IN VITRO INHIBITORY EFFECTS OF TURMERIC EXTRACTS AGAINST BIOFILM FORMATION AND VIRULENCE OF *FLAVOBACTERIUM OREOCHROMIS*

Mr. Sirawich Ngernson

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2022

ผลการยับยั้งในหลอดทดลองของสารสกัดขมิ้นต่อการสร้างไบโอฟิล์มและความรุนแรงของเชื้อฟลาโว แบคทีเรียม โอริโอโครมิส

นายสิรวิชญ์ เงินสอน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	*IN VITRO* INHIBITORY EFFECTS OF TURMERIC EXTRACTS AGAINST BIOFILM FORMATION AND VIRULENCE OF *FLAVOBACTERIUM OREOCHROMIS*
By	Mr. Sirawich Ngernson
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

(Assistant Professor PATHARAPOL PIAMSOMBOON, D.V.M., Ph.D.)

สิรวิชญ์ เงินสอน : ผลการยับยั้งในหลอดทดลองของสารสกัดขมิ้นต่อการสร้างไบโอฟิล์มและความรุนแรงของเชื้อฟลาโวแบคทีเรียม โอริโอโครมิส. (*IN VITRO* INHIBITORY EFFECTS OF TURMERIC EXTRACTS AGAINST BIOFILM FORMATION AND VIRULENCE OF *FLAVOBACTERIUM OREOCHROMIS*) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : ชุมพร สุวรรณยาน

Flavobacterium oreochromis เป็นแบคทีเรียฉวยโอกาศที่เป็นสาเหตุของโรคคอลัมนาริสในปลาน้ำจืด การเกิดโรคคอลัมนาริสนั้นแบคทีเรียชนิดนี้จะลงเกาะและสร้างไบโอฟิล์มบนผิวหนังของปลา แบคทีเรียในไบโอฟิล์ม จะผลิตและปล่อยเอนไซม์ที่ทำลายเนื้อเยื่อเกี่ยวพันและเนื้อเยื่อบริเวณอื่นๆ ซึ่งส่งผลให้เกิดแผลบนผิวปลาที่ติดเชื้อ การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบประสิทธิภาพของสารสกัดขมิ้นที่สกัดโดยใช้ตัวทำละลายสามชนิดคือ เฮกเซน เอทานอล และเมทานอล ในการการยับยั้งการสร้างไบโอฟิล์มและการแสดงออกของยีนที่เกี่ยวข้องในการสร้างไบโอฟิล์มของเชื้อ *F. oreochromis* โดยนำสารสกัดจากขมิ้นจากทั้งสามตัวทำละลายมาทดสอบประสิทธิภาพในการยับยั้งการเจริญเติบโตและการสร้างไบโอฟิล์มของเชื้อแบคทีเรีย *F. oreochromis* จากการทดสอบพบว่าสารสกัดขมิ้นโดยตัวทำละลายเฮกเซนมีประสิทธิภาพมากสุดในการยับยั้งการเจริญเติบโตและการสร้างไบโอฟิล์มของเชื้อ *F. oreochromis* ทั้ง 17 isolate จากนั้นก็ทำการทดสอบเพื่อหาค่า MIC และ MBIC ของสารสกัดขมิ้นที่ใช่เฮกเซนเป็นตัวทำละลาย ก่อนจะเลือกความเข้มข้นที่ยับยั้งการสร้างไบโอฟิล์มแต่ที่ไม่ส่งผลกระทบต่อการเจริญเติบของเชื้อแบคทีเรีย (1 ไมโครกรัม/มิลลิลิตร) ไปทดสอบเพื่อดูการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างไบโอฟิล์มของเชื้อ *F. oreochromis* สายพันธุ์ที่ 15 ซึ่งเป็นสายพันธุ์ที่สร้างไบโอฟิล์มได้หนาและมีความรุนแรงในการก่อโรคต่อปลานิล โดยการศึกษาการแสดงออกของยีนใช้วิธี quantitative real-time PCR ซึ่งพบว่าการแสดงออกยีนที่เกี่ยวข้องกับ iron acquisition, type IX secretion, และ quorum sensing (luxR) systems ในไบโอฟิล์มของเชื้อ *F. oreochromis* ที่ได้รับสารสกัดมีการแสดงออกลดลงทั้งสามเวลา (24 ชั่วโมง, 48 ชั่วโมง, 78 ชั่วโมง) หลังการบ่ม โดยสรุปสารสกัดขมิ้นโดยตัวทำละลายเฮกเซนมีประสิทธิภาพในการยับยั้งการสร้างไบโอฟิล์มของเชื้อ *F. oreochromis* โดยการไปลดการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างไบโอฟิล์มเช่น iron acquisition (alcB, rhbC, sido, tonB), type IX secretion system (gldL, sprA, porV), และ luxR

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6278014831 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY

KEYWORD: Biofilm; Biofilm inhibitors; Biofilm associated genes; Flavobacterium oreochromis; Turmeric extract

Sirawich Ngernson : *IN VITRO* INHIBITORY EFFECTS OF TURMERIC EXTRACTS AGAINST BIOFILM FORMATION AND VIRULENCE OF *FLAVOBACTERIUM OREOCHROMIS*. Advisor: Assoc. Prof. CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P Co-advisor: Chumporn Soowannayan, Ph.D.

Flavobacterium oreochromis is an opportunistic bacteria and causative agent of columnaris disease that affects freshwater fish worldwide. To initiate infection, the bacteria are required to attach and form biofilm on the fish's skin. The bacteria cells in the biofilm then produce toxins that can degrade fish connective tissue, resulting in lesions and ulcers. This study aimed to evaluate the efficacy of turmeric (*Curcuma longa L.*) extract by three organic solvents (hexane, ethanol, and methanol) in biofilm inhibition and modulation of biofilm-associated genes of *F. oreochromis*. Turmeric extracts by three different organic solvents were tested for their ability to inhibit the growth and biofilm formations of *F. oreochromis*. The results showed that the hexane extract of turmeric was the most potent inhibitor of the growth and biofilm formation of *F. oreochromis*. To determine the effect of hexane extract of turmeric on biofilm-associate genes in *F. oreochromis* isolate no. 15, the qPCR method was used. To do this minimal inhibition concentration (MIC) and minimal biofilm inhibition concentration (MBIC) of this extract were determined for this bacterial isolate, and the concentration of extract that inhibited the bacterial biofilm but not the growth was used (1 µg/ml). The obtained results showed that turmeric extract strongly downregulated the expression of genes in the iron acquisition, type 9 secretion, and quorum sensing (*luxR*) systems in *F. oreochromis* isolate number 15 at all three-time points (24h, 48h, 72h) studied. In summary, turmeric extract inhibited biofilm formation of *F. Oreochromis* through the downregulations of genes in the iron acquisition (*alcB, rhbC, sido, tonB*), type 9 secretion (*gldL, sprA. porV*) and quorum sensing (luxR) systems.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

This research work was financially supported by the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej and The Agricultural Research Development Agency (ARDA).

In this opportunity, I would like to thank Associate Professor Dr Channarong Rodkum as my principal advisor for every support and guidance during the time that I study in the VST program. I would like to give special thanks to Dr. Chumporn Soowannayan as my co-advisor that always have been supported, encouraged, and motivated me in this journey. Also, I am very grateful for his patience with my writing skills.

I would like to thank all of my thesis committee members Assistant Professor Dr Ha Thanh Dong, Associate Professor Dr Nopdon Pirarat, and Assistant Professor Dr Patharapol Piamsomboon. Their comment, advice, and suggestion have built my own perspective about being a better student.

I would like to thank to Farras, Pent, P'Name, colleagues, and my friend especially in room 435 at CENTEX shrimp, Faculty of Science of Mahidol University for kindly support, cheerfulness, and help. Also, I would like to thank my colleagues in the Center of Excellent in Fish Infectious Disease (CEFID), Veterinary Science and Technology (VST) program of Chulalongkorn University, and all the staff for their assistance.

Last but not least, I would like to thank my family that always supported me during this Master's degree journey.

Sirawich Ngernson

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1. Importance and Rationale

Tilapia culture is one of the most important finfish species to the economies of many Asian countries including Thailand. Because the fish is a good protein source and because of the ease of its production, tilapia culture has gained a lot of attention from many farmers and tilapia farming has an established industry in many countries (El-Sayed, 2019). Many farmers adopted or converted their farms into intensive farms by increasing stock density and other farm management practices to increase tilapia farm productivity (Chitmanat et al., 2016; El-Sayed, 2019). However, high stock density coupled with poor management usually brings in multiple problems, such as low water quality due to waste accumulation from uneaten feed and feces, increased fish stress in high-density ponds, and disease outbreaks due to the ease of transmission between the fish living in close proximity (Suomalainen et al., 2005b).

Among the most serious bacterial pathogens in intensive tilapia farming systems, *Flavobacterium columnare*(*oreochromis*), the causative agent of Columnaris disease, is the most prevalent, especially during the juvenile stage (Decostere and Haesebrouck, 1999; Decostere, 2002; Declercq et al., 2013). Columnaris disease causes mass mortality in infected fish, which could lead to tremendous economic losses. Transmission of the bacteria does not require direct contact between infected fish (fish-fish contact), it can spread via the water column, and dead fish is considered an essential source for the outbreak (Declercq et al., 2013).

Columnaris disease causes several external lesions such as gill, fin, or skin ulcers . In Thailand, Columnaris disease outbreaks have been reported during periods of sudden change of temperature or fish experiences stress which occur in both river-based cages and ponds (Chitmanat et al., 2016). Farmers normally apply antibiotics to control the outbreak (Chitmanat et al., 2016). However, inappropriate use of antibiotics results in several problems such as antibiotic residual in fish products and in the environment which could lead to or enhance the development of antibiotic

resistant bacteria (Alderman and Hastings, 1998). Antibiotic resistant bacteria are a serious concern in both human as well as environmental health. To the aquaculture industry, antibiotic- resistant bacteria mean more expenses, time, and labor needed to prevent or treat.

Apart from the development of antibiotic-resistant bacteria associated with antibiotic use, biofilm, a preferred mode of living for most bacteria can also promote bacterial virulence (Davies, 2003; Hossain et al., 2017). The majority of bacterial pathogens form biofilms to initiate infection, *F. oreochromis*, included (Cai et al., 2013; Cai and Arias, 2017; Lange et al., 2020a). Biofilm is a complex community of microbes living together under a multi-layer of extracellular polymeric substances (EPS). EPS are essential for the bacterial biofilm and bacterial cells living within it by supporting the biofilm structure, adhesion, nutrient source, retention of water, and exchange of genetic materials (Flemming and Wingender, 2010). EPS are barriers that provide bacterial protection from specific and nonspecific host immune factors, hazardous environments, and antibiotics (Flemming and Wingender, 2010; Ghannoum et al., 2020). Moreover, bacterial cells in the biofilm release toxins that can degrade host tissues, such as Chondroitin AC lyase, Proteases, and collagenase (Dalsgaard, 1993; Bernardet and Bowman, 2006b).

Because of the emergence of antibiotic-resistant bacteria, conventional approaches such as antibiotic usage, physical cleaning, and chemical treatments are no longer working (Zhu, 2020). A new strategy is necessary to prevent bacterial diseases and eradicate bacterial biofilms. Plant products have gained a lot of attention in the past ten years because they possess a large variety of secondary metabolites with different bioactivities that can be applied in the aquaculture industries, such as antibacterial activity (Reverter et al., 2014; Zhu, 2020). Turmeric (*Curcuma longa L.*) extracts have been reported to possess antibacterial activities against gram-positive and gram-negative bacteria (Sharma et al., 2005; Gul and Bakht, 2015). For example, alkaloids and flavonoids from *Curcuma* species have been shown to inhibit the growth and biofilm formed by *Bacillus subtilis*, *staphylococcus epidermidis*, and *staphylococcus aureus* (Jain and Parihar, 2018a). For many antibiotics or extracts with antibiotic properties, many bacteria can develop resistant

to them easily if they are not properly used (Alderman and Hastings, 1998; Mazel and Davies, 1999). So, compounds or molecules that interfere with the formation of biofilm and virulence factors without killing the bacteria or affecting their growth are desirable (Soowannayan et al., 2019b). The mechanism behind biofilm formation is the quorum-sensing system, which is bacterial intercellular communication that involves the detection of small signaling molecules called autoinducers (Lazdunski et al., 2004). Quorum sensing regulation genes control many bacterial group behaviors and activities such as bioluminescence, motility, virulent factors expression, drug-resistant, and biofilm formation. Natural products or plant extracts with their abundant secondary metabolites with diverse bioactivities present a great potential to be used as quorum quenching or quorum sensing inhibitor candidates. For instance, flavonoids and curcumin produced by *Curcuma* species have been shown to inhibit biofilm formation and virulence of *Acinetobacter baumannii*, the bacteria that causes wound infection, bloodstream infection, and urinary tract infection in human (Raorane et al., 2019). Additionally, ethanol extract of *Piper betle L.* has been shown to effectively suppress quorum sensing regulation genes resulting in less bioluminescence production and biofilm formation of *Vibrio harveyi* that cause luminescent disease in shrimp (*Penaeus vannamei*) (Guzman et al., 2022a). The ethanolic extract of *P. betle L.* has also been shown to protect the shrimp from the bacterial infection when supplemented to the shrimp feed, from the same study.

Since quorum sensing (QS) has been shown to influence or regulate *F. oreochromis* infection process, which involves the bacteria gliding motility and biofilm formation, a crucial part of initiating columnaris disease and toxin and protease productions that lead to ulcer/pathological lesions (Declercq et al., 2013; Li et al., 2017; Thunes et al., 2022), therefore, this QS can potentially be used as a target for the disease prevention and treatment. Therefore, this study aims to determine the efficacy of turmeric extracts as *F. oreochromis* quorum sensing inhibitors and their abilities to inhibit virulent gene expressions and biofilm formation.

2. Objectives of the study

1. To determine the efficacy of turmeric extracts against biofilm formation of *F. oreochromis*.

2. To determine expression levels of virulence-related genes in *F. oreochromis* after exposure to turmeric extracts.

3. Hypothesis

Turmeric extracts can reduce biofilm formation of *F. oreochromis*.

