P, Gullstrand CW, Roets JE, Powers JL, Kulkarni SS, Erbes DH, García JC, Nie P and McBride MJ 2017. The Type IX Secretion System Is Required for Virulence of the Fish Pathogen *Flavobacterium columnare*. Appl. Environ. Microbiol. 83(23).

Lisiecki P, Wysocki P and Mikucki J 2000. Occurrence of Siderophores in Enterococci. Zentralblatt für Bakteriologie. 289(8): 807-815.

Livak KJ and Schmittgen TD 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 25(4): 402-408.

Lulijwa R, Rupia EJ and Alfaro AC 2020. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. Rev. Aquacult. 12(2): 640-663.

Lynch D, O'Brien J, Welch T, Clarke P, ÓCuív P, Crosa JH and O'Connell M 2001. Genetic Organization of the Region Encoding Regulation, Biosynthesis, and Transport of Rhizobactin 1021, a Siderophore Produced by *Sinorhizobium meliloti*. J.

Bacteriol. 183(8): 2576-2585.

McBride MJ and Zhu Y 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum bacteroidetes. J. Bacteriol. 195(2): 270-278.

Melo TA, Dos Santos TF, de Almeida ME, Junior LAGF, Andrade EF, Rezende RP, Marques LM and Romano CC 2016. Inhibition of *Staphylococcus aureus* biofilm by *Lactobacillus* isolated from fine cocoa. BMC Microbiol. 16(1): 250-250.

Miller MB and Bassler BL 2001. Quorum Sensing in Bacteria. Annu. Rev. Microbiol. 55(1): 165-199.

Montoya Vallejo C, Flórez Restrepo MA, Guzmán Duque FL and Quintero Díaz JC 2021. Production, characterization and kinetic model of biosurfactant produced by lactic acid bacteria. Electron. J. Biotechnol. 53: 14-22.

Noinaj N, Guillier M, Barnard TJ and Buchanan SK 2010. TonB-dependent transporters: regulation, structure, and function. Annu. Rev. Microbiol. 64: 43-60.

O'Toole GA 2011. Microtiter Dish Biofilm Formation Assay. JoVE. (47): e2437.

Olivares-Fuster O, Bullard SA, McElwain A, Llosa MJ and Arias CR 2011. Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge. Dis Aquat Organ. 96(3): 221-227.

Olivares-Fuster O, Shoemaker CA, Klesius PH and Arias CR 2007. Molecular typing of isolates of the fish pathogen, *Flavobacterium columnare*, by single-strand conformation polymorphism analysis. FEMS Microbiol. Lett. 269(1): 63-69.

Oliveira F, Lima T, Correia A, Silva AM, Soares C, Morais S, Weißelberg S, Vilanova M, Rohde H and Cerca N 2021. Siderophore-Mediated Iron Acquisition Plays a Critical Role in Biofilm Formation and Survival of *Staphylococcus epidermidis* Within the Host. Frontiers in Medicine. 8(2550).

Ordal EJ and Rucker RR 1944. Pathogenic myxobacteria. Proceedings of the Society for Experimental Biology and Medicine. 56(1): 15-18.

Penttinen R, Hoikkala V and Sundberg L-R 2018. Gliding Motility and Expression of Motility-Related Genes in Spreading and Non-spreading Colonies of *Flavobacterium columnare*. Front. Microbiol. 9: 525-525.

Prior BS 2020. Exploration of the Anti-biofilm Effects of Fish Antimicrobial Peptides Against Common Aquaculture Pathogens. Auburn University, Auburn, Alabama.

Rossoni RD, Velloso MDS, de Barros PP, de Alvarenga JA, Santos JDD, Santos Prado A, Ribeiro FC, Anbinder AL and Junqueira JC 2018. Inhibitory effect of probiotic *Lactobacillus* supernatants from the oral cavity on *Streptococcus mutans* biofilms. Microb. Pathog. 123: 361-367.

Ruwandeepika HAD, Defoirdt T, Bhowmick PP, Karunasagar I, Karunasagar I and Bossier P 2011. In vitro and in vivo expression of virulence genes in Vibrio isolates belonging to the Harveyi clade in relation to their virulence towards gnotobiotic brine shrimp (Artemia franciscana). Environ. Microbiol. 13(2): 506-517.