CHAPTER II

LITERATURE REVIEW

1. General introduction to *Flavobacterium columnare*

Flavobacterium columnare has recently been reclassified based on their amino acid profiles and a multigene sequencing method into four species, including *F. columnare* sp., *F. covae* sp. nov., *F. davisii* sp. nov., and *F. reochromis* sp. nov. (LaFrentz et al., 2022). The bacteria have been known by many different names after it was first discovered by Davis in 1922 during an outbreak of the disease later called columnaris disease (Davis, 1922). The bacteria was first named Bacillus columnaris based on its column-like rod shape (Davis, 1922). Later in 1944, the bacterium was found to produce a fruiting body and microcyst (resting cells) on infected tissue of sockeye salmon (*Onchorhynchus nerka*) upon wet mount examination and was re-named *Chondrococcus columnaris* (Ordal and Rucker, 1944). However, the microcyst was found absent in a study of infected by Garnjobst (Garnjobst, 1945); thus, the bacteria was re-named *Cytophaga columnaris*. In the year 1989, Bernardet and Grimont performed an experiment to determine the relatedness of the bacteria based on their 16 sRNA gene sequences and their phenotypic characters; the results showed that the bacterium with rhizoid colony that can adhere to Anecker agar medium were found to be similar to *Flavobacterium* spp, however, the G+C content of *C. columnaris* was excluded from *Flavobacterium* spp. Thus, the name of the bacterium was changed to *Flexibacter columnaris* (Bernardet and Grimont, 1989). In the late 19TH century, the name of the bacterium was altered again to *Flavobacterium columnare* based on their DNA-rRNA hybridizations and fatty acid profiles (Bernardet et al., 1996). The authors described the main characteristic of *F. columnare* as a gram-negative rod shape bacteria with a yellow colony commonly found in a freshwater environment that can decompose polysaccharides with a G+C content range of 32 to 37% (Bernardet et al., 1996). *F. columnare* was Later categorized into five genomovars (I, II, II-B, I/II, and III) based on the restriction fragment length polymorphism (RFLP) assay of their genomic DNA (LaFrentz et al., 2014). Nevertheless, a bacterium and host association study provided information

which differentiated genomovars into four genetic groups (LaFrentz et al., 2018). Genetic group 1 is normally found in cold water, which mainly affects Salmonidae, Cyprinidae, and other cold-water fish species. Genetic groups 2 and 3 mainly affect Ictaluridae and other warm-water fish species. Genetic group 4 mainly affects tilapia (LaFrentz et al., 2018). However, the latest study revealed that all four genetic groups showed similarities in their physiological and biochemical characteristics (LaFrentz et al., 2022). The authors reclassified the bacterium by characterizing the fatty acids profile using Matrix-Assisted Laser Desorption/Ionization Time- of-Flight (MALDI-TOF) and calculated the average of nucleotide identity / digital DNA-DNA hybridization (ANI/GGC). Finally, the authors were able to differentiate between genetic groups and reclassified genetic groups 1, 2, 3, and 4 into distinct species named *F. columnare* sp., *F. covae* sp. nov., *F. davisii* sp. nov., and *F. reochromis* sp. nov. respectively (LaFrentz et al., 2022).

As with other genetic groups of the former *F. columnare*, *F. oreochromis* (*F. columnare* genetic group 4) is a Gram-negative rod shape bacteria and is the causative agent of columnaris disease in cichlidae group (mainly tilapia) (LaFrentz et al., 2018; LaFrentz et al., 2022). The bacterium produces yellow pigments on rhizoid or non-rhizoid colonies. In addition, the bacterium shows gliding motility on agar medium and produces polysaccharide degradation enzyme. *F. oreochromis* typically affects tilapia and is found in warm water (28 ℃ to 30 ℃). Columnaris disease causes mass mortality in infected fish, which could lead to tremendous economic losses. To initiate the infection, the bacteria are required to colonize and form a biofilm on the susceptible host. To form a biofilm, there are three steps involved, including attraction between the bacterial cells and the host mucus, adhesion of the bacterial cells and host surface to form a microcolony, and aggregation and growth of the bacterial cells to create a larger colony and form biofilm (Declercq et al., 2013).

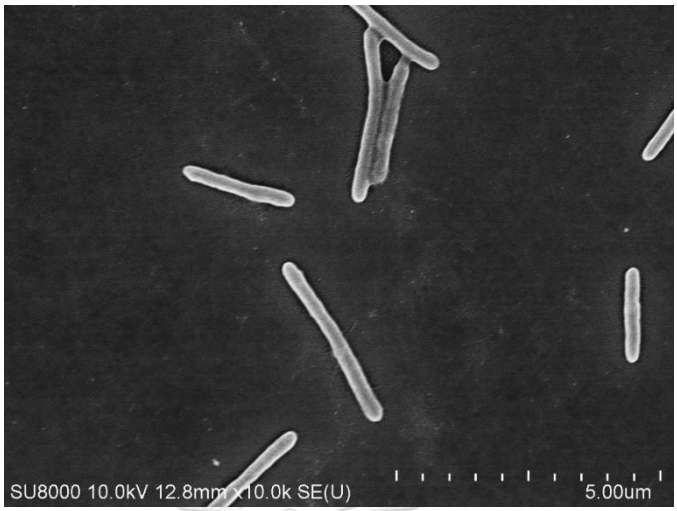

Figure 1 Planktonic cells or free-living cells of *F. oreochromis* isolate 15 under a scanning electron microscope (SEM).

2. Columnaris disease and biofilm formation

Columnaris disease or cotton wool or peduncle disease is a well-known and major disease outbreak that affects worldwide freshwater fish such as carp, channel catfish, goldfish, salmonids, red tilapia, and tilapia. Juvenile fish are generally more susceptible to the disease than other life stages and from many reports gill is a primary target organ for the bacteria infection (Declercq et al., 2013). Accumulated F. oreochromis on the gill filaments and gill lamellae which is the site where O2/CO2 exchange occurs, resulted in significant interference to the respiratory system of the fish. Lesions on the gill structure led to inflammatory cell infiltration and fusion of the gill filament and lamellae. Finally, complete fusion of the gill results in respiratory failure and follows with a high mortality rate (Pacha and Ordal, 1967; Foscarini, 1989; Dalsgaard, 1993; Declercq et al., 2013). In addition, several symptoms, such as lesions on the back of the fish that result in skin discoloration, sometimes called saddle-back patterns, peduncle erosion, and mouth rot, are shown during chronic cases that take longer to develop than gill lesions (Declercq et al., 2013). Saddle-back symptom occurs during the accumulation of *F. oreochromis* on fish skin around the base of a dorsal fin. The affected area shows pale skin color and small

lesions are noticed. The lesion can be advanced and extended out from the base of the dorsal fin to surround the dorsal fin (Pacha and Ordal, 1967; MORRISON et al., 1981; Bernardet and Bowman, 2006a). In addition, fin rot often occurs together with the saddle-back symptom (Decostere and Haesebrouck, 1999). Peduncle lesion is typically found in rainbow trout the sign starts with a lesion at the adipose fin and progresses deeper into the muscle or skin layer leading to a severe ulcer. The peduncle lesion is normally covered with yellowish mucus (Decostere, 2002; Bernardet and Bowman, 2006a). In addition, mouthrot causes death to the fish because of starvation. This symptom can lead to secondary infection by bacteria or fungi (Decostere and Haesebrouck, 1999).

Figure 2 Gill lesion of Nile tilapia 6 hours after being challenged with *F. oreochromis* isolate 15.

Bacteria prefer to inhabit in a sessile from more than the planktonic stage therefore it adheres to the biotic and abiotic surface beginning to colonize and form biofilm. This colonization referred to a community of bacteria that build their own supportive environment during the development of biofilm formation. Biofilm formation is composed of five phases. The initial phases begin from planktonic cells beginning to attach to the surface substrate, followed by aggregation and forming a microcolony in phase II. The monolayer was formed during phase III and produced an extracellular polymeric substance (EPS). Phase IV is a mature biofilm covered by EPS with a multidimensional layer. Phase V is the dispersion of planktonic cells a crucial part of biofilm development, these planktonic cells are seeds of new biofilm colonization. Mature biofilms produced EPS which consists of many types of biopolymers such as polysaccharides, nucleic acid, lipids, and proteins. These

components are helping to support biofilm architecture or biofilm stability and provide advantages to bacteria such as retention of water, nutrient source, and protective barrier. The bacteria within a biofilm are protected by EPS allowing it to tolerate a hazardous environment such as specific and nonspecific host immune systems, disinfectants, and antibiotics (Davies, 2003; Flemming and Wingender, 2010). EPS play an important role against antibiotic agent by absorbing the agent results in an incapacity to penetrate to biofilm. However, the ability to tolerate antibiotic agents depend on antimicrobial agent and microorganism within the biofilm (Stewart, 2002). More importantly, EPS helps bacteria initiate communication between bacteria to bacteria called quorum sensing (Flemming and Wingender, 2010). In addition, biofilm formed by *F. oreochromis* releases several toxins such as chondroitin AC lyase and proteases which can degrade a connective tissue in animals (gelatin, tyrosine, and casein) (Dalsgaard, 1993; Bernardet and Bowman, 2006b). *F. oreochromis* not only releases these polysaccharide degradations to enter host tissue but it's also released bactericidal to compete with other bacteria as well (Anacker and Ordal, 1955).

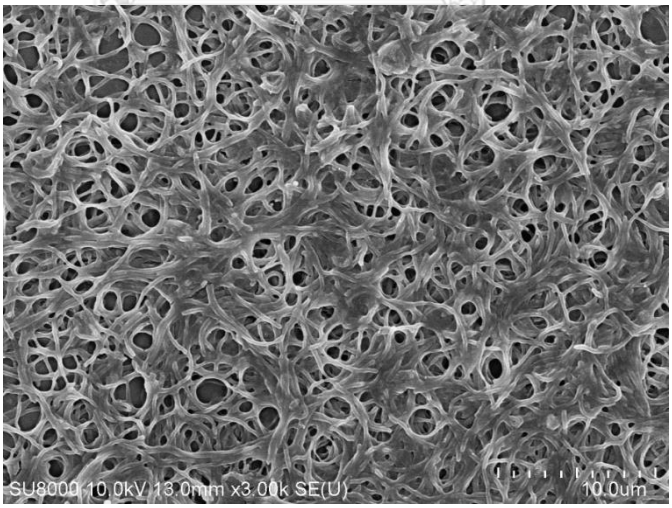

Figure 3 Biofilm formation of *F. oreochromis* isolate 15 under scanning electron microscope (SEM).

3. Virulence factors involved in *Flavobacterium oreochromis*

The ability to cause columnaris disease is dependent on the expression level of virulence factors. There are three major virulence factors involved in biofilm formation and columnaris disease infection. Iron acquisition is important for bacteria growth and metabolic pathway. During infection, bacteria face limitations of iron. In the environment or host, iron is present in two forms of ferrous (Fe^{2+}) or ferric (Fe^{3+}) which are compound-binding proteins. To acquire iron from the environment, bacteria produce siderophores to bind to the iron and transport it into the bacteria cell via active transport pathways (Sheldon et al., 2016). The genes responsible for siderophores synthesis are composed of alcB, rhbC, and Sido. The genes responsible for iron transportation are tonB (Cai et al., 2019; Lange et al., 2020b). Moreover, in the biofilm found that these genes were upregulated when compared to the planktonic cells (Cai et al., 2019).

Type XI secretion systems (T9SS) are important for bacteria survival and play two major roles such as secreting protein and gliding motility for initiating disease infection (Lasica et al., 2017). The gliding motility ability help *F. oreochromis* move toward to fish or suitable host and the responsible gene are gldL, gldK, and gldM. The secreting proteins from the type IX system are diverse in function such as adhesin protein or toxin and the gene responsible for adhesin protein is sprA, the gene responsible for toxin release is porV (Li et al., 2017; Penttinen et al., 2018). These components together help *F. oreochromis* to initiate infection by gliding to the host surface and secreting adhesin protein to form biofilm. Finally, during the development of biofilm formation *F. oreochromis* release toxins that destroy host tissue, thus, columnaris disease occur (Declercq et al., 2013).

The quorum-sensing system (QS) is known as a mechanism behind biofilm formation, it's an important system to alter the planktonic stage to the biofilm stage. Quorum sensing is a cell-to-cell communication of bacteria, which assists them to reach a certain population density via the production of small molecules called autoinducers (Ais). In gram-negative bacteria, *N*-acyl homoserine lactone (AHL) is present as the signal molecule (Lazdunski et al., 2004). *F. oreochromis* QS system required two important proteins for regulation. I-protein and R-protein, the signal

molecule is synthesized by enzyme I-protein which detect by R-protein. After I-protein detects by R-protein, the signal molecule will be sent to outside of the cell, after it reaches a certain density, they bind to LuxR the response regulator. LuxR bind to signaling molecule that stimulates the transcriptional of quorum sensing regulates genes (Lazdunski et al., 2004; Defoirdt et al., 2013; Declercq et al., 2021). Furthermore, QS is involved in various virulence factors besides biofilm formation such as drug resistance, motility, virulence factors expression, and bioluminescence (Hossain et al., 2017).

4. Prevention and treatment of columnaris disease

There are numerous approaches to prevent columnaris disease infection which depend on farmer strategies. Management practices have been useing nowadays since the bacteria respond well to fish when it experiences stress conditions, thus, controlling the ecosystem of the pond is important (Austin et al., 2007). Therefore, reducing suitable hosts for the bacteria in the rearing system is a promising practice for the prevention of columnaris disease (Suomalainen et al., 2005b). Moreover, controlling water quality by removing the accumulated feed or waste in the system also prevents *F. oreochromis* to adhere to the host and avoid fish from being exposed to ammonia toxins (Decostere et al., 1999). In addition, water treatment using ozone which is sufficient to reduce *F. columnare* in the water column (Conrad et al., 1975). Temperature is proven to be one of the factors that trigger columnaris disease infection. Many reports show that columnaris infection usually occurs during low temperatures or sudden changes in environmental conditions. Thus, controlling the temperature would prevent fish from columnaris disease infection and the survival ability of fish increased was noticed during high temperatures (Suomalainen et al., 2005b).

Chemical agents can also apply to prevent the outbreak, several chemical agents are developed for fish treatment such as potassium permanganate (KMnO4) or copper sulfate (CuSO4). The study from Davis suggested that treating fish using CuSO4 or apply to the pond would prevent columnaris disease infection (Davis, 1922). A study from Rogers suggested that dipping the fish into KMnO4 also prevents

columnaris disease infection (Rogers, 1971; Riley, 2000). In addition, adding artificial salt into the pond also prevents columnaris disease by reducing bacterium growth, adhesin, and motility (Suomalainen et al., 2005a).

Vaccination trials have been developed to administer many infections including columnaris disease (Austin et al., 2007; Declercq et al., 2013). Live attenuated vaccines such as heat-killed, and formalin-killed have been proven to be useful against columnaris disease infection. Formalin-killed vaccine was used by intraperitoneal injection increased the relative survival rate of grass carp up to 70.9% relative percent survival (RPS) compared to the uninfected group after the challenge with columnaris infection (Ransom, 1975). Heat-killed vaccines also provide an effective result, the vaccine can protect salmon (*Oncorhynchus kisutch*) from columnaris disease infection throughout 17 weeks by feed regimen (Fujihara and Nakatani, 1971). Considering that columnaris disease infection causes several lesions along the fish body, therefore, a bath immersion vaccine was introduced. Mucoadhesive nanovaccine coated with chitosan and pathogen-like (*F. columnare*) was able to protect tilapia after challenge with columnaris disease infection with a relative percent survival (RPS) greater than 60% when compared to whole cell bacterial vaccine (Kitiyodom et al., 2019). More importantly, the modified of mucoadhesive nanovaccine gives a satisfying result, the vaccine can protect red tilapia after challenge with columnaris infection up to 78% relative percent survival (RPS) and stimulate the mucosal immune response of fish as well (Kitiyodom et al., 2021).

Probiotics bacteria are commonly used in aquaculture to prevent many diseases and infections. Probiotics are the use of microorganisms that provide beneficial effects for the host such as growth promoter, stress tolerance, nutrient digestibility, reproduction improvement, water quality, and anti-pathogenic. The administration of probiotic can be immersion or feed supplementation which depend on farmer strategies (Verschuere et al., 2000; Lara-Flores, 2011; Dawood et al., 2019). For example, supplementation feed with probiotic bacteria *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* and yeasts protects shrimp (*Penaeus vannamei*) from acute hepatopancreas necrosis disease (AHPND) (Pooljun et al., 2020). In

addition, bacterial isolate from fish (*Salvelinus fontinalis*) skin was found to be effective against *F. columnare* and *F. psychrophilum*. These bacteria were reported to protect Salvelinus fontinalis from columnaris disease infection via feed supplementation or immersion into the water (Boutin et al., 2012). However, vaccines and probiotics are required for further studies because vaccination trials are not always successful, and probiotic selection criteria are difficult along with feeding strategies on an industrial scale (Declercq et al., 2013).

Antimicrobial agents are successfully controlled many diseases outbreak including columnaris disease, especially in Thailand. In Thailand, many antimicrobial agents are applied by immersing the agent in water and mixed with feed if the disease goes advanced (Rico et al., 2014). For instance, the study from Darwish showed that mixing florfenicol with fish feed prevents fish from *Aeromonas hydrophila* and *F. columnare* infection (Darwish et al., 2012). The commonly used antimicrobial agents in Thailand are Enrofloxacin, Oxytetracycline, Amoxicillin, Sulfadiazine, Trimethoprim, Penicillin, and Tetracycline (Rico et al., 2014). However, inappropriate or massive use of antibiotics leads to several problems such as residual in fish products and antibiotic resistance. More importantly, the aquatic environment receives the spread of antibiotic resistance from multiple sources such as antimicrobial use in farm animals, antimicrobial use in agriculture, and antimicrobial use in humans (Karunasagar, 2020). The spread of antibiotic-resistant bacteria in several regions causes ineffectiveness and limited preventive measure, thus, a new strategy is necessary.

5. Biofilm inhibitors and plant extracts

The emergence of antibiotic-resistant bacteria (AMR) has been causing global concern throughout decades. Therefore, new strategies that impede the virulence factors are needed. Biofilm formation control strategies that didn't affect bacteria growth provide an option against the AMR situation. Alternative strategies such as enzyme-based detergent, phages, microbial interaction, and plant extracts are proposed in many industries that affect by biofilm problems (Simões et al., 2010). Dairy industry is affected by bacteria biofilms such as *Lactobacillus bulgaricus*, *Lactobacillus lactis*, and *Streptococcus thermophilus*. The control strategy used is enzyme together with a surfactant, this approach proved to be sufficient to degrade biofilm structure (Oulahal-Lagsir et al., 2003). However, enzyme-based detergents are expensive and limited to big factories due to being patent-protected. Bacteriophage infections were used to destroy the biofilm structure; however, this method was rarely successful because phages required specific conditions of environmental factors (Simões et al., 2010). Unfortunately, these two approaches are not suited for the aquaculture industry. Microbial interaction and plant extracts have been showing significant results in inhibited biofilm from many fields including aquaculture. Microbial interaction is the use of metabolite of interspecies to compete or interfere with bacteria biofilm mechanism. For example, a metabolite produced by *Lactococcus latis* presents an anti-adherence ability which was interfering with the biofilm formation process (Rodrigues et al., 2004). In addition, *Bacillus subtilis* produce a surfactant that affects biofilm formation and biofilm disperses of *Salmonella enterica*, *E. coli*, and *Proteus mirabilis* without influent on bacterial growth (Mireles et al., 2001). Plant extract has been gaining attention from scientists because of their large variety of primary and secondary metabolites that contributes abundant benefit to the therapeutic approach (Zhu, 2020).

Plant extracts present a great potential benefit in aquaculture due to the secondary metabolites such as alkaloids, steroids, saponins, terpenoids, phenolics, tannins, flavonoids, glycosides, or essential oils (Reverter et al., 2014). Secondary metabolite shows many mechanisms against biofilm formation such as inhibition of biofilm formation, biofilm eradication, disruption of biofilm structure, and inhibition

of biofilm recovery. However, this mechanism depends on the plant's phytochemicals (Ali and Neelakantan, 2022). Apart from the anti-pathogenic properties of plant extracts, there are reported that plant extracts contribute to an immunostimulant, appetite stimulator, and growth promoter. For example, the supplementary diet of *Glycyrrhiza uralensis* prevents *Flavobacterium columnare* infection together with elevated growth, and immune responses in yellow catfish (*Pelteobagrus fulvidraco*) (Wang et al., 2020). Moreover, Methanol extracts of *Oscimum sanctum* show the ability to inhibit the growth of *Staphylococcus aureus* as well (Khaliq et al., 2018b).

Turmeric (*Curcuma longa L.*) is a local spice that is normally found in Asia and Southeast Asia including Thailand. Turmeric gains attention from scientists because of its nutritional and medicinal properties. In Thailand, turmeric is also used to treat illness both inside and outside the body. For example, turmeric powder mixed with lime water, it can be used as an external treatment for skin wounds, and rashes or eaten to relieve diarrhea. Apart from its medicinal use, scientists have been studying turmeric for antibacterial activity by extracting it phytochemical composition (Labban, 2014). Turmeric extract is effective in inhibiting the growth and biofilm formation of both gram-negative and gram-positive bacteria. For example, the presence of alkaloids and flavonoids produce by Curcuma species, there were successfully inhibited growth and biofilm formation by *Bacillus subtilis* and *Staphylococcus aureus* (Jain and Parihar, 2018b). In addition, essential oil from turmeric showed great potential against adherence ability and biofilm formation of *Staphylococcus epidermis* (Jardak et al., 2021). However, the ideal preventive measure that impedes virulence factors without influencing growth is desirable (Hossain et al., 2017; Soowannayan et al., 2019a). Several studies were successful in using alternative measures to inhibit biofilm formation or virulence factors without affecting bacterial growth. For example, extracts from ginger and fermented soybean were accomplished to inhibit biofilm formation without affecting the growth of bacteria and protect shrimp (*Penaeus vannamei*) from *Vibrio parahaemolyticus* and *Vibrio harveyi* infection (Yatip et al., 2018; Soowannayan et al., 2019a). *Piper betle* extract was able to inhibit quorum sensing expression genes resulting in less

bioluminescence production and biofilm formation together with protecting shrimp (*Penaeus vannamei*) from *V. harveyi* infection (Guzman et al., 2022a). More importantly, curcumin is a major constituent of bioactive compounds from turmeric (Sharma et al., 2005). The study from Raorane and the team were shown that curcumin at 50 µg/ml able inhibits biofilm formation and surface movement of *Acinetobacter baumannii* without affecting bacterial growth (Raorane et al., 2019).

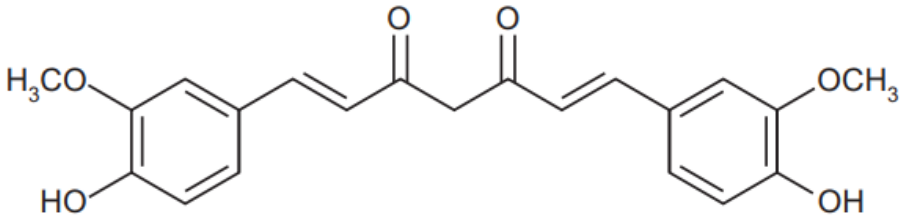

Figure 4 Curcumin; major compound of *Curcuma longa L*. (Sharma et al., 2005).

CHAPTER III

MATERIAL AND METHODS

3.1 Conceptual framework

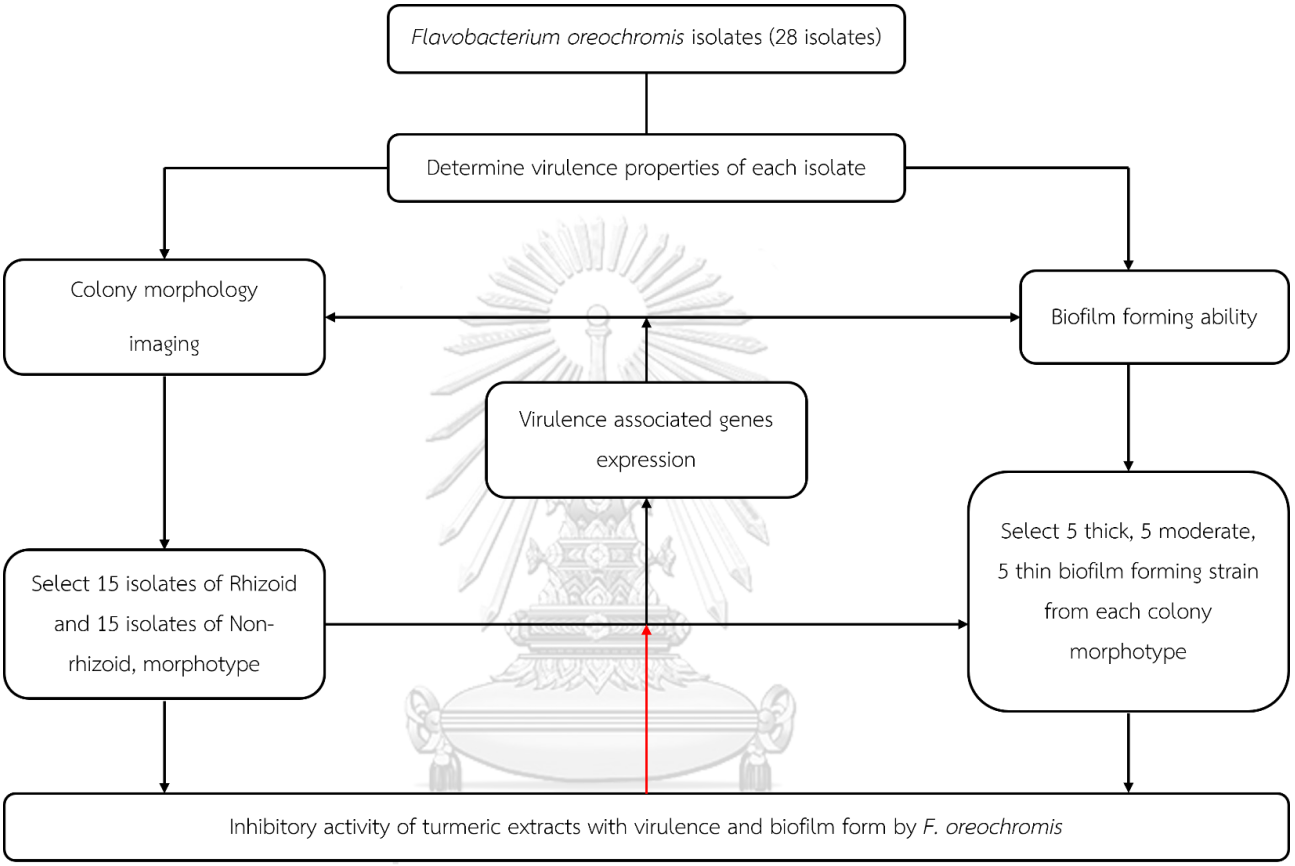

Figure 5 Conceptual framework of the study.

3.2 Bacteria strains

3.2.1 Bacteria strains, growth condition and colony morphology imaging

Seventy-eight isolates of *Flavobacterium columnare* were obtained the Center of Excellence in Fish Infectious Disease (CEFID) Chulalongkorn University. The bacteria were grown on Tryptone Yeast Extract Salts (TYES) agar and incubated overnight at 28 ℃. Colony morphology was visualized using a stereomicroscope. To prepare the bacteria starter cultures, single colony of each bacterial isolates were grown overnight in TYES broth and incubated at 28 ℃ with 200 rpm agitation. Optical density at 600 ($O.D_{600}$) of the overnight culture of each bacterial isolate were measured and the culture was diluted to 0.5 and then diluted 10 time further with new TYES medium. The diluted culture was used as starter cultures for biofilm-forming ability, growth, and biofilm inhibition assays.

3.3 Biofilm formation assays

To determine biofilm-forming ability of each of the 28 *F. oreochromis* isolate, the experiment was conducted using 96-well polystyrene plates, as described by (Yatip et al., 2018). Briefly, the 96-well plates were first coated with 1% tilapia gelatin solution and allowed to dry overnight in sterile lamina flow chamber. The coated plates were sterilized by 30 minutes expose to UV. Starter culture (200 µl) prepared as above was seeded into each well of the coated plates and incubated for 72 h at 28 ℃ in an incubator without agitation. After 72 h of incubation, unattached cell in the biofilm was removed and washed using distilled water and the attached cells in the biofilm was allowed to dry at room temperature. The dried biofilm was stained with 0.3% crystal violet for 15 minutes and excess stain was washed three times with tap water and stained biofilm on the plates were allowed to dry at room temperature. To quantify the formed biofilm thickness, the biofilm was dissolved by adding 200 µl of 33% acetic acid to each well of the 96-well plates, allowed to dissolve for 15 minutes at room temperature before their optical densities (O.D. 600) at 600 nm was measured using a microplate reader. This experiment was performed in eight replicates for each bacterial isolate.

3.4 Ribonucleic acid (RNA) extraction from biofilm

Total RNA was extracted from each bacteria isolate described as followed. The RNA extraction from biofilm was performed using 12-well polystyrene plates coating with 1% tilapia gelatin solution. Starter culture (2000 µl) was prepared as above and seeded to 12-well plates and incubated for 72 h at 28 °C without agitation. To extract the RNA from bacterial biofilm, bacteria culture supernatant was first removed from the biofilm culture than the biofilm on the plate was washed with distilled water. To extract RNA sample, form the washed biofilms, 500 µl of Trizol reagent (Invitrogen, USA) was added to each well of the bacteria biofilm and the biofilm will be scraped using a plastic pestle and the biofilm was allowed to solubilize with Trizol on ice for 5 minutes than transferred suspended solid to new microcentrifuge tube. After that, 100 µl of chloroform was seeded to each well and mixed using vortex mixer follow with incubated on ice for 15 minutes. Next, centrifuged at 12, 000 rpm at 4 °C for 15 minutes. Then transferred supernatant into new microcentrifuge and added 250 µl of isopropanol than mixed with vortex mixer following with 10 minutes incubation on ice. Next, centrifuged at 12, 000 rpm at 4 °C for 10 minutes and removed supernatant. After that, RNA pellet was washed 2 times with 500 µl of 75% ethanol and centrifuged at 7,500 rpm at 4 °C for 5 minutes. Finally, RNA pellet was dried at room temperature and dissolved with diethylpyrocarbonate (DEPC) water 10-50 µl. RNA sample was measured the quality using nano drop one (Thermo scientific) and stored at -80 °C.

Table 1 Oligonucleotide primer use in this study.

Primers	Forward	Reverse	References
sprA	AGCCGTGCAGAAGATAAAGC	ACGCTTCTAATGCGGGTACAA	(Penttinen et al., 2018)
gldL	GCTTCTGTACCTAAACCAGCA	TTGGTGCGGCAGTAGTAATC	(Declercq et al., 2019)
tonB	TGCAATGCAAGCCTATCTTATTC	TGCTGAACCCTTCCGTTATATT	(Lange et al., 2020a)
rhbC	GAATTGGGAGACGAAACCATTG	CCAAGGATGTGTTGGCATAAAG	(Lange et al., 2020a)
alcB	TCGTGTAGTTGTAGAACCTGATG	GAGCATTGTAGTATTGTTGTCTT GT	(Lange et al., 2020a)
PorV	GTGCCAACTCCTAAAACAGCC	AAACCTCCTGGAGCATCACC	(Penttinen et al., 2018)
LuxR	ACCTTTCGCACACACAGAAA	GCAATGTCGTTCTTTAGGCTGT	(Cai et al., 2019)
Sido	TCAGAGCGCAGCAGAAGTTT	TGATCTCGTTTGGCTTCTGGT	(Cai et al., 2019)
16s	ACGATCAAACGGCCATTG	AGTAACCTGCCTTCGCAATC	(Declercq et al., 2019)

3.5 Synthesis Complementary DNA (cDNA)

To synthesize cDNA, RNA from biofilm was synthesized using RNA-dependent DNA polymerase enzyme by Promega. The cDNA synthesis reaction was performed as described by the company, briefly, 5X buffer was added to PCR tube 4 µl along with RNA extract from biofilm 100 ng and adjusted volume using PCR grad water up to 20 µl. Then the solution was mixed by vortex mixer followed by 30 minutes of incubation using a water bath at 37 ℃, the PCR tubes were transferred to the PCR machine (Thermo Fisher Scientific). The PCR reaction was performed at 5 minutes of annealing at 25 ℃, 60 minutes of extension at 42 ℃, 15 minutes of heat-inactivated the ImProm-II™ reverse transcriptase, after that, cDNA was stored at -20 ℃ for further experiment.