Sambrook J, Fritsch EF and Maniatis T 1989. Molecular cloning: a laboratory manual. In: Cold spring harbor laboratory press.

Saputra F, Shiu Y-L, Chen Y-C, Puspitasari AW, Danata RH, Liu C-H and Hu S-Y 2016. Dietary supplementation with xylanase-expressing *B. amyloliquefaciens* R8 improves growth performance and enhances immunity against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol. 58: 397-405.

Seghouani H, Garcia-Rangel C-E, Füller J, Gauthier J and Derome N 2017. Walleye Autochthonous Bacteria as Promising Probiotic Candidates against *Flavobacterium columnare*. Front. Microbiol. 8(1349).

Shrivastava A, Johnston JJ, van Baaren JM and McBride MJ 2013. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell surface gliding motility adhesins SprB and RemA. J. Bacteriol. 195(14): 3201-3212.

Suomalainen L-R, Tiirola M and Valtonen ET 2006. Chondroitin AC lyase activity is related to virulence of fish pathogenic *Flavobacterium columnare*. J. Fish Dis. 29(12): 757-763.

Suomalainen LR, Tiirola M and Valtonen E 2005. Influence of rearing conditions on *Flavobacterium columnare* infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 28(5): 271-277.

Tan HY, Chen S-W and Hu S-Y 2019. Improvements in the growth performance, immunity, disease resistance, and gut microbiota by the probiotic

Rummeliibacillus stabekisii in Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol. 92: 265-275.

Thompson JD, Higgins DG and Gibson TJ 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22): 4673-4680.

Tomova A, Ivanova L, Buschmann AH, Rioseco ML, Kalsi RK, Godfrey HP and Cabello FC 2015. Antimicrobial resistance genes in marine bacteria and human uropathogenic *Escherichia coli* from a region of intensive aquaculture. Environ. Microbiol. Rep. 7(5): 803-809.

Triyanto and Wakabayashi H 1999. Genotypic Diversity of Strains of *Flavobacterium columnare* from Diseased Fishes. Fish Pathol. 34(2): 65-71.

Wasfi R, Abd El-Rahman OA, Zafer MM and Ashour HM 2018. Probiotic *Lactobacillus* sp. inhibit growth, biofilm formation and gene expression of caries-inducing *Streptococcus mutans*. J. Cell. Mol. Med. 22(3): 1972-1983.

Woo J and Ahn J 2013. Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. Lett. Appl. Microbiol. 56(4): 307-313.

Yan X, Gu S, Cui X, Shi Y, Wen S, Chen H and Ge J 2019. Antimicrobial, anti-adhesive and anti-biofilm potential of biosurfactants isolated from Pediococcus acidilactici and Lactobacillus plantarum against Staphylococcus aureus CMCC26003. Microb. Pathog. 127: 12-20.

Yang KM, Kim J-S, Kim H-S, Kim Y-Y, Oh J-K, Jung H-W, Park D-S and Bae K-H 2021. *Lactobacillus reuteri* AN417 cell-free culture supernatant as a novel antibacterial agent targeting oral pathogenic bacteria. Scientific Reports. 11(1): 1631.

Yatip P, Nitin Chandra Teja D, Flegel TW and Soowannayan C 2018. Extract from the fermented soybean product Natto inhibits *Vibrio* biofilm formation and reduces shrimp mortality from *Vibrio harveyi* infection. Fish Shellfish Immunol. 72: 348-355.

Zhu W, Yang G, Zhang Y, Yuan J and An L 2012. Generation of Biotechnology-Derived

Flavobacterium columnare Ghosts by PhiX174 Gene *E*-Mediated Inactivation and the Potential as Vaccine Candidates against Infection in Grass Carp. J. Biomed. Biotechnol. 2012: 760730.

VITA

NAME	FARRAS DAFFA IMTIYAZ
DATE OF BIRTH	21 November 1997
PLACE OF BIRTH	Bandung, Indonesia
INSTITUTIONS ATTENDED	Diponegoro University, Indonesia
HOME ADDRESS	Jalan Sekemerak No 17A, Bandung, Indonesia