3.6 Polymerase Chain Reaction (PCR) assay

To examine the presence of biofilm-associated genes of *F. oreochromis* isolate number 15, the experiment was examined by using conventional PCR. The PCR assay was carried out in a total volume of 25 µl containing 5 µl of master mix 5X, 0.5 µl of each forward and reverse primer, and 1 µl of template RNA. The PCR amplification was performed in 1 cycle of denaturation at 94 ℃ for 7 minutes; melting, annealing, and the extension was performed in 35 cycles at 94 ℃ for the 30s, 55 ℃ 30S, and 72 ℃ for 1 minute respectively. The final extension was performed at 72 ℃ for 10 minutes. PCR products were detected by 1.2% agarose gel electrophoresis (Chockmangmeepisan et al., 2020).

Table 2 The component of the PCR reaction

Component	Final volume (µl)	Final concentration
5X buffer	5	1X
25 mM $MgCl_2$	2	1.0-4.0 mM
dNTP	2	0.2 mM
Forward primer	0.5	0.1-1.0 mM
Reverse primer	0.5	0.1-1.0 mM
DNA Polymerase	0.25	1.25 u
Template DNA	1	0.5 mg/ml
Adjust with water to final volume	25	

3.7 Identification of Flavobacterium oreochromis isolate number 15

To identify the *Flavobacterium columnare* into four distinct species, the experiment was done using 16s rRNA sequencing. The PCR product were cloned into pGem-T Easy Vector and ligation process was done in PCR machine. Then the vector was transferred to *Escherichia coli* DH5α, then the competent cells were incubated on ice 20 minutes after that incubated the competent cell at 42 ℃ for 90 sec. Then the competent cells were incubated on ice for 2 minutes before incubated at room temperature for 2 minutes. The competent cells were culture in LB (Luria-Bertani) broth at 37 ℃ for 1 hr with agitation. Before picking up the white colony of competent cells, the LB+AMP (Luria-Bertani with ampicillin) agar were prepared by spread Xgal and IPTG, then competent cells were spread on the prepared agar and incubate 37 ℃ for overnight. The competent cells with white colony were picked and incubated into LB+AMP broth with agitation at 37 ℃ for overnight. The plasmids were extracted form competent cell before sent to Macrogen for sequencing.

3.8 Quantitative real time PCR of biofilm-associated genes

To determine the difference between virulence-associated genes expression of each of *F. oreochromis* isolates, the experiment was conducted as follow. Complementary DNA (cDNA) was synthesized using Promega, briefly, cDNA 1 µl was mixed with Master mix 10 µl, Forward and Reverse primer 0.4 µl, and adjusted volume using PCR-grade water up to 20 µl in 96-well plate PCR (BIO-RAD). After that, PCR plate was performed and analyzed by qPCR machine (BIO-RAD) and qPCR conditions were conducted as followed in table 3 using KAPA SYBR. The results were interpreted by the delta-delta Ct ($2^{-\Delta\Delta CT}$) method using the following formula.

$$\Delta\Delta CT = \Delta CT(\text{a target sample}) - \Delta CT(\text{a reference sample})$$

3.9 Turmeric extracts activities

3.9.1 Turmeric extracts preparation

To prepare turmeric (*Curcuma longa L*) crude extracts the experiment were performed as described (Khaliq et al., 2018a) with slight adjusted. Fresh turmeric 2 kg were brought from TalaadThai (Phathumthani province). Turmeric was washed and cut into small pieces and dried in a hot air oven at 60 ℃ until dry. Dry turmeric was ground to a fine powder by blender and stored in a refrigerator at 4 ℃ before use. Extraction method in this study was performed using the Soxhlet apparatus with three organic solvents (Hexane, Ethanol, Methanol) sequentially increased polarity from low to high. For the turmeric extracts preparation, 60 g of turmeric powder was added to a distillation flask followed by organic solvents. The temperature was adjusted depending on the boiling point of each organic solvent using a heat source and the extraction was continued overnight or solvents show colorless. The warm solvents of crude extract were collected and concentrated by using a rotary vacuum evaporator. The crude extract solvents were added to the rotary bottle and adjusted temperature using a heating water bath pot. Before the drying process, crude extracts were kept at -20 ℃ for overnight. The drying process was operated by a freeze dryer machine. Finally, dry crude extracts were weighed and stored in a refrigerator at 4 ℃ before adjusted concentration. The dry crude extracts were mixed with dimethyl sulfoxide (DMSO) and adjusted the concentration using distilled water. The final concentration was set up at 1 g/ml and kept at 4 ℃ until use. The percent yield of the turmeric extract was obtained using the following equation.

$$\text{Percent yield of extract} = (w_1 \times 100) / w_2$$

W_1 is the weight of the extract after drying process

W_2 is the weight of the dried plant powder

3.9.2 Growth inhibition effect of turmeric extracts

To determine the effect of turmeric extracts on *F. oreochromis* growth the experiment was used 96-well polystyrene plates without coating as described by (Yatip et al., 2018). The experiment was done using a 2-fold dilution which followed a CLSI regulation with a concentration range from 512 – 0.25 µg/ml. Starter culture (200 µl) was prepared as above and seeded on 96-well plates followed by 200 µl of turmeric extracts using a multi-channel pipette. The microplates were incubated at 28 °C overnight with 200 rpm agitation. The control groups were placed by using TYES medium, distilled water, and DMSO with the same amount of starter culture and turmeric extracts. To quantify the effect of turmeric extracts on bacteria growth, overnight culture plate was measured the optical density ($O.D_{600}$) at 600 nm by a microplate reader. This experiment was conducted in eight replicates. The growth inhibiting activity was calculated as percent relative inhibition by the following formula (Soowannayan et al., 2019b).

$$\text{Mean\% Relative Inhibition} = [1 - (\frac{\text{Mean } OD_{600} \text{ of test}}{\text{Mean } OD_{600} \text{ of Neg Cont}})]*100$$

3.9.3 Biofilm inhibition effect of turmeric extracts

To determine the effect of turmeric extracts on biofilm formation the experiment was carried out as described (Yatip et al., 2018) with a slight adjustment. Briefly, 96-well polystyrene plates were be coated with 1% tilapia gelatin solution and allowed to dry overnight with 15 minutes UV before use. The experiment was done using a 2-fold dilution which followed a CLSI regulation with a concentration range from 512 – 0.25 µg/ml. Stater culture (200 µl) was prepared as above and seeded on 96-well plates followed by 200 µl of turmeric extracts using a multi-channel pipette. The control groups were placed by using TYES medium, distilled water, and DMSO with the same amount of starter culture and turmeric extracts. The microplates were incubated at 28 °C for 72 h without agitation. After 72 h of incubation, unattached cells in the biofilm were removed and washed using distilled water and allowed attached to dry at room temperature. Biofilm was stained with

0.3% crystal violet for 15 minutes and excess stain were washed three times with tap water and allowed stained biofilm on the plate to dry at room temperature. To quantify the formed biofilm thickness, 200 µl of 33% acetic acid was added to 96-well plates, allowing acetic acid to dissolve the stained biofilm for 15 minutes at room temperature before measuring the optical density (O.D.) at 600 nm by a microplate reader. This experiment was performed in eight replicates. The biofilm inhibiting activity was calculated as percent relative inhibition by the following formula (Soowannayan et al., 2019b).

$$\text{Mean\% Relative Inhibition} = \left[1 - \left(\frac{\text{Mean } OD_{600} \text{ of test}}{\text{Mean } OD_{600} \text{ of Neg Cont}}\right)\right]*100$$

3.9.4 Effect of turmeric extracts against virulence of *F. oreochromis*

To determine the effect of turmeric extracts on the virulence of *F. oreochromis*, the experiment was be performed as described by (Guzman et al., 2022b) with adjustment using 12-well polystyrene plates coated with 1% tilapia gelatin solution. Briefly, stater culture (1800 µl) was be prepared as above and seeded into each coated 12-well plate followed by 200 µl of turmeric extracts each concentration along with 72 h of incubated at 28 °C in an incubator without agitation. After incubation, unattached cells in the biofilm were removed using an auto pipette and washed with distilled water. To extract RNA from biofilm after expose to turmeric extracts the experiment was conducted as described above in RNA extract from the biofilm. To quantify the difference between virulence-associated genes expression of *F. columnare* after exposed to turmeric extracts, cDNA was be synthesized using Promega and cDNA was be used as DNA template for qPCR analysis using KAPA SYBR. The experiment was done in triplicate.

3.10 Statistical analysis

The data was combined and represented in means ± standard deviation (SD). The correlation of virulence between colony morphology and biofilm-forming ability was calculated using point biserial correlation. The efficiency of crude turmeric extracts was calculated using Pearson correlation. Statistical analyses were done using R program (Student version). The differences of growth and biofilm were using one-way ANOVA with Turkey's multiple comparison test. The differences of mean gene expression were compared among groups and the difference between biofilm-forming ability were performed by using student t-test. A p-value set at 0.05 for statistically significant differences.

CHAPTER IV

RESULTS

4.1 Colony morphology, Biofilm forming ability

4.1.1 Colony morphology

In this study, twenty-eight isolates of *F. columnare (F. oreochromis)* isolated from affected/diseased tilapia and seabass from outbreak farms in Kanchanaburi province, west of Bangkok, were obtained from the Center of Excellence in Fish Infectious Disease (CEFID) Chulalongkorn University. All isolates formed yellow colonies but with two different colony morphologies (morphotypes), rhizoid and non-rhizoid, as shown in Figure 6. Among the 28 isolates, 21 isolates were found to form rhizoid colonies, while 7 isolates were found to form non-rhizoid colonies. The rhizoid colonies (Figure 6A) usually have a raised center with several processes surrounding it, which is in contrast to the non-rhizoid colonies, which display a smooth edge in a circular shape without appendages/or processes, as shown in Figure 6B.

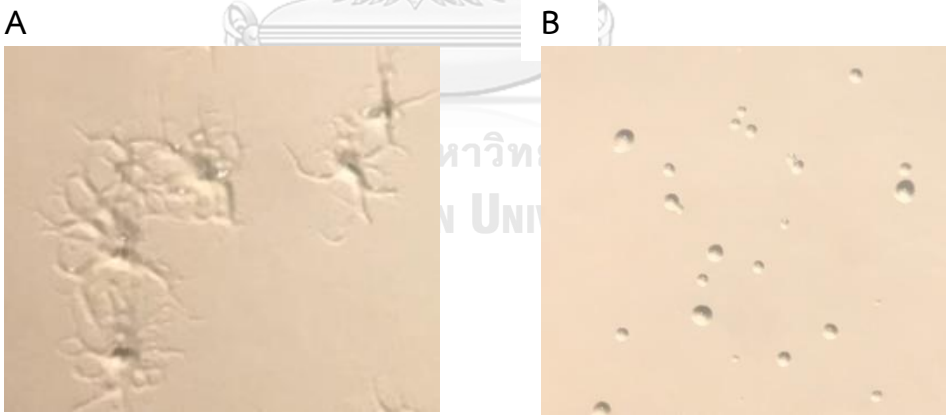

Figure 6 Rhizoid (A) and non-rhizoid (B) colonies of F. oreochromis

4.1.2 Biofilm-forming ability

To determine the biofilm-forming ability of each isolate, 200 µl of a starter culture of each bacterial isolate was seeded into eight wells of a 96-well plate coated with tilapia gelatin. The plates were then incubated without shaking for 72 hours to allow the bacterial biofilms to form and grow. After incubation, the planktonic cells were removed, and the formed biofilms were stained with crystal violet. The stained biofilms were dissolved in acetic acid before the optical density of the stained, and dissolved biofilms were measured. Biofilm-forming ability of each *F. oreochromis* isolate was found to be varied (Fig. 7). Many isolates were found to form thick biofilms (O.D reading at 600 nm ranges from 4.00 to 3.00), and some were found to form biofilms with moderate thicknesses (O.D reading at 600 nm ranges from 2.90 to 2.00), and some were found to form thin biofilms (O.D reading at 600 nm ranges from 1.00 to 0.00).

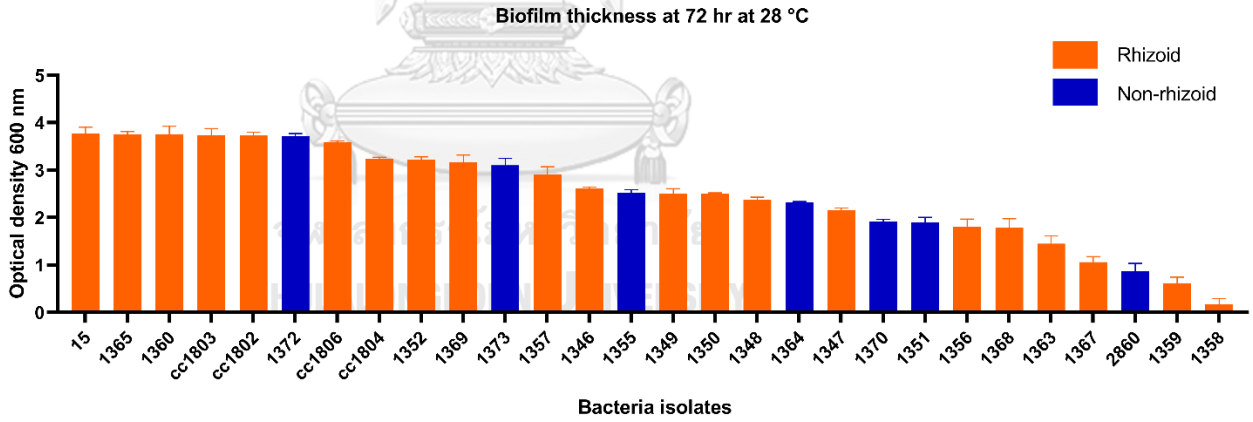

Figure 7 Absorbance at 600 nm of crystal violet-stained biofilms indicative of biofilm thicknesses of 28 isolates of *F. oreochromis* that form rhizoid (orange bars) and non-rhizoid (blue bars) colonies. Bars represent mean OD600 readings of crystal violet-stained biofilms from 8 replicate wells ± standard deviation.

4.1.3 Correlation between colony morphology and biofilm-forming ability

To determine the correlation between colony morphology and biofilm-forming ability, Point Biserial correlation coefficient was used to calculate the correlation between colony morphology and biofilm thickness. Among the 28 isolates, 21 were found to form rhizoid colonies and 7 isolates were found to form non-rhizoid colonies. The colony morphology and biofilm thicknesses of *F. oreochromis* were shown in figure 8. The correlation score between these two variables was -0.1140, which indicates a weak negative correlation between these two variables.

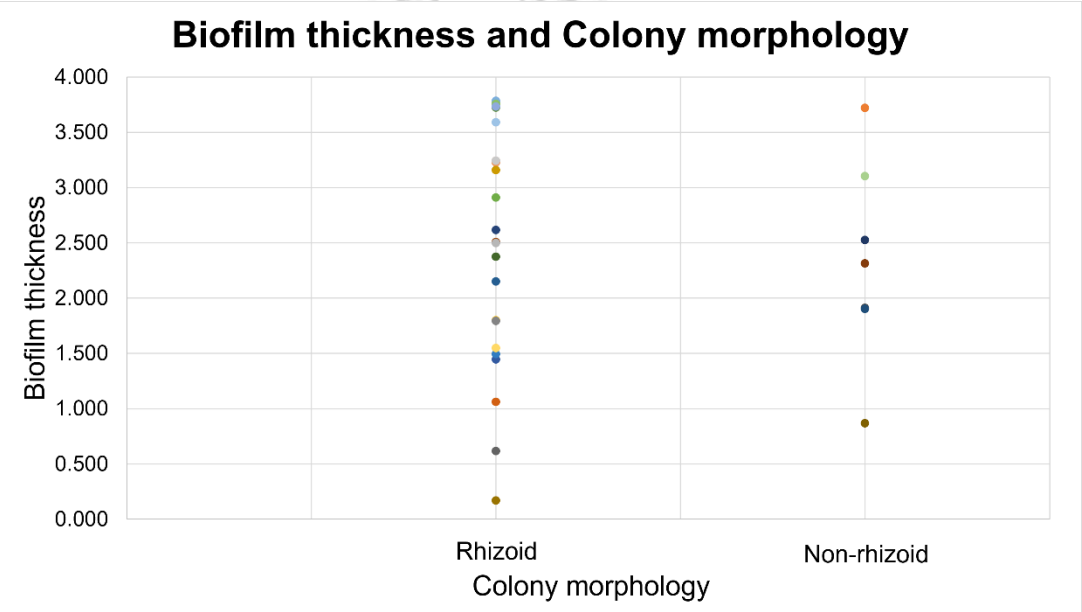

Figure 8 The graph shows colony morphotypes (Rhizoid and non-rhizoid) and absorbances at 600nm of crystal violet-stained biofilms indicative of biofilm-forming ability of 28 isolates of *F. oreochromis*.

4.2 **Identity *Flavobacterium columnare* isolate number 15**

To identify *Flavobacterium columnare* isolate number 15, the isolate chosen for gene expression study. The bacteria was first grown in TYES medium before its DNA sample was extracted as used as a template for PCR amplification using primer targeting 16sRNA gene. The amplified product was cloned into pGEMT-easy and sent for sequencing (Macrogen, Korea). From 16sRNA sequence analysis, the sequence of *F. columnare* isolate number 15 was found to share 100% identity to the sequences from several isolates of *F. oreochromis*. The phylogenetic tree was constructed using 16s rRNA on the GenBank database with MEGA X software (Figure 9).

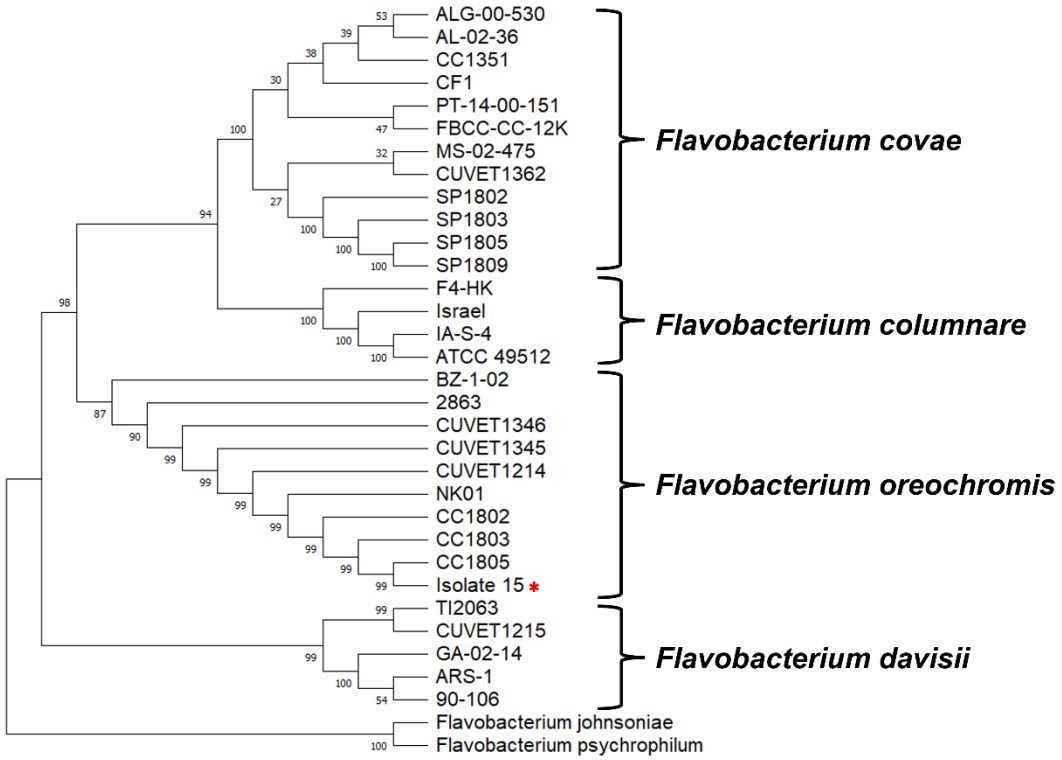

Figure 9 *Flavobacterium columnare* isolate number 15 was classified as *Flavobacterium oreochromis*.

4.3 Yields of turmeric extract by three different organic solvents

To extract phytochemical components from turmeric rhizome three organic solvents (hexane, ethanol, and methanol) with increasing polarities were tested using a Soxhlet apparatus set at 69 ℃, 78 ℃, and 65 ℃ respectively. The obtained extracts from each solvent were then concentrated using a rotary evaporator and then freeze-dried. The dried products were weighed and dissolved in DMSO to 1 g/ml. The hexane extraction of 60 g of turmeric powder produces 16.3 g of brown powder after freeze-dried. The ethanol extraction of 60 g of turmeric powder produces 7.3 g of brown powder after freeze-dried. The methanol extraction of 60 g of turmeric powder produces 8 g of brown powder. The percent yields of turmeric extracts using three organic solvents are shown in table 3.

Table 3 Percent yields of turmeric extracts using three organic solvents

No	Solvent	Polarity index	Boiling point	Percent yields
1	Hexane	0.1	69	27.1
2	Ethanol	5.2	78	12.1
3	Methanol	5.1	65	13.3

4.4 Effect of turmeric extracts against *F. oreochromis* growth and biofilm formation

To determine the minimal inhibitory concentration (MIC) of turmeric extracts , 12 concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml) of each of the three turmeric extracts were tested on 17 isolates of *F. oreochromis.* To do this, starter cultures (180 µl) of each bacterial isolates were grown in TYES broth medium and seeded into 8 wells of a 96-well plate. Then, the extracts (20 µl) were added to each of the 8 wells of the 96-well plate ,and the plate was incubated at 28 c° overnight. After incubation, the absorbance at 600 nm, indicative of bacterial growth (growth inhibition by turmeric extracts) was measured using a microplate reader. The results are shown in table 4, which represents Minimum Inhibitory Concentration

(MIC). To determine Minimum Biofilm Inhibitory Concentration (MBIC) of turmeric extracts , 12 concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml) of each of the three turmeric extracts were tested on 17 isolates of *F. Oreochromis.* To do this, starter cultures (180 µl) of each bacterial isolates were grown in TYES broth medium and seeded into 8 wells of a 96-well plate coated with tilapia gelatin. Then, the extracts (20 µl) were added to each of the 8 wells of the 96-well plate ,and the plate was incubated at 28 c° for 72 h. After incubation, the planktonic cells were removed, and the biofilms were stained with crystal violet. The stained biofilms were dissolved in acetic acid before the optical density of the stained, and dissolved biofilms were measured. The results are shown in table 4, which represents Minimum Biofilm Inhibitory Concentration (MBIC).

Table 4 MIC and MBC responses on bacterial growth and biofilm formation. The concentration of the extracts was rage 0.25-512 (µg/ml).

Bacteria isolates	Colony morphology	Biofilm thickness	MIC (µg/ml)			MBIC (µg/ml)		
			Hexane	Ethanol	Methanol	Hexane	Ethanol	Methanol
1353	Rhizoid	3.787	128	128	256	512	512	512
22		3.779	128	256	512	128	128	256
15		3.763	128	128	256	128	128	256
1365		3.762	64	64	64	128	64	64
1360		3.759	256	256	512	256	256	256
1357		2.912	128	128	512	128	128	256
1346		2.616	64	64	64	16	32	64
41		2.603	32	128	128	16	64	128
1349		2.510	16	16	32	8	16	32
1359		0.618	128	128	128	16	32	128
1358		0.169	128	128	512	64	64	256

1372		3.721	128	128	256	128	128	256
1373		2.526	64	32	32	64	64	64
1355		2.316	32	32	32	8	2	16
1364	Non-Rhizoid	1.104	64	32	512	16	32	128
1370		1.013	32	32	128	16	2	128
2860		0.869	32	32	128	8	8	128

4.4.1 Effect of hexane extract of turmeric on growth and biofilm of *F. oreochromis* isolate 15

Hexane extract of turmeric was the most potent inhibitor of growth and biofilm formation of *F. oreochromis*. The results of growth inhibition are shown in Figure 10A, which shows that the extract inhibited the growth of *F. oreochromis* in a dose-dependent manner. At 128 µg/ml of the extract successfully inhibited the bacteria growth by 98%, while at 2 µg/ml the extract significantly inhibited the growth of the bacteria by 13%. The results of biofilm inhibition are shown in Figure 10B, which, the extract strongly inhibited the biofilm formation of *F. oreochromis*, also in a dose dependent manner. At concentrations between 16 µg/ml and 512 µg/ml, the extract successfully inhibited biofilm formation of *F. oreochromis* by 90%. While at the lowest concentration of 0.25 µg/ml, it inhibited biofilm formation by 20%, which is still significant difference to that of the negative control. It is interesting to note that the extract at concentrations between 0.25 µg/ml and 1 µg/ml were found to inhibit biofilm formation without affecting the bacterial growth.

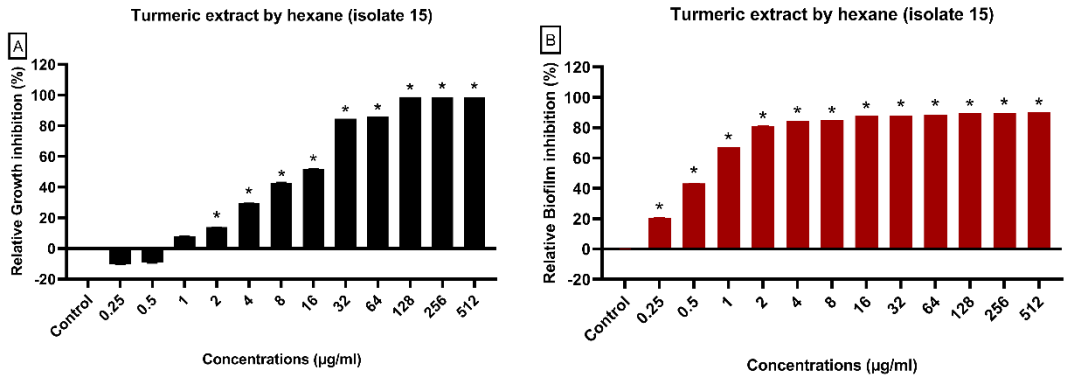

Figure 10 Relative percent inhibition of growth (A) and Biofilm (B) by hexane extract of turmeric on *F. oreochromis* isolate 15. As shown in figure 10A, the extract significantly ($p < 0.05$) inhibited the growth on *F. oreochromis* by 13.95% to 98.66%. As shown in figure 10B, the extract was significantly inhibited biofilm formation on *F. oreochromis* by 20.59% to 90.24%. Bars represent mean relative growth and biofilm ± standard deviation of eight replicates and the asterisks (*) represent the significant difference at $p < 0.05$ when compared to control group (one-way ANOVA; $p < 0.05$).

4.4.2 Ethanolic extract of turmeric on growth and biofilm of *F. oreochromis* isolate 15

Ethanolic extract of turmeric was shown inhibitor of growth and biofilm formation of *F. oreochromis*. The results of growth inhibition are shown in Figure 11A, which shows that the extract inhibited the growth of *F. oreochromis* in a dose-dependent manner. At 128 µg/ml of the extract successfully inhibited the bacteria growth by 98%, while at 4 µg/ml the extract significantly inhibited the growth of the bacteria by 33%. The results of biofilm inhibition are shown in Figure 11B, which, the extract inhibited the biofilm formation of *F. oreochromis*, also in a dose dependent manner. At concentrations between 64 µg/ml and 512 µg/ml, the extract successfully inhibited biofilm formation of *F. oreochromis* by 90%. While at the lowest concentration of 0.5 µg/ml, it inhibited biofilm formation by 59%, which is still significant difference to that of the negative control.

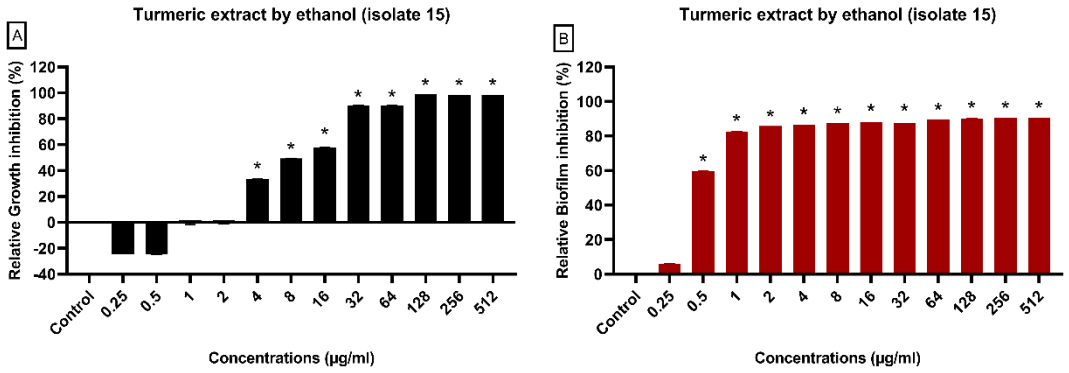

Figure 11 Relative percent inhibition of growth (A) and Biofilm (B) by ethanolic extract of turmeric on *F. oreochromis* isolate 15. As shown in figure 11A, the extract significantly ($p < 0.05$) inhibited the growth on *F. oreochromis* by 33.20% to 98.66%. As shown in figure 11B, the extract was significantly inhibited biofilm formation on *F. oreochromis* by 59.61% to 90.48%. Bars represent mean relative growth and biofilm ± standard deviation of eight replicates and the asterisks (*) represent the significant difference at $p < 0.05$ when compared to control group (one-way ANOVA; $p < 0.05$).

4.4.3 Methanolic extract of turmeric on growth and biofilm of *F. oreochromis* isolate 15

Methanolic extract of turmeric was shown inhibitor of growth and biofilm formation of *F. oreochromis*. The results of growth inhibition are shown in Figure 12A, which shows that the extract inhibited the growth of *F. oreochromis* in a dose-dependent manner. At 256 µg/ml of the extract successfully inhibited the bacteria growth by 98%, while at 2 µg/ml the extract significantly inhibited the growth of the bacteria by 30%. The results of biofilm inhibition are shown in Figure 12B, which, the extract inhibited the biofilm formation of *F. oreochromis*, also in a dose dependent manner. At concentrations between 32 µg/ml and 512 µg/ml, the extract successfully inhibited biofilm formation of *F. oreochromis* by 80%. While at the lowest concentration of 1 µg/ml, it inhibited biofilm formation by 42%, which is still significant difference to that of the negative control.

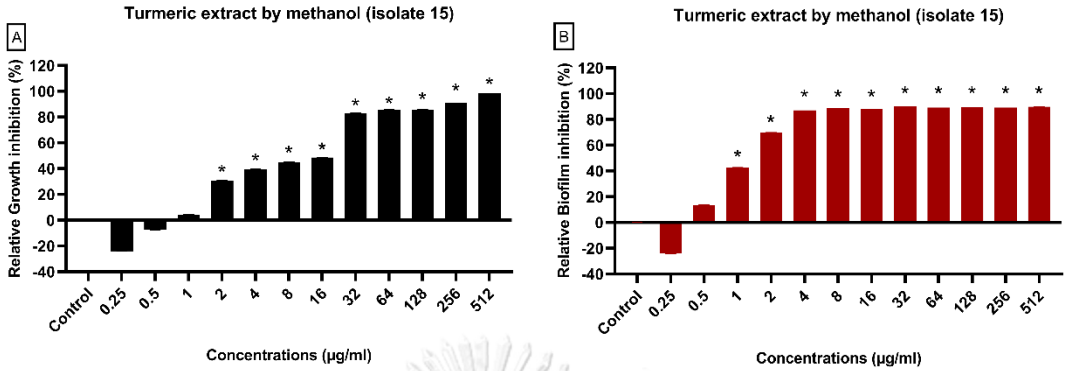

Figure 12 Relative percent inhibition of growth (A) and Biofilm (B) by methanolic extract of turmeric on *F. oreochromis* isolate 15. As shown in figure 12A, the extract significantly ($p < 0.05$) inhibited the growth on *F. oreochromis* by 30.76% to 98.66%. As shown in figure 12B, the extract was significantly inhibited biofilm formation on *F. oreochromis* by 42.57% to 89.73%. Bars represent mean relative growth and biofilm ± standard deviation of eight replicates and the asterisks (*) represent the significant difference at $p < 0.05$ when compared to control group (one-way ANOVA; $p < 0.05$).

4.5 The biofilm-associated genes in *F. oreochromis* isolate number 15

To determine the effect of turmeric extract on the expressions of biofilm and virulence-associated genes in *F. oreochromis* isolate number 15, quantitative RT-PCR was used (see Table 1 for the genes and primer sequences). To obtain RNA sample from the biofilm, 2000 µl of starter culture was seeded into each well of a 12-well plate coated with tilapia gelatin. The plates were incubated at 28 °C for 72 h before the RNA sample was extracted. The obtained RNA was reverse transcribed to cDNA before used as templates for PCR amplifications. The results of PCRs are shown in figure 13. The figure revealed that 9 genes of biofilm-associated were detected in isolate 15, which consist of *gldL, sprA, porV, alcB, rhbC, Sido, tonB*, and *luxR*.

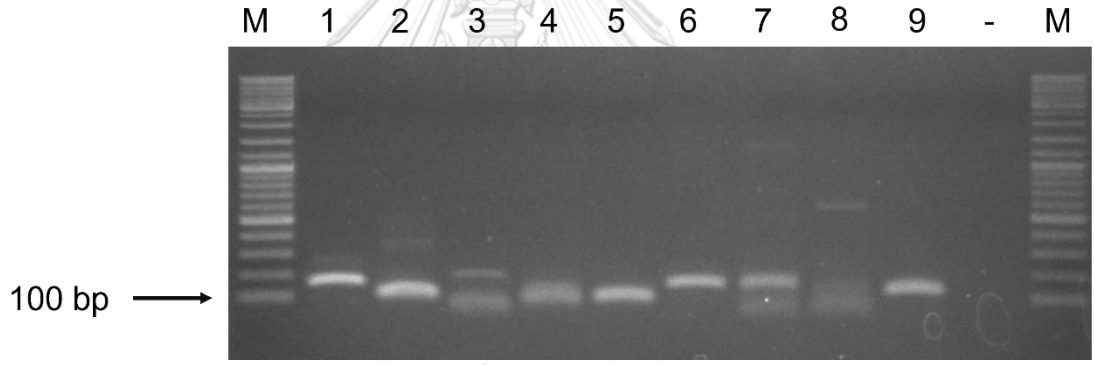

Figure 13 Detection of biofilm-associated genes of *Flavobacterium oreochromis* isolate number 15. In the figure, well 1 is alcB, well 2 is rhbC, well 3 is sido, well 4 is tonB, well 5 is gldL, well 6 sprA, well 7 porV, well 8 is luxR, well 9 is 16s rRNA, and – is negative control.

4.6 Effect of turmeric extracts on *F. oreochromis* virulence and biofilm-associated genes.

In order to study the interference of hexane extract of turmeric on the bacterial virulence and biofilm-associated genes, *F. orepchromis* isolate 15 biofilm was grown in the presence and absence of the turmeric extract (1 µg/ml, at which it does not affect the growth but inhibit the biofilm) and the RNA samples were collected at three different time points (24, 48, and 72 h). The obtained RNA samples were then used as templates for qRT-PCR using primers targeting biofilm and virulence-associated genes. These genes were selected based on their involvement in the bacterial pathogenesis, toxin secretion, and quorum sensing system. The biofilm-associated genes studied were composed of genes in iron inquisition system (*alcB, rhbC, tonB*, and *Sido*), T9SS (*sprA, gldL*, and *PorV*), and quorum sensing genes (*luxR*). To normalize data, primers targeting 16S rRNA, a house-keeping gene was used. The experiments were done in triplicates.

4.6.1 Expression of iron acquisition genes after exposure to turmeric extract

Iron acquisition is a crucial part of the bacteria growth and metabolism. Bacteria face limitations of iron during initiating infection. Therefore, bacteria produce molecules called siderophores to acquire free iron (Fe3+) from the surrounding cell.

The alcB, rhbC, and Sido genes are responsible for the biosynthesis of the siderophore which produces to trap iron. In this study, the effect of turmeric extracts on siderophores synthetase was shown in Figure 14. The results of turmeric on *alcB* gene expression were shown in figure 14A, the picture revealed that the expression levels were downregulated (log 2-fold change) by -2.0, -1.6, and -1.8 at 24h, 48, and 72h respectively. Similar results were observed for *rhbC* and *Sido* genes expression as shown in figure 14B and 12C. Figure 11B revealed that the expression levels of *rhbC* were downregulated (log 2-fold change) by -2.0, -2.1, and -2.6 at 24h, 48, and 72h respectively. Figure 14C revealed that the expression levels of *Sido* were downregulated (log 2-fold change) by -1.9, -1.9, and -2.0 at 24h, 48, and 72h respectively. The result indicates that turmeric extract was shown efficiency significantly suppressed the expression of *alcB, rhbC*, and *Sido* genes of *F. oreochromis*.

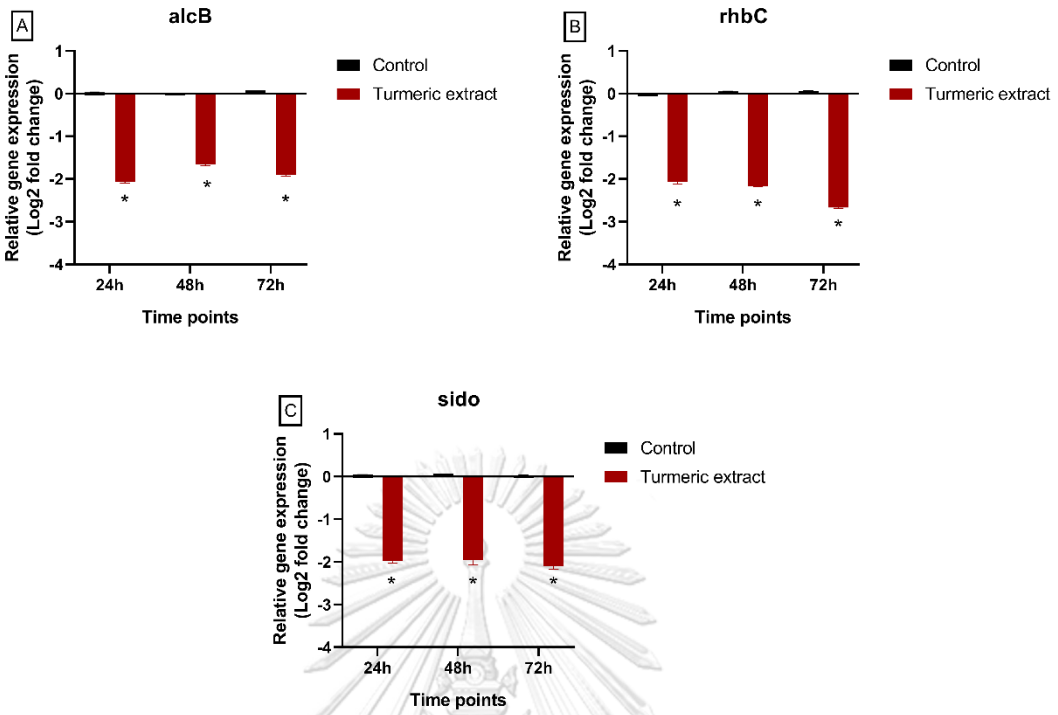

Figure 14 Relative iron acquisition gene expressions in the biofilm of *Flavobacterium oreochromis* (isolate 15) after exposure to turmeric extract at three time points (24h, 48h, and 72h). (A) Turmeric extract downregulated *alcB* gene expression at all three-time points. (B) Turmeric extract downregulated *rhbC* gene expression at all time points. (C) Turmeric extract downregulated *Sido* gene expression at three-time points. Bars represent the mean relative expression ± standard deviation of three replicates, and the asterisks (*) denote the significant difference at $p < 0.05$ when compared to the control group (student t-test; $p < 0.05$).

The *tonB* gene is responsible for the iron transport. In this study, the effect of turmeric extracts on *tonB* gene was shown in figure 15. The figure revealed that turmeric extract downregulated *tonB* gene expression (log 2-fold change) by -2.1, -2.4, and -2.6 at 24h, 48, and 72h respectively. The picture shows the highly efficient turmeric extract to disrupt siderophore transport at three-time points. The obtained result shows that the extract significantly downregulated *tonB* gene expression of *F. oreochromis* compared to the control group.

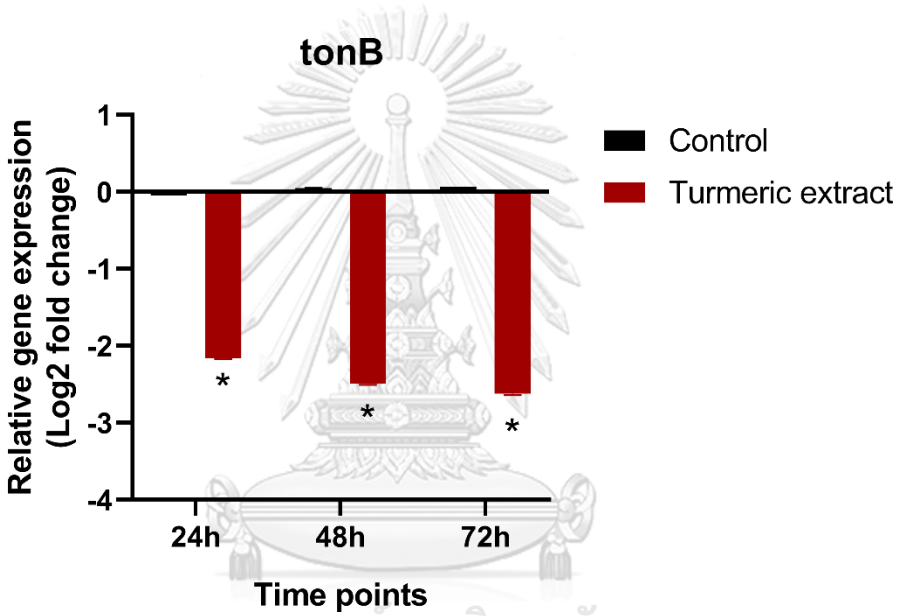

Figure 15 Relative iron acquisition gene expressions in the biofilm of *Flavobacterium oreochromis* (isolate 15) after exposure to turmeric extract at three time points (24h, 48h, and 72h). Turmeric extract downregulated *tonB* gene expression at three-time points. Bars represent mean relative expression ± standard deviation of three replicates and the asterisk (*) a represent the significant difference at $p < 0.05$ when compared to control group (student t-test; $p < 0.05$).

4.6.2 Expression of type IX secretion system (T9SS) genes after exposed to turmeric extract

Type IX secretion system is involved in biofilm formation and bacterial virulence. In this study, the effect of turmeric extracts was investigated on the type IX secretion system which includes bacteria gliding motility, bacteria adhesin, and toxin release. The result of T9SS gene expression after being exposed to turmeric extract was shown in Figure 16.

The *gldL* gene is responsible for the bacteria gliding motility. The effect of turmeric extract on bacteria gliding motility was shown in figure 16A. The picture revealed that turmeric extract downregulated *gldL* gene expression (log 2-fold change) by -2.1, -3.1, and -4.5 at 24h, 48, and 72h respectively. The obtained result indicates that turmeric extract shows the potential to inhibit biofilm at an early stage of formation by impeding the bacteria movement.

The *sprA* is responsible for bacteria adhesin. The effect of turmeric extract on bacteria adhesin was shown in figure 16B. The picture revealed that the extract significantly downregulated *sprA* gene expression (log 2-fold change) by -3.1, -2.9, and -3.9 at 24h, 48, and 72h respectively. The obtained result indicates that turmeric extract plays an important role to destroy biofilm structures by affecting their adherence ability.

The *porV* is responsible for bacteria toxin secretion. The effect of turmeric extracts on toxin secretion was shown in figure 16C. The figure revealed that turmeric extract significantly downregulated *porV* gene expression (log 2-fold change) by -2.8, -3.2, and -3.8 at 24h, 48, and 72h respectively. The obtained results indicates that turmeric extract highly accomplishes against the virulence of *F. oreochromis* by downregulating the expression genes of the type IX secretion system.

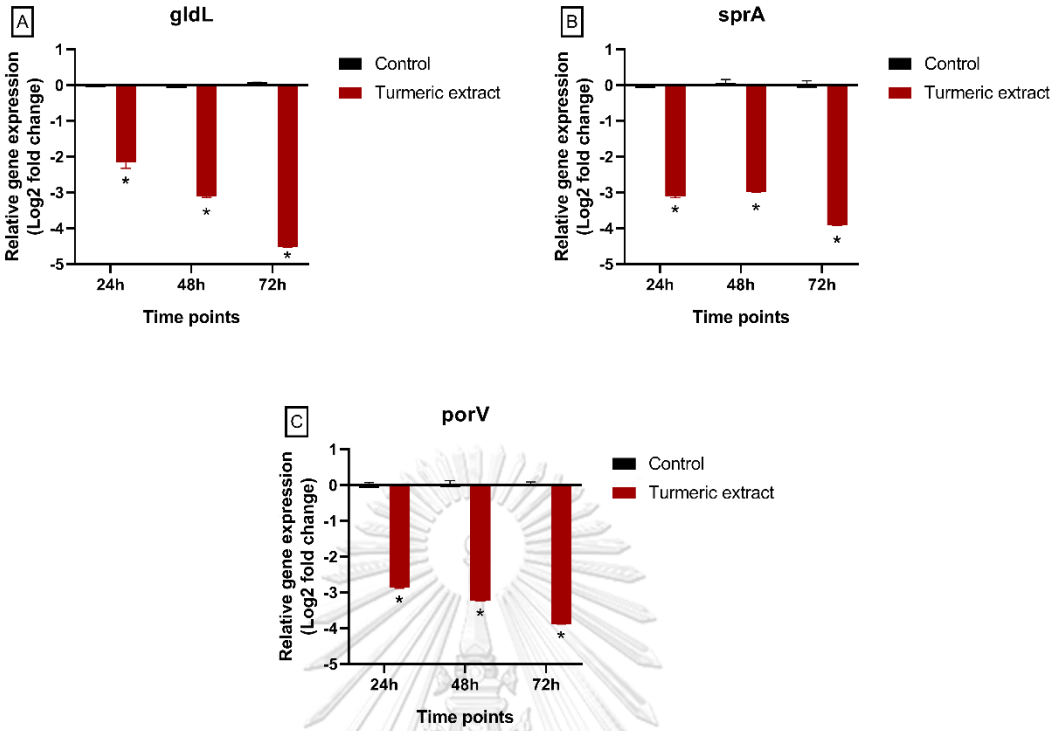

Figure 16 Relative T9SS gene expressions in the biofilm of *Flavobacterium oreochromis* (isolate 15) after exposure to turmeric extract at three time points (24h, 48h, and 72h). (A) Turmeric extract downregulated *gldL* gene expression at all time points. (B) Turmeric extract downregulated *sprA* gene expression at three-time points. (C) Turmeric extract downregulated *porV* gene expression at three-time point. Bars represent mean relative expression ± standard deviation of three replicates and the asterisk (*) represent the significant difference at $p < 0.05$ when compared to control group (student t-test; $p < 0.05$).

4.6.3 Expression of *luxR* gene after exposed to turmeric extract

The luxR is the master regulator for the quorum sensing system. The QS system is major control for biofilm formation and bacteria virulence. In this study, *luxR* gene expression was tested after exposure to turmeric extract. The effect of the extracts on *luxR* expression was shown in figure 17. The figure revealed that turmeric extract downregulated *luxR* gene expression (log 2-fold change) by -2.4, -2.9, and -3.7 at 24h, 48, and 72h respectively. The obtained result indicates that turmeric extracts exhibit a great potential for anti-biofilm formation and virulence of *F. oreochromis*.

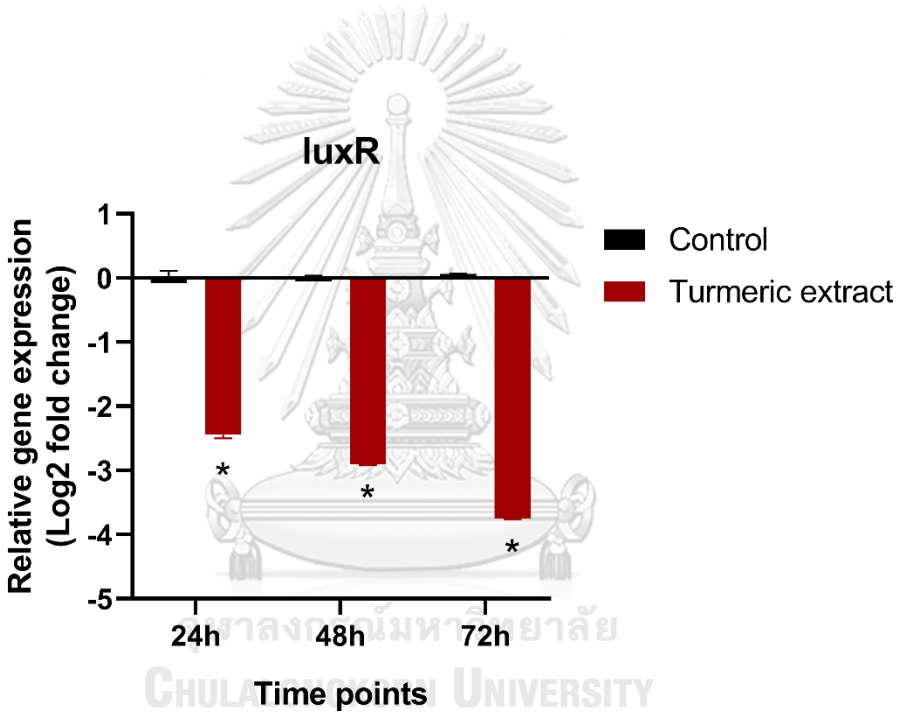

Figure 17 Relative *luxR* gene expressions in the biofilm of *Flavobacterium oreochromis* (isolate 15) after exposure to turmeric extract at three time points (24h, 48h, and 72h). Turmeric extract shown downregulated of *luxR* gene expression at three-time point. Bars represent mean relative expression ± standard deviation of three replicates and asterisk (*) represent the significant difference at $p < 0.05$ when compared to control group (student t-test; $p < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSIONS

1. Discussion

From this study, *F. oreochromis* grown on TYES medium exhibit two types of colonies, rhizoid and non-rhizoid. Similar results were also obtained from the study in which the bacteria were grown in the same medium (Dong et al., 2016; Penttinen et al., 2018). However, four colony morphotypes were observed in another studies in which the bacteria were grown in other media (shieh agar) (Kunttu et al., 2009; Dong et al., 2016). These results suggest that culture media and conditions can influence colony formation.

Interestingly, among the 28 isolates that originated from columnaris disease-affected fish studied, more rhizoid colonies were observed than their non-rhizoid counterparts. Since all these isolates were derived from infected fish, both colony morphotypes can likely cause the disease. These suggested both colony morphotypes are pathogenic) The results here agree with our infection study, in which several isolates of the bacteria of both morphotypes were used to infect the fish and both morphotypes were found to cause the disease. However, the results obtained here disagree with a previous report which shows that only the rhizoid colony morphotypes is pathogenic (Dong et al., 2016; Penttinen et al., 2018). It is possible that the bacteria changed or adapted to culture media and culture conditions in that lab, and some isolates that form rhizoid colonies might have converted to non-rhizoid forming isolates (Arias et al., 2012).

It has been known or generally accepted that the biofilm-forming ability of bacteria has some association with their pathogenicity and virulence (Declercq et al., 2013). In this study, 28 isolates of *F. oreochromis* were tested for their ability to form biofilm in TYES medium on 1.0 % tilapia gelatin-coated polystyrene plate. From the results obtained, all bacteria isolates can form biofilm on the coated plate as expected. Although their biofilm-forming abilities vary. Most of these isolates formed thick biofilm (OD. Reading of crystal violet-stained biofilm of between 3.0-3.7), following moderate biofilm thickness (OD. Reading of crystal violet-stained biofilm of

between 2.0-2.9), and low biofilm thickness (OD. Reading of crystal violet-stained biofilm of between 0.0-1.9).

This study observed no correlation between colony morphology and biofilm-forming ability. But since, these two phenotypic characteristics have been known to correlate with the pathogenicity and virulence of these bacteria (Decostere, 2002; Kunttu et al., 2009; Dong et al., 2016; Penttinen et al., 2018). It is possible that the correlation between one of these characteristics with virulence may not be an accurate or genuine association, which is very likely to be the colony morphology.

Bacteria preferred to live in the biofilm form more than a planktonic lifestyle because it protected bacteria from hazardous environments such as UV, host immunity, and antibiotic agents (Stewart, 2002; Flemming and Wingender, 2010). Eradication of biofilm typically requires a high concentration of antibiotic agents because of the extracellular polymeric substances (EPS). These substances play an important role against antibiotic agents by absorbing the agent resulting in an incapacity to penetrate biofilm. The use of antibiotic agents at high concentration cause concern about food safety; and the development of antibiotic resistance bacteria. Therefore, new strategies that impede the virulence factors of bacteria without influent on bacteria growth are necessary. In this study, turmeric extracts from three solvents (hexane, ethanol, methanol) were tested and found to inhibit both the growth and biofilm formations of 17 isolates of *F. oreochromis* in dose-dependent manners. The crude hexane extract of turmeric was found to be the most potent inhibitor against *F. oreochromis* growth and biofilm formation. Therefore, it was chosen to be used in the gene expression study of *F. oreochromis* isolate 15 after turmeric extract treatment. The extract was found to downregulate the expressions of genes involved in iron acquisition (*alcB*, *sido*, *rhbC*, and *tonB*), type 9 secretion system (*sprA*, *gldL*, and *porV*), and quorum sensing (*luxR*) of the *F. oreochromis* isolate 15.

This QS system helps bacteria alter the planktonic lifestyle to the biofilm lifestyle via autoinducer (Ais) production (Jiang et al., 2018). It is known that the quorum sensing systems (QS) regulate various virulence genes expression, including type IX secretion system and iron acquisition (Van Kessel et al., 2013; Hossain et al.,

2017). This study analyzed the effect of crude hexane extract of turmeric on the virulence of *F. oreochromis* isolate 15. Three major bacteria virulence factors were chosen based on their involvement in biofilm formation and pathogenesis of the bacteria that results in columnaris disease. The concentration of crude hexane extract of turmeric used was below minimal inhibition concentration (MIC), so it did not affect the growth of bacteria. Iron acquisition is vital for bacteria growth and metabolisms. However, during infection, bacteria usually encounter with insufficient supply of iron (Sheldon et al., 2016; Lange et al., 2020a). Iron is present in two forms in the environment or host, i.e., ferrous (Fe2+) or ferric (Fe3+) compound-binding proteins. To acquire iron from the environment, bacteria produce siderophores to trap or bind with iron and transport it into the bacteria cell via active transport pathways (Sheldon et al., 2016). Since *F. oreochromis,* the iron acquisition system is associated with biofilm formation (Cai et al., 2019; Lange et al., 2020a). In this study, I investigated the effects of crude hexane extracts of turmeric on four iron acquisition gene expressions: *sido*, *rhbC*, *alcB*, which are the genes responsible for siderophore synthesis, and *tonB*, the gene responsible for iron-bound siderophore transport into the bacteria cell (Cai et al., 2019; Lange et al., 2020a). I found that all three siderophores as well as the *tonB* gene expressions of *F. oreochromis* isolate 15 were strongly downregulated by the crude hexane extract of turmeric. The downregulations of these genes would mean less siderophore proteins are being produced and available to trap iron ions. At the same time, the downregulation of *tonB* gene expression would also reduce siderophore-iron transportation into the cells resulting in reduced iron available for the bacteria to use for their growth and other activities such as biofilm formation (Giardina et al., 1997; Lynch et al., 2001). The results showed that the downregulation of iron acquisition genes might contribute to the failure of biofilm formation.

Type IX secretion (T9SS) system plays a vital role in gliding motility and biofilm formation (Lasica et al., 2017; Li et al., 2017; Thunes et al., 2022). Type IX secretion system of *F. oreochromis* has been reported to be responsible for the secretion of several enzymes, adhesins, and other proteins important in biofilm formation and virulence, such as chondroitin AC lyase, collagenase, and protease

(Declercq et al., 2013; Li et al., 2017; Thunes et al., 2022). Therefore, interfering with the type IX secretion system would result in less virulent bacteria (Li et al., 2017). In this study, I found that crude hexane extract of turmeric efficiently reduced the expression of all three genes (*sprA*, *gldL*, and *porV*) at all three time points after incubation in T9SS of *F. oreochromis* isolate 15 studied. In a study by Thunes and colleagues (Thunes et al., 2022) in *F. columnare* mutant with deletion of *gldN,* and *porV* genes, bacteria protein secretion and virulence were found to be affected, suggesting these genes have roles in bacterial virulence. Inhibitions of these genes would result in decreased ability to attach to the host surface, reduced bacterial ability to glide towards other bacterial cells to form microcolonies and produce biofilm, and decreased chondroitin AC lyase release to destroy the host tissues. Downregulation of sprA has been shown to lead to biofilm production failure in *Flavobacterium columnare* (Penttinen et al., 2018). Quorum sensing is a cell-cell communication system of bacteria via the production of molecules called autoinducers (Ais). The QS system in Gram-negative bacteria is well documented, especially for *Vibrio* spp (Lazdunski et al., 2004). Three different autoinducers are found to regulate the QS system in *Vibrio* spp, namely cholerae autoinducer 1 (CAI-1), harveyi autoinducer 1 (HAI-1), and autoinducer 2 (AI2) which are synthesized by *cqs*A, *lux*m, and *lux*S, respectively (Cao and Meighen, 1989; Chen et al., 2002; Higgins et al., 2007). Unfortunately, *F. oreochromis* quorum sensing circuits are not yet fully characterized, only two studies report that *lux*R (One of the master regulators of quorum sensing of Gram negative bacteria) is present in the regulatory system (Lange et al., 2018; Cai et al., 2019). In the present study, crude hexane extract of turmeric strongly downregulated *luxR* in *F. oreochromis* at all three-time points. In Gram-negative bacteria (*Vibrio harveyi*), *luxR* and *aphA* genes, the master quorum-sensing regulator genes at high and low cell densities, work together and control multiple quorum-sensing regulated genes *(Van Kessel et al., 2013)*. Many studies have shown that luxR is associated with bacteria virulence. For example, the study from (Croxatto et al., 2002) revealed that the wild type *Vibrio anguillarum* produced more biofilm when compared to the *luxR* mutated strain. Therefore, downregulation of *luxR* gene expression results in less biofilm production.

Our results agree with previous studies that plant phytochemicals such as curcumin can reduce the virulence determinants of many pathogenic bacteria. For example, the study by Packiavathy and colleagues (Packiavathy et al., 2013) found that curcumin strongly inhibits bioluminescence, bacteria movement, biofilm production, and other virulence aspects of *Vibrio* spp. In addition, flavonoids produced by *Curcuma spp.* have been shown to inhibit the biofilm and surface motility of *Acinetobacter baumannii* (Raorane et al., 2019). These findings indicate that turmeric extracts can be used as an alternative strategy against biofilm formation and virulence of *Flavobacterium oreochromis*.

2. Conclusion and suggestion

The emergence of antibiotic-resistant bacteria causes global concern in many fields, including aquaculture. Turmeric extracts show great potential against both Gram-negative and Gram-positive bacteria. In this study, turmeric extracts were found to inhibit the biofilm formation of *F. oreochromis*. This biofilm inhibition is associated with the downregulation of genes in iron acquisition, the T9SS secretion, and the quorum sensing systems. The downregulation of these genes is also associated with less biofilm production, which is one of the virulent determinants of the bacteria.

3. Advantages of the study

The study has shown the efficiency of turmeric extracts against biofilm formation and virulence of *F. oreochromis*. If proven effective in the fish experiment, the results from this study can be applied to prevent Nile tilapia against columnaris disease.

Appendix

Instruments

The instruments used in this study are listed below:

Incubator

Shanking incubator

Stereo microscope

Spectrophotometer

Microplate reader

Blender

Hot air oven

Digital balance

Soxhlet apparatus

Evaporator machine

Freez dry machine

Nanodrops spectrophotometer

PCR machine

Gel electrophoresis system

Gel documentation

Real-time PCR machine

Centrifuge

Spin-down centrifuge

Vortex mixer

Refrigerator

Water bath

Chemicals and reagent

Culture medium (Tryptone Yest Extract Salts : TYES 1000 ml)

Tryptone	4.0 g
Yeast extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Agar	10 g
Distilled water	1000 ml

Chemicals and Reagents

Glycerol

Hexane

Ethanol

Methanol

Dimethyl sulfoxide (DMSO)

Ethidium bromide

Diethylpyrocarbonate (DEPC)

Agarose

Aceti acid

Crystal violet

Tilapia gelatin

TRIzol reagent

Deoxynucleoside triphosphates (dNTP)

DNA ladder

Tris-Borate EDTA

REFERENCES

Alderman D and Hastings T 1998. Antibiotic use in aquaculture: development of antibiotic resistance–potential for consumer health risks. International journal of food science & technology. 33(2): 139-155.

Ali IA and Neelakantan P 2022. Antibiofilm activity of phytochemicals against Enterococcus faecalis: A literature review. Phytotherapy Research.

Anacker RL and Ordal EJ 1955. Study of a bacteriophage infecting the myxobacterium Chondrococcus columnaris. Journal of Bacteriology. 70(6): 738-741.

Arias CR, LaFrentz S, Cai W and Olivares-Fuster O 2012. Adaptive response to starvation in the fish pathogen Flavobacterium columnare: cell viability and ultrastructural changes. BMC microbiology. 12(1): 1-12.

Austin B, Austin DA and Munn C 2007. Bacterial fish pathogens: disease of farmed and wild fish. Vol 26. In: Springer.

Bernardet J-F and Bowman JP 2006a. The genus flavobacterium. The prokaryotes. 7: 481-531.

Bernardet J-F and Bowman JP 2006b. The Genus Flavobacterium. In: The Prokaryotes. 481-531.

Bernardet J-F and Grimont PA 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. International Journal of Systematic and Evolutionary Microbiology. 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). International Journal of Systematic and Evolutionary Microbiology. 46(1): 128-148.

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. Veterinary Microbiology. 155(2-4): 355-361.

Cai W and Arias CR 2017. Biofilm Formation on Aquaculture Substrates by Selected Bacterial Fish Pathogens. J Aquat Anim Health. 29(2): 95-104.

Cai W, De La Fuente L and Arias CR 2013. Biofilm formation by the fish pathogen Flavobacterium columnare: development and parameters affecting surface attachment. Appl Environ Microbiol. 79(18): 5633-5642.

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 microbiology. 19(1): 1-11.

Cao J-G and Meighen E 1989. Purification and structural identification of an autoinducer for the luminescence system of Vibrio harveyi. Journal of Biological Chemistry. 264(36): 21670-21676.

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.

Chitmanat C, Lebel P, Whangchai N, Promya J and Lebel L 2016. Tilapia diseases and management in river-based cage aquaculture in northern Thailand. Journal of Applied Aquaculture. 28(1): 9-16.

Chockmangmeepisan P, Sakulworakan R, Dong HT, Kayansamruaj P, Rung-ruangkijkrai T, Pirarat N and Rodkhum C 2020. Virulence properties and pathogenicity of Flavobacterium columnare in hybrid red tilapia (Oreochromis sp.). The Thai Journal of Veterinary Medicine. 50(1): 103-108.

Conrad J, Holt R and Kreps T 1975. Ozone disinfection of flowing water. The Progressive Fish-Culturist. 37(3): 134-136.

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. Journal of bacteriology. 184(6): 1617-1629.

Dalsgaard I 1993. Virulence mechanisms in Cytophaga psychrophila and other Cytophaga-like bacteria pathogenic for fish. Annual Review of Fish Diseases. 3:

127-144.

Darwish A, Bebak J and Schrader K 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. Journal of fish diseases. 35(9): 637-647.

Davies D 2003. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov. 2(2): 114-122.

Davis HS 1922. A New Bacterial Disease of Fresh-water Fishes. In: US Government Printing Office.

Dawood MA, Koshio S, Abdel-Daim MM and Van Doan H 2019. Probiotic application for sustainable aquaculture. Reviews in Aquaculture. 11(3): 907-924.

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, Haesebrouck F, Van den Broeck W, Bossier P and Decostere A 2013. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Veterinary research. 44(1): 1-17.

Declercq AM, Tilleman L, Gansemans Y, De Witte C, Haesebrouck F, Van Nieuwerburgh F, Smet A and Decostere A 2021. Comparative genomics of Flavobacterium columnare unveils novel insights in virulence and antimicrobial resistance mechanisms. Vet Res. 52(1): 18.

Decostere A 2002. Flavobacterium columnare infections in fish: the agent and its adhesion to the gill tissue. Verhandelingen-Koninklijke Academie voor Geneeskunde van België. 64(6): 421-430.

Decostere A and Haesebrouck F 1999. Outbreak of columnaris disease in tropical aquarium fish. The Veterinary Record. 144(1): 23-24.

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. Journal of fish diseases. 22(1): 1-11.

Defoirdt T, Brackman G and Coenye T 2013. Quorum sensing inhibitors: how strong is the evidence? Trends Microbiol. 21(12): 619-624.

Dong H, 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. Journal of Fish Diseases. 39(6): 649-655.

El-Sayed A-FM 2019. Tilapia culture. In: Academic Press.

Flemming HC and Wingender J 2010. The biofilm matrix. Nat Rev Microbiol. 8(9): 623-633.

Foscarini R 1989. Induction and development of bacterial gill disease in the eel (Anguilla japonica) experimentally infected with Flexibacter columnaris: pathological changes in the gill vascular structure and in cardiac performance. Aquaculture. 78(1): 1-20.

Fujihara M and Nakatani R 1971. Antibody production and immune responses of rainbow trout and coho salmon to Chondrococcus columnaris. Journal of the Fisheries Board of Canada. 28(9): 1253-1258.

Garnjobst L 1945. Cytophaga columnaris (Davis) in pure culture: a myxobacterium pathogenic to fish. Journal of Bacteriology. 49(2): 113-128.

Ghannoum M, Parsek M, Whiteley M and Mukherjee PK 2020. Microbial biofilms. In: John Wiley & Sons.

Giardina PC, Foster L-A, 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.

Gul P and Bakht J 2015. Antimicrobial activity of turmeric extract and its potential use in food industry. Journal of food science and technology. 52(4): 2272-2279.

Guzman JPMD, Yatip P, Soowannayan C and Maningas MBB 2022a. Piper betle L. leaf extracts inhibit quorum sensing of shrimp pathogen Vibrio harveyi and protect Penaeus vannamei postlarvae against bacterial infection. Aquaculture. 547: 737452.

Guzman JPMD, Yatip P, Soowannayan C and Maningas MBB 2022b. Piper betle L. leaf extracts inhibit quorum sensing of shrimp pathogen Vibrio harveyi and protect

Penaeus vannamei postlarvae against bacterial infection. Aquaculture. 547.

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.

Hossain MA, Lee SJ, Park NH, Mechesso AF, Birhanu BT, Kang J, Reza MA, Suh JW and Park SC 2017. Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. Sci Rep. 7(1): 10618.

Jain A and Parihar D 2018a. Antibacterial, biofilm dispersal and antibiofilm potential of alkaloids and flavonoids of Curcuma. Biocatalysis and agricultural biotechnology. 16: 677-682.

Jain A and Parihar DK 2018b. Antibacterial, biofilm dispersal and antibiofilm potential of alkaloids and flavonoids of Curcuma. Biocatalysis and Agricultural Biotechnology. 16: 677-682.

Jardak M, Mnif S, Ayed RB, Rezgui F and Aifa S 2021. Chemical composition, antibiofilm activities of Tunisian spices essential oils and combinatorial effect against Staphylococcus epidermidis biofilm. Lwt. 140: 110691.

Jiang T, Zhu P, Du L and Li M 2018. Identification of AI-2 quorum sensing inhibitors in Vibrio harveyi through structure-based virtual screening. In: Quorum Sensing. Springer. 353-362.

Karunasagar I 2020. Complexities Involved in Source Attribution of Antimicrobial Resistance Genes Found in Aquaculture Products. Asian Fisheries Science. 33S.

Khaliq T, Waseem MA, Lone AM and Hassan QP 2018a. Oscimum sanctum extract inhibits growth of Gram positive and Gram negative bacterial strains. Microb Pathog. 118: 211-213.

Khaliq T, Waseem MA, Lone AM and Hassan QP 2018b. Oscimum sanctum extract inhibits growth of Gram positive and Gram negative bacterial strains. Microbial pathogenesis. 118: 211-213.

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 Immunology. 112: 81-91.

Kitiyodom S, Yata T, Yostawornkul J, Kaewmalun S, Nittayasut N, Suktham K, Surassmo S, Namdee K, Rodkhum C and Pirarat N 2019. Enhanced efficacy of immersion vaccination in tilapia against columnaris disease by chitosan-coated "pathogen-like" mucoadhesive nanovaccines. Fish & shellfish immunology. 95: 213-219.

Kunttu HM, Suomalainen L-R, Jokinen EI and Valtonen ET 2009. Flavobacterium columnare colony types: connection to adhesion and virulence? Microbial pathogenesis. 46(1): 21-27.

Labban L 2014. Medicinal and pharmacological properties of Turmeric (Curcuma longa): A review. Int J Pharm Biomed Sci. 5(1): 17-23.

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. Frontiers in microbiology. 9: 452.

LaFrentz BR, Králová S, Burbick CR, Alexander TL, Phillips CW, Griffin MJ, Waldbieser GC, García JC, de Alexandre Sebastião F and Soto E 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. Systematic and Applied Microbiology. 45(2): 126293.

LaFrentz BR, Waldbieser G, Welch T and Shoemaker C 2014. Intragenomic heterogeneity in the 16 S r RNA genes of F lavobacterium columnare and standard protocol for genomovar assignment. Journal of Fish Diseases. 37(7): 657-669.

Lange MD, Farmer BD and Abernathy J 2018. Catfish mucus alters the Flavobacterium columnare transcriptome. FEMS microbiology letters. 365(22): fny244.

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

Lange MD, Farmer BD and Abernathy J 2020b. Vertebrate mucus stimulates biofilm development and upregulates iron acquisition genes in Flavobacterium columnare. Journal of fish diseases. 43(1): 101-110.

Lara-Flores M 2011. The use of probiotic in aquaculture: an overview. Int Res J

Microbiol. 2(12): 471-478.

Lasica AM, Ksiazek M, Madej M and Potempa J 2017. The type IX secretion system (T9SS): highlights and recent insights into its structure and function. Frontiers in cellular and infection microbiology. 7: 215.

Lazdunski AM, Ventre I and Sturgis JN 2004. Regulatory circuits and communication in Gram-negative bacteria. Nat Rev Microbiol. 2(7): 581-592.

Li N, Zhu Y, LaFrentz BR, Evenhuis JP, Hunnicutt DW, Conrad RA, Barbier P, Gullstrand CW, Roets JE and Powers JL 2017. The type IX secretion system is required for virulence of the fish pathogen Flavobacterium columnare. Applied and environmental microbiology. 83(23): e01769-01717.

Lynch D, O'Brien J, Welch T, Clarke P, ÓCuív Pr, 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. Journal of bacteriology. 183(8): 2576-2585.

Mazel D and Davies J 1999. Antibiotic resistance in microbes. Cellular and Molecular Life Sciences CMLS. 56(9): 742-754.

Mireles JR, Toguchi A and Harshey RM 2001. Salmonella enterica serovar Typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. Journal of bacteriology. 183(20): 5848-5854.

MORRISON C, Cornick J, Shum G and Zwicker B 1981. Microbiology and histopathology of ‘saddleback’disease of underyearling Atlantic salmon, Salmo salar L. Journal of Fish Diseases. 4(3): 243-258.

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

Oulahal-Lagsir N, Martial-Gros A, Bonneau M and Blum LJ 2003. “Escherichia coli-milk” biofilm removal from stainless steel surfaces: synergism between ultrasonic waves and enzymes. Biofueling. 19(3): 159-168.

Pacha R and Ordal E 1967. Histopathology of experimental columnaris disease in young salmon. Journal of Comparative Pathology. 77(4): 419-423.

Packiavathy IASV, Sasikumar P, Pandian SK and Veera Ravi A 2013. Prevention of

quorum-sensing-mediated biofilm development and virulence factors production in Vibrio spp. by curcumin. Applied microbiology and biotechnology. 97(23): 10177-10187.

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. Frontiers in microbiology. 9: 525.

Pooljun C, Daorueang S, Weerachatyanukul W, Direkbusarakom S and Jariyapong P 2020. Enhancement of shrimp health and immunity with diets supplemented with combined probiotics: application to Vibrio parahaemolyticus infections. Diseases of Aquatic Organisms. 140: 37-46.

Ransom DP 1975. Immune responses of salmonids: a) Oral immunization against Flexibacter columnaris: b) Effects of combining antigens in parenterally administered polyvalent vaccines.

Raorane CJ, Lee J-H, Kim Y-G, Rajasekharan SK, García-Contreras R and Lee J 2019. Antibiofilm and antivirulence efficacies of flavonoids and curcumin against Acinetobacter baumannii. Frontiers in microbiology. 10: 990.

Reverter M, Bontemps N, Lecchini D, Banaigs B and Sasal P 2014. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. Aquaculture. 433: 50-61.

Rico A, Oliveira R, McDonough S, Matser A, Khatikarn J, Satapornvanit K, Nogueira AJ, Soares AM, Domingues I and Van den Brink PJ 2014. Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. Environ Pollut. 191: 8-16.

Riley TA 2000. Treatment of Flavobacterium columnare and toxicity of chloramine-T in channel catfish, Ictalurus punctatus. Auburn University.

Rodrigues L, Van der Mei H, Teixeira JA and Oliveira R 2004. Biosurfactant from Lactococcus lactis 53 inhibits microbial adhesion on silicone rubber. Applied microbiology and biotechnology. 66(3): 306-311.

Rogers WA 1971. Principal diseases of catfish: how to identify and fight them. In: Watt Publishing Company.

Sharma R, Gescher A and Steward W 2005. Curcumin: the story so far. European journal

of cancer. 41(13): 1955-1968.

Sheldon JR, Laakso HA and Heinrichs DE 2016. Iron acquisition strategies of bacterial pathogens. Microbiology spectrum. 4(2): 4.2. 05.

Simões M, Simões LC and Vieira MJ 2010. A review of current and emergent biofilm control strategies. LWT-Food Science and Technology. 43(4): 573-583.

Soowannayan C, Boonmee S, Puckcharoen S, Anatamsombat T, Yatip P, Ng W-K, Thitamadee S, Tuchinda P, Munyoo B and Chabang N 2019a. Ginger and its component shogaol inhibit Vibrio biofilm formation in vitro and orally protect shrimp against acute hepatopancreatic necrosis disease (AHPND). Aquaculture. 504: 139-147.

Soowannayan C, Chandra Teja DN, Yatip P, Mazumder FY, Krataitong K, Unagul P, Suetrong S, Preedanon S, Klaysuban A, Sakayaroj J and Sangtiean T 2019b. Vibrio biofilm inhibitors screened from marine fungi protect shrimp against acute hepatopancreatic necrosis disease (AHPND). Aquaculture. 499: 1-8.

Stewart PS 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol. 292(2): 107-113.

Suomalainen L-R, Tiirola M and Valtonen E 2005a. Treatment of columnaris disease of rainbow trout: low pH and salt as possible tools? Diseases of Aquatic Organisms. 65(2): 115-120.

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

Thunes NC, Conrad RA, Mohammed HH, Zhu Y, Barbier P, Evenhuis JP, Perez-Pascual D, Ghigo J-M, Lipscomb RS and Schneider JR 2022. Type IX secretion system effectors and virulence of the model Flavobacterium columnare strain MS-FC-4. Applied and environmental microbiology. 88(3): e01705-01721.

Van Kessel JC, 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. Journal of bacteriology. 195(3): 436-443.

Verschuere L, Rombaut G, Sorgeloos P and Verstraete W 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and molecular biology

reviews. 64(4): 655-671.

Wang Q, Shen J, Yan Z, Xiang X, Mu R, Zhu P, Yao Y, Zhu F, Chen K and Chi S 2020. Dietary Glycyrrhiza uralensis extracts supplementation elevated growth performance, immune responses and disease resistance against Flavobacterium columnare in yellow catfish (Pelteobagrus fulvidraco). Fish & Shellfish Immunology. 97: 153-164.

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 F 2020. A review on the application of herbal medicines in the disease control of aquatic animals. Aquaculture. 526.

VITA

NAME	Sirawich Ngernson
DATE OF BIRTH	11 january 1996
PLACE OF BIRTH	Pathum Thani
INSTITUTIONS ATTENDED	Bachelor of Science (2019) Burapha University
HOME ADDRESS	69/580, Bueng Yitho, Thanyaburi, Pathum Thani 12130
PUBLICATION	Ngernson S 2022. Inhibitory effects of herbal extracts against Flavobacterium oreochromis biofilm. Thai J Vet Med. 52(suppl): 237-239.
AWARD RECEIVED	Scholarship-Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej