EFFECTS OF *Bacillus velezensis* S141 SUPPLEMENTATION ON GROWTH, IMMUNE RESPONSES, AND DISEASE RESISTANCE OF *Litopenaeus vannamei*

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ผลของอาหารเสริม *Bacillus velezensis* S141 ต่อการเจริญเติบโต, การตอบสนองของระบบภูมิคุ้มกันและความต้านทานโรคของกุ้งขาว

นางสาวตวงรัก สืบกงแสง

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

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คำสำคัญ: *Bacillus velezensis* โปรไบโอติก กุ้งขาว *Enterocytozoon hepatopenaei* (EHP) โรคตัวแดงดวงขาว (WSSV) *Vibrio parahaemolyticus* (AHPND)

ในอุตสาหกรรมการเพาะเลี้ยงกุ้งขาว (*Litopenaeus vannamei*) เผชิญกับปัญหาการระบาดของโรคติดเชื้ออย่างต่อเนื่อง จึงมีความเป็นเร่งด่วนในการพัฒนาวิธีการใหม่ ๆ เพื่อควบคุมและป้องกันโรค การศึกษานี้มีวัตถุประสงค์เพื่อวิเคราะห์ประสิทธิภาพของ *Bacillus velezensis* S141 ที่ผสมในอาหารที่ระดับความเข้มข้น 10^2, 10^4, และ 10^6 CFU/g ต่อการตอบสนองทางภูมิคุ้มกัน ประสิทธิภาพการเจริญเติบโต และความต้านทานต่อโรค โดยเน้นการป้องกันไวรัส White Spot Syndrome Virus (WSSV), เชื้อ *Vibrio parahaemolyticus* (AHPND) และเชื้อ *Enterocytozoon hepatopenaei* (EHP) ผลการศึกษาแสดงให้เห็นว่า กุ้งที่ได้รับอาหารเสริม *B. velezensis* S141 มีน้ำหนักเพิ่มขึ้น (Weight Gain, WG) การเพิ่มน้ำหนักเฉลี่ยต่อวัน (Average Daily Gain, ADG) และอัตราการเจริญเติบโตจำเพาะ (Specific Growth Rate, SGR) สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ นอกจากนี้ กุ้งที่ได้รับ *B. velezensis* S141 มีอัตราการรอดตายหลังติดเชื้อ WSSV สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ อีกทั้ง *B. velezensis* S141 secretion ร่วมกับ WSSV ช่วยเพิ่มอัตราการรอดตายและลดปริมาณไวรัสในเหงือกได้อย่างมีนัยสำคัญ ซึ่งบ่งชี้ถึงความสามารถในการต้านการติดเชื้อ WSSV เพิ่มขึ้น นอกจากนี้การเสริม *B. velezensis* S141 ในอาหารยังช่วยลดอัตราการตายสะสมของกุ้งที่ติดเชื้อ VP_{AHPND} และลดจำนวนของเชื้อ EHP เมื่อเทียบกับกลุ่มควบคุม จากผลการวิเคราะห์การแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันระบบ Toll/IMD pathway (Toll, IMD, LYZ1, LYZ-C, PEN4, ALF1, Relish), JAK/STAT pathway (STAT และ GILT), Vago pathway (Vago4, Vago5), NF-KB pathway (NF-kappa), TLR pathway (RPX และ DOME), รวมถึง CathC และ α2M พบว่า การแสดงออกของยีนเพิ่มขึ้นอย่างมีนัยสำคัญในกลุ่มที่ได้รับอาหารที่เสริม *B. velezensis* S141 ความเข้มข้น 10^6 CFU/g เมื่อเทียบกับกลุ่มควบคุม การศึกษานี้เน้นย้ำถึงศักยภาพของ *B. velezensis* S141 ในรูปแบบโปรไบโอติกที่เสริมสร้างภูมิคุ้มกันและเพิ่มความต้านทานต่อโรค

WSSV, EHP และ VP_{AHPND} ในกุ้ง *L. vannamei* ซึ่งเป็นแนวทางที่มีศักยภาพสำหรับการเพาะเลี้ยงกุ้งอย่างยั่งยืน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ศศิวรัก กิ่งกาญจน์

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา

TUANGRAK SEABKONGSENG : EFFECTS OF *Bacillus velezensis* S141 SUPPLEMENTATION ON GROWTH, IMMUNE RESPONSES, AND DISEASE RESISTANCE OF *Litopenaeus vannamei.* THESIS ADVISOR : ASST. PROF. PAKPOOM BOONCHUEN, Ph.D., 96 PP.

Keywords: *Bacillus velezensis*/Probiotic/*Enterocytozoon hepatopenaei* (EHP)/White spot syndrome virus (WSSV)/*Vibrio parahaemolyticus* (AHPND)

The *Litopenaeus vannamei* aquaculture industry is continually facing outbreaks of infectious diseases. This urgent situation requires the development of innovative strategies for disease control and prevention. This study evaluated the efficacy of *Bacillus velezensis* S141, supplemented in feed at concentrations of 10^2, 10^4, and 10^6 CFU/g, on growth performance, immune responses, and disease resistances against the White Spot Syndrome Virus (WSSV), *Vibrio parahaemolyticus* (VP_{AHPND}), and *Enterocytozoon hepatopenaei* (EHP). The shrimp fed with *B. velezensis* S141-supplemented feeds exhibited significant enhanced weight gain (WG), average daily gain (ADG), and specific growth rate (SGR) compared to the control group. Moreover, shrimp supplemented with *B. velezensis* S141 had significantly higher survival rates post-WSSV infection. Co-injection of *B. velezensis* S141 secretion with WSSV significantly improved survival rates and reduced WSSV copy numbers in the gill tissues, indicating enhanced resistance to the WSSV infection. Administering *B. velezensis* S141 reduced cumulative mortality during the VP_{AHPND} challenge and decreased EHP copy number compared to the control group. Furthermore, the gene expression analyses revealed significant upregulation of immune-related genes in the gill tissue of shrimp fed with *B. velezensis* S141, including genes in the Toll/IMD pathway (Toll, IMD, LYZ1, LYZ-C, PEN4, ALF1, Relish), JAK/STAT pathway (STAT and GILT), Vago pathway (Vago4, Vago5), NF-**K**B pathway (NF-kappa), TLR pathway (RPX and DOME), CathC, and **α**2M. This study emphasizes the potential of *B. velezensis* S141 as a probiotic supplement for enhancing shrimp immune responses and disease

resistance against WSSV, EHP, and VP_{AHPND} in *L. vannamei*, providing promising prospects for sustainable shrimp aquaculture practices.

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LIST OF ABBREVIATIONS

°C	=	Degree celsius
µ	=	Micro
×g	=	Relative centrifugal force
ADG	=	Average Daily Gain
AHPND	=	Acute hepatopancreatic necrosis disease
ALFs	=	Anti-lipopolysaccharide factors
ANOVA	=	Analysis of Variance
CAT	=	Catalase
CFU	=	Colony-Forming Unit
cDNA	=	Complementary DNA
DNA	=	Deoxyribonucleic acid
DOME	=	Domeless
EF-1α	=	Elongation factor-1 alpha
EHP	=	*Enterocytozoon hepatopenaei*
GILT	=	Gamma-Interferon-Inducible Lysosomal Thiol Reductase
g	=	Gram
gDNA	=	Genomic DNA
h	=	Hour
hpi	=	Hour post-infection
IMD	=	Immune Deficiency Pathway
JAK	=	Janus Kinase
l	=	Liter
LYZ	=	Lysozyme
LYZ-C	=	Lysozyme-C
m	=	Milli
M	=	Molar
min	=	Minute

LIST OF ABBREVIATIONS (Continued)

n	=	Nano
NaCl	=	Sodiam cholride
NF-**K**B	=	Nuclear Factor Kappa B
PCR	=	Polymerase Chain Reaction
PEN	=	Penaeidin
pH	=	Potential of hydrogen ion
PPO	=	Phenoloxidase
PRX	=	Peroxiredoxin
q	=	Quantitative
RNA	=	Ribonucleic Acid
RPX	=	Reactive Peroxide Activity
RNA	=	Ribonucleic Acid
RPX	=	Reactive Peroxide Activity
SD	=	Standard Deviation
SGR	=	Specific Growth Rate
STAT	=	Signal Transducer and Activator of Transcription
TLR	=	Toll-Like Receptor
TNF**α**	=	Tumor necrosis alpha
TOR	=	Target of Rapamycin
TRAF	=	Tumor Necrosis Factor Receptor-Associated Factor
VP	=	*Vibrio parahaemolyticus*
WSSV	=	White spot syndrome virus
α	=	Alpha
β	=	Beta
%	=	Percent

CHAPTER I
INTRODUCTION

1.1 Background

Litopenaeus vannamei, commonly known as the Pacific white shrimp, holds significant importance in shrimp farming and serves as a vital source of income globally. However, the problem of infectious diseases in shrimp farming is intensifying due to an increasing variety of pathogens. Numerous infections pose a threat to the shrimp farming (Chiu et al., 2007; Neiland et al., 2001). To combat these infections, farmers often resort to antibiotics. Still, while effective for treating various ailments, the overuse of antibiotics has raised concerns about environmental contamination and potential human health risks. This is largely because such overuse could contribute to the emergence of antibiotic-resistant pathogens in the future (Dawood et al., 2020).

Disease outbreaks have long been a devastating blow to the shrimp farming industry (Amaya et al., 2007; Jin et al., 2018). The White Spot Syndrome Virus (WSSV) is a large, enveloped double-stranded DNA virus linked to the *Whispovirus* genus within the *Nimaviridae* virus family. It affects a wide range of crustaceans, with mortality rates in shrimp reaching up to 100% within 3 to 5 days post-infection (Pradeep et al., 2012; Sánchez-Martínez et al., 2007). Furthermore, the bacterium *Vibrio parahaemolyticus*, which causes acute hepatopancreatic necrosis disease (AHPND), can produce pore-forming proteins known as Photorhabdus insect-related toxins or Pir. These are formed from the oligomerization of two heterodimeric proteins, PirA and PirB. When these toxins are produced within infected shrimp, they target and destroy hepatopancreas cells, which results in acute pancreatitis and liver disease. As a result, infected shrimp can experience swift mortality, with death rates reaching up to 100% within 48 (De Schryver et al., 2014; Prachumwat et al., 2019). Additionally, *Enterocytozoon hepatopenaei* (EHP) – a microsporidian pathogen – affects the hepatopancreas of its host and has been linked to poor growth in aquaculture settings (Chaijarasphong et al., 2021).

Currently, probiotics and prebiotics are being used to enhance host immunity, decrease disease susceptibility, and improve the overall health of aquatic organisms (Zhu et al., 2021). In the aquaculture industry, probiotics are increasingly being considered as an alternative approach to treating aquatic diseases (Ninawe & Selvin, 2009). For instance, the use of probiotic *Bacillus coagulans* ATCC 7050 (BC) in shrimp diets can enhance growth, intestinal health, immune response, and resistance to *V. parahaemolyticus* infections (Amoah et al., 2019). Additionally, *Bacillus* PC465 probiotic supplementation at $1x10^9$ CFU/g has shown beneficial effects on growth, digestive health, gut microbiota, and improved resistance to WSSV infection of *L. vannamei* shrimp (Chai et al., 2016). Upon implementing feed containing a mixture of bacteria such as *Staphylococcus hemolyticus* and *Pediococcus pentosaceus*, the mortality of WSSV infection and IHHNV-induced hematopoietic necrosis infection was found to decrease in shrimp fed with this formulated feed (Leyva-Madrigal et al., 2011). Moreover, a basal diet with continuous supplementation of *Rhodotorula paludigena* CM33 was shown to efficiently increase shrimp growth, bacterial disease resistance, and the activities of superoxide dismutase (SOD) and catalase (CAT), which are molecular biomarkers for assessing the oxidative stress status (Sriphuttha et al., 2023; Yang et al., 2015). Therefore, the application of probiotics in aquatic farming is emerging as an instrumentally alternative strategy to improve animal health and survival rates while noticeably reducing the environmental impacts posed by the use of antibiotics.

B. velezensis is a microorganism that has been extensively studied for its biocontrol properties in plants, suggesting that it can inhibit bacterial and fungal diseases, thereby promoting plant development in the rhizosphere (Gu et al., 2017). Moreover, the potential of *B. velezensis* as a probiotic in shrimp has been established. It was discovered that *B. velezensis* CPA1–1 could inhibit the growth of *V. cholerae* non-O1175, which causes disease in *Macrobrachium nipponense*, and stimulates the immune system (Zhu et al., 2021). In *L. vannamei*, the introduction of *B. velezensis* BV007 in shrimp feed could enhance the growth performance of shrimp and reduce *V. parahaemolyticus* infection (Chen et al., 2021). *B. velezensis* S141 has previously been isolated and studied for its anti-fungal properties against plant pathogens, attributable to its secretory molecules (Sibponkrung et al., 2017; Songwattana et al., 2023).

However, no research has been reported on the effects of *B. velezensis* on detailed immune responses and resistance to other pathogens (WSSV and EHP) in shrimp. Moreover, no reports exist on the application of *B. velezensis* S141 as a probiotic in shrimp, despite the possibility that this bacterial strain may secrete various antimicrobial molecules that might benefit shrimp. Therefore, this study focuses on the benefits of supplementing *L. vannamei* shrimp feed with *B. velezensis* S141, assessing the probiotics' efficacy in delaying infection onset and reducing the cumulative mortality rate when confronted with WSSV, VP_{AHPND}, and EHP. This study explores the potential of probiotics in shrimp aquaculture, emphasizing their role as a sustainable dietary supplement that can significantly reduce the reliance on antibiotics. The results highlight the ability of probiotics to enhance overall shrimp health, improve immune responses, and mitigate disease outbreaks, establishing them as an essential alternative for promoting eco-friendly and resilient shrimp farming practices.

1.2 Research objectives

The objectives of this study are as below:

1.2.1 To investigate the beneficial properties of *B. velezensis* S141 towards the growth performance and survival rate of shrimp fed with the probiotic-formulated feed.

1.2.2 To determine the disease resistance of *B. velezensis* S141-received shrimp against various pathogens including WSSV, VP_{AHPND}, and EHP.

1.2.3 To disclose an insight into the immunogenic perspectives of *B. velezensis* S141 to shrimp's immune system.

1.2.4 To purify the sVP28 protein derived from White Spot Syndrome Virus (WSSV) for generate specific antibodies.

CHAPTER II
LITERATURE REVIEW

2.1 *Litopenaeus vannamei* shrimp

Litopenaeus vannamei, also referred to as the white leg shrimp or Pacific white shrimp stands as a globally esteemed species in aquaculture circles. Its widespread cultivation owes to a trifecta of attributes: swift growth, adaptability to diverse environmental settings, and a soaring market appetite. Characterized by a pale, translucent physique adorned with striking white streaks along its carapace and tail, this species finds its origins along the Pacific coast of Latin America, stretching from Mexico to Peru (Bondad-Reantaso et al., 2005). In Thailand's aquaculture industry, it has an extensive history. This species was introduced in the late 1990s and became recognized because of its quick rate of development, capacity to adapt to many environmental conditions, and strong demand in the market. Thai shrimp farmers strategically introduced *L. vannamei* to diversify their farming methods and reduce the impact of diseases that had affected native shrimp species, including *Penaeus monodon* (Engle et al., 2017) .

L. vannamei thrives in coastal waters and estuaries with temperatures between 20°C and 30°C (68°F and 86°F), it is a tough inhabitant of aquatic environments. It is recognized for developing quickly; given the right conditions, it may achieve market readiness in just 3 to 6 months (González et al., 2010). When it comes to sexual propagation, during spawning episodes, each female contributes thousands to millions of eggs. The lifecycle complexity of the species is highlighted by the developmental stages that are experienced throughout the evolution from egg to juvenile. Moreover, *L. vannamei* shrimp play an important role in aquaculture projects around the worldwide, developing in large-scale and high-intensity farming environments. Their proliferation in supported by cutting-edge methods such as recirculating aquaculture systems (RAS) and biofloc technologies (Furtado et al., 2015; Suantika et al., 2020), w

hich are used for anything from raceways and tanks to pond culture. proliferation in supported by cutting-edge methods such as recirculating aquaculture systems (RAS) and biofloc technologies (Furtado et al., 2015; Suantika et al., 2020), which are used for anything from raceways and tanks to pond culture. Although hardy, these shrimps are not immune to illness; parasitic, bacterial, and viral risks are significant. Consequently, effective disease control becomes an essential component of shrimp farming activities. Apart from their importance to aquaculture, L. vannamei shrimp's delicate taste, solid texture, and culinary adaptability make them a highly sought-after product in international seafood markets, drawing interest from North America, Europe, and Asia (Fig. 1) (Bondad-Reantaso et al., 2005)

Figure 2.1 *Litopenaeus vannamei* shrimp (Bondad-Reantaso et al., 2005).

2.2 Morphology of *L. vannamei*

The organs of *L. vannamei* shrimp function collaboratively to maintain vital physiological processes. The stomach plays a critical role in the mechanical breakdown and initial enzymatic digestion of ingested food, which is subsequently transferred to the hepatopancreas. The hepatopancreas serves as the primary site to produce

digestive enzymes, absorption of nutrients, and storage of energy reserves. The intestine completes the digestive process by absorbing residual nutrients and excreting waste. The heart, located dorsally, pumps hemolymph throughout the open circulatory system, ensuring the distribution of oxygen and nutrients to tissues. The brain, situated in the cephalothorax, coordinates neural control of sensory and motor functions, including antennae movement and pereopod locomotion (Fig. 2). Together, these organs integrate complex physiological processes to support the shrimp's growth, metabolism, and survival in aquatic environments (Li et al., 2008). The external morphology of *Litopenaeus vannamei* demonstrates specialized anatomical adaptations. Key morphological features include:

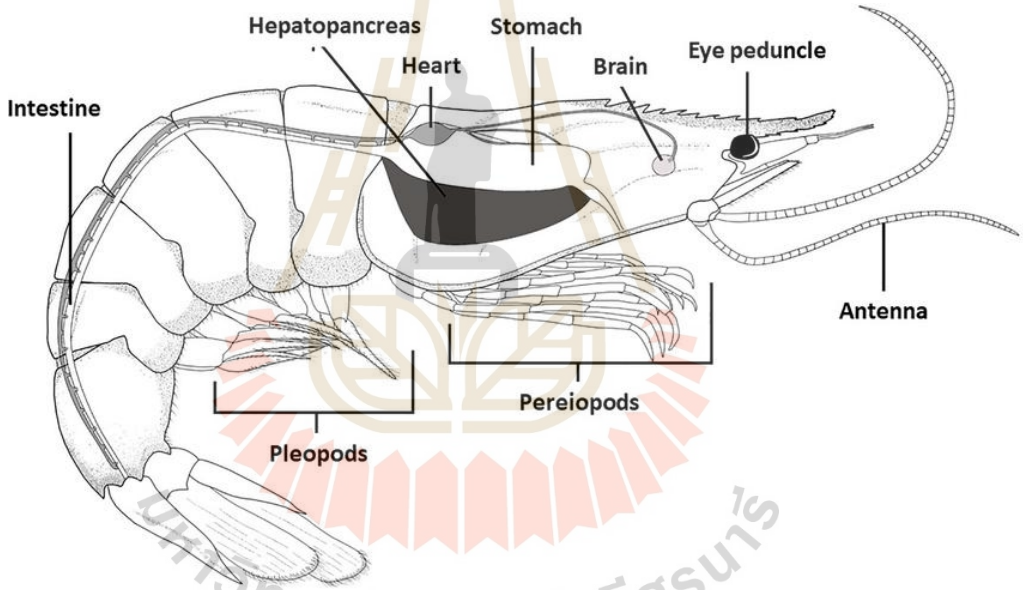


Figure 2.2 Anatomy of *L. vannamei* (Duarte-Restrepo et al., 2020)

2.2.1 Digestive system

The digestive system comprises three main sections including foregut, midgut, and hindgut, each playing distinct roles (Fig. 3). The foregut includes a gastric mill in its anterior chamber, responsible for the mechanical breakdown of food through cuticular tooth-like structures. This mechanical processing is complemented by enzymatic action in the midgut, which contains the hepatopancreas multifunctional

gland responsible for the secretion of digestive enzymes and the absorption of nutrients. The midgut is lined with epithelial cells that produce a peritrophic membrane, which protects the gut lining while facilitating selective nutrient absorption. The hindgut, extending into the pleon, manages waste expulsion and is a site for bacterial colonization, which aids in the breakdown of complex organic matter and enhances nutrient bioavailability. This sophisticated digestive system underscores *L. vannamei* is ability to thrive in diverse aquaculture settings, making it a model species for studying crustacean physiology and aquaculture practices (Dugassa & Gaetan, 2018).

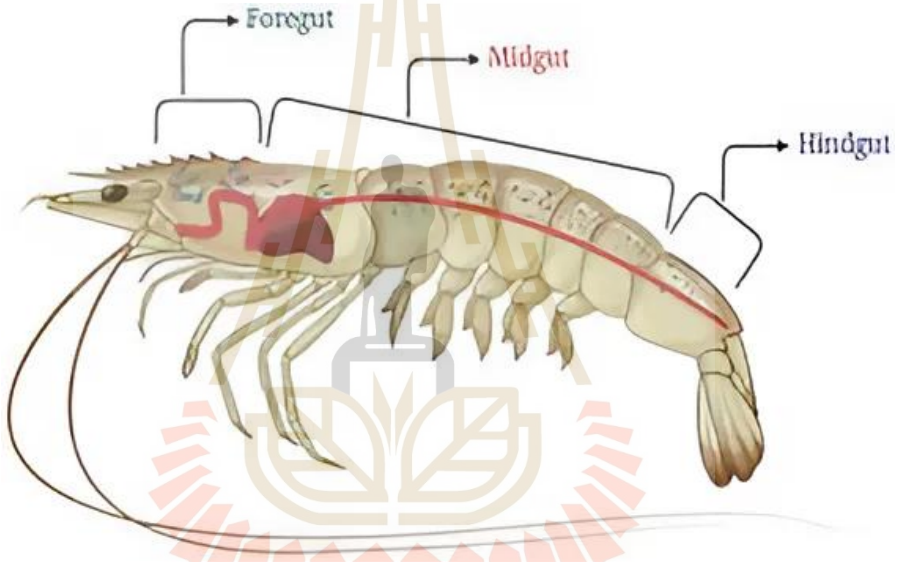


Figure 2.3 Digestive system of *L. vannamei* shrimp (Hembrom et al., 2024)

Additionaly, there are still studies that have highlighted the role of microbial communities in the digestive tract, emphasizing their contribution to the shrimp's health and growth. For example, identified dominant bacterial phyla, such as Proteobacteria and Firmicutes, that support metabolic and environmental adaptability. The presence of these bacteria suggests a symbiotic relationship crucial for efficient nutrient cycling and disease resistance. Moreover, the findings contribute to understanding the symbiotic relationships between shrimp and their intestinal microbiota, which are vital for optimizing growth performance and disease resistance (Cheng et al., 2021)

2.2.2 Respiratory system

The respiratory system of *L. vannamei* is specialized for efficient gas exchange and maintaining homeostasis in aquatic environments. This system primarily relies on dendrobranchiate gills, which are highly developed and structurally adapted for respiratory and osmoregulatory functions. Each gill consists of paired lateral branches emanating from a central axis, with numerous secondary rami that maximize surface area for gas exchange. The gill lamellae are responsible for oxygen absorption and the release of carbon dioxide, facilitated by a thin water-blood barrier composed of a cuticle, epithelium, and basal lamina. The gills also play a crucial role in ionic and osmotic regulation, ammonia excretion, and calcium uptake, vital for maintaining ionic equilibrium in varying salinity conditions. Hemolymph, the shrimp's circulatory fluid, is transported to the gills through afferent vessels and oxygenated within the lamellae before returning to the body via efferent vessels (Cheng et al., 2021; Dugassa & Gaetan, 2018).

2.2.3 Nervous System

The nervous system of the *L. vannamei* is a highly specialized network essential for coordinating physiological processes and behavioral responses. It comprises a central nervous system (CNS), peripheral nervous system (PNS), and sensory organs that collectively enable the shrimp to interact with its environment effectively. The CNS includes the supraesophageal ganglion, often referred to as the brain, and the subesophageal ganglion. These structures are interconnected by a pair of circumesophageal connectives and are responsible for higher-order processing and integration of sensory input. The ventral nerve cord extends longitudinally from the CNS, consisting of a series of ganglia and interconnecting nerves that innervate various body regions. The PNS encompasses nerves branching from the CNS and ventral nerve cord, which innervate muscles, appendages, and visceral organs. These nerves facilitate the transmission of motor commands and sensory information. Sensory inputs are detected by specialized receptors located on antennae, antennules, and appendages, enabling the perception of stimuli such as chemical cues, vibrations, and tactile sensations (Dugassa & Gaetan, 2018).

2.2.4 Integument System

The integumentary system of the *L. vannamei*, serves as a multifunctional interface between the organism and its aquatic environment, playing a critical role in protection, osmoregulation, sensory perception, and structural support. It is primarily composed of a chitinous exoskeleton, epidermis, and associated cuticular structures that undergo periodic molting to accommodate growth and maintain functionality. The exoskeleton is a complex, multilayered structure composed of epicuticle, exocuticle, and endocuticle layers, each enriched with chitin and sclerotized proteins. This rigid framework provides mechanical protection against predation and environmental stress while serving as an attachment site for muscles that enable locomotion. Calcium carbonate is deposited within the cuticle, enhancing rigidity and structural integrity. Beneath the cuticle lies the epidermis, a single-layered epithelial tissue responsible for secreting cuticular components and maintaining the structural renewal of the exoskeleton. The epidermis also hosts specialized gland cells that secrete enzymes during molting, facilitating the separation of the old cuticle from the underlying epidermis (Dugassa & Gaetan, 2018). The process of molting, regulated by hormones such as ecdysteroids produced by the Y-organ, is a critical aspect of the integumentary system. Molting allows for growth and the repair of cuticular damage but renders the shrimp temporarily vulnerable due to the soft, newly formed exoskeleton. The molting cycle involves several stages, including pre-molt, ecdysis, post-molt, and intermolt phases, each characterized by distinct physiological and biochemical changes (Corteel et al., 2012). In addition to its protective functions, the integument system is integral to osmoregulation. The epicuticle's lipid-rich composition minimizes water permeability, aiding the shrimp in maintaining ionic balance in hypo- or hyperosmotic environments. Sensory setae distributed across the exoskeleton further enhance the shrimp's ability to detect tactile and chemical cues, supporting behaviors critical to survival.

2.2.5 Circulatory System

The circulatory system is an open circulatory system, characterized by the presence of a hemolymph fluid that circulates freely within the body cavity (Fig. 4). Hemolymph, a combination of blood and interstitial fluid, is pumped by the heart

and flows through various sinuses, bathing the internal organs and tissues directly composed of heart, arteries, and hemolymph sinuses. The heart is located in the thorax and is connected to the pericardial sinus, from which hemolymph is pumped into the arteries. The arteries then carry the fluid to different parts of the body, including the gills where gas exchange occurs, and back into the sinuses. The system lacks veins and capillaries, making it fundamentally different from the closed circulatory systems seen in many vertebrates (Dugassa & Gaetan, 2018). Hemolymph circulation serves multiple vital functions, including the distribution of nutrients, hormones, and metabolic waste removal. It is also involved in immune responses, as hemolymph contains a variety of cells such as hemocytes, which play a role in defending the shrimp against pathogens. Furthermore, the circulatory system aids in temperature regulation and supports the functioning of the shrimp's other organ systems, particularly in response to environmental changes such as temperature fluctuations (Dugassa & Gaetan, 2018; Z. Wang et al., 2019).

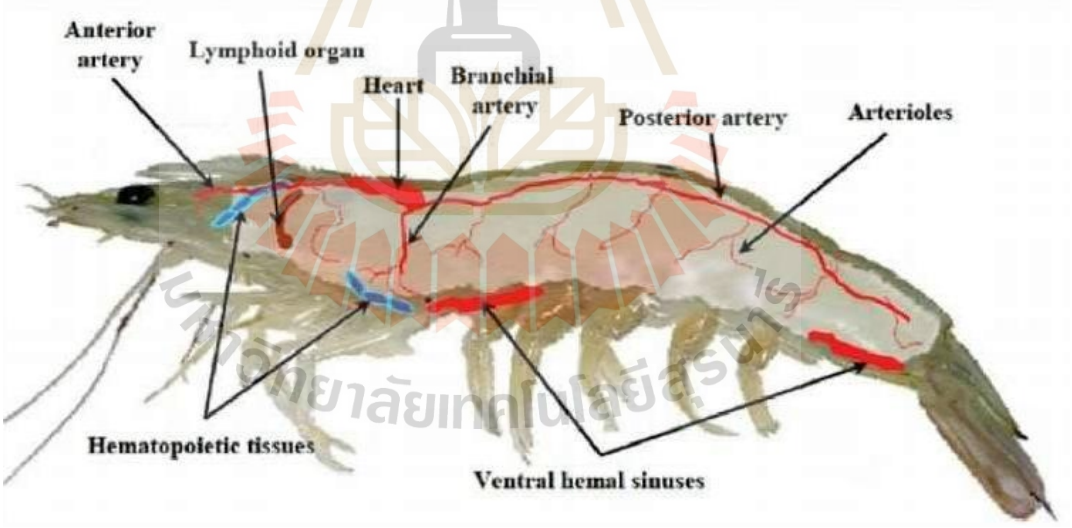


Figure 2.4 Circulatory System of *L. vannamei* shrimp (Dugassa & Gaetan, 2018)

2.3 Infectious diseases in shrimp

Infectious diseases in shrimp represent one of the major challenges in aquaculture, significantly affecting both farm productivity and the global shrimp industry. The pathogens involved in these diseases vary widely, with viral, bacterial,

fungal, and parasitic infections being the primary causes of mortality. Among the viral diseases, White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Yellowhead Virus (YHV), and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) are particularly notorious, leading to massive die-offs in affected shrimp populations. The impacts of these viruses are not limited to high mortality rates; they also influence growth rates, reproductive success, and overall health, leading to severe economic losses (Cuéllar-Anjel et al., 2010).

2.3.1 Viral diseases

2.3.1.1 White spot syndrome virus (WSSV)

White Spot Syndrome Virus (WSSV) is a highly contagious and lethal virus that affects shrimp, particularly penaeid shrimp species like the *L. vannamei* and the *Penaeus monodon*. It is one of the most devastating pathogens in shrimp aquaculture, causing significant economic losses worldwide WSSV. Belongs to the genus *Whispovirus* within the virus family *Nimaviridae* WSSV is classified as a dsDNA (double-stranded DNA) virus (Sánchez-Martínez et al., 2007). The virus contains a circular dsDNA genome of approximately 300 kb in size and encodes over 180 open reading frames (ORFs) involved in various aspects of viral replication, transcription, morphogenesis, and modulation of host immune responses (Oakey & Smith, 2018). WSSV exhibits a broad host range encompassing various crustaceans, and its host range includes decapods and other crustaceans, while non-decapod hosts can act as carriers by harboring latent infections in the absence of disease. Furthermore, non-crustacean carriers such as polychaete worms and oysters have been found to harbor WSSV (Jane Oakey & Smith, 2017). The virus can cause mortality rates of up to 100% within 3 to 5 days following infection in shrimp (Pradeep et al., 2012). WSSV-infected shrimp can quickly develop white spots on the exoskeleton, arms, legs, and epidermis, usually ranging from 0.5–3.0 mm in diameter. However, the presence of these spots alone is not always suggestive of WSSV infection, as similar spots can also be caused by certain bacteria, high alkalinity levels, and stress. As a result, relying solely on the observation of white spots is not a dependable way to make a preliminary diagnosis of this disease (Joseph et al., 2015; Sánchez-Paz, 2010). In addition to the development of white spots, WSSV-infected shrimp may exhibit additional clinical signs such as slowness,

reduced appetite, and abnormal swimming habits as the infection progresses, systemic consequences become increasingly noticeable as the infection spreads, possibly resulting in organ damage and high mortality rates (Chen & He, 2019).

The entry of the WSSV into shrimp host cells involves a complex process that is still not fully understood. WSSV proteins are involved with host receptors during viral entry into the cell, causing Clathrin-mediated endocytosis. As WSSV passes through endosomes, the maturation process induces the pH to decrease, signaling the virus's readiness to exit the endosomes. This stage likely involves an interaction between protein VP28 and Rab7. Nevertheless, the mechanism by which WSSV enters the nuclear envelope remains unknown. Processivity factors and other host machinery are required for WSSV DNA replication during intracellular interaction in the host cell. Through E2F1, WSSV can prevent the cell cycle in the S-phase, enhancing their availability. Unfolded protein response (UPR) pathways may be activated by increased viral protein synthesis, which can lead to endoplasmic Reticulum (ER) stress. Transcription factors of the UPR can, in turn, activate the expression of viral genes, which may inhibit translation through Eukaryotic initiation factor 2 (eIF2). Iron is one of the critical elements required for WSSV replication. WSSV can prevent iron from attaching to ferritin, counteracting any potential iron withholding by the host. Furthermore, WSSV can control the signaling involved in apoptosis through the suppression of initiator caspases by miRNA or the inhibition of effector caspase activity by viral proteins (Escobedo-Bonilla et al., 2008; Verbruggen et al., 2016). This is illustrated graphically by the schematic presented in Fig. 5

The WSSV viral envelope comprises at least 35 different proteins, the most abundant of which are VP28 and VP26, which comprise about 60% of the envelope. As the major envelope protein, VP28 is encoded by open reading frame (ORF) 421 (wsv421). Several studies suggest that VP28 plays a critical role in the early phases of systemic WSSV infection in shrimp. Additionally, it is suggested that WSSV VP28 functions as an important attachment protein during the infection process, helping the virus bind to shrimp cells and enter the cytoplasm. With possible glycosylation sites present, it is hypothesized that VP28 may play a major role in receptor recognition at the shrimp cell surface (Sánchez-Paz, 2010; Tang et al., 2007).

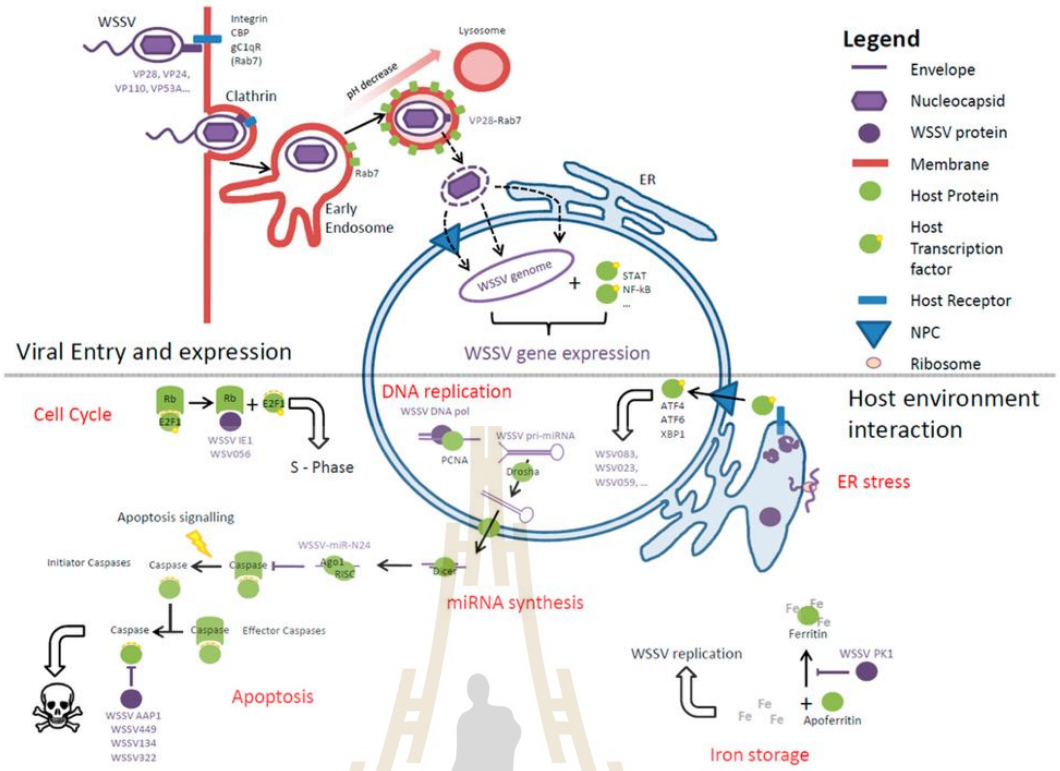


Figure 2.5 Molecular mechanisms of WSSV entry into the host cell (Verbruggen et al., 2016).

2.3.1.2 Yellow head disease (YHD)

Yellow head disease (YHD) is among the most severe viral threats to the penaeid aquaculture industry, predominantly affecting key shrimp species such as *P. monodon* and *P. vannamei*. The causative agent, yellow head virus (YHV), YHV is a rod-shaped RNA virus belonging to the Roniviridae family. Initially identified in Thailand during the early 1990, YHD has since posed a recurring challenge to shrimp farming across Southeast Asia and other regions involved in shrimp production. YHV primarily attacks the shrimp's lymphoid organs, gills, and hemocytes, resulting in a rapid, systemic infection. Characteristic symptoms of YHD include a yellowish tint on the cephalothorax, sluggish behavior, reduced appetite, and a mortality rate that can surpass 90% within a few days of the infection's onset. Such severe outbreaks have dire consequences for shrimp aquaculture, which relies heavily on the health and sustainability of cultivated stocks (Munro & Owens, 2007; Senapin et al., 2010). The advancement of diagnostic tools has played a crucial role in the control of YHD.

Modern molecular techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) and loop-mediated isothermal amplification (LAMP), have enabled the early and accurate detection of YHV, even in shrimp that do not yet display symptoms. Early detection allows for prompt implementation of measures like isolating infected shrimp and applying strict biosecurity protocols, which help mitigate losses. Effective management strategies for YHD require an integrated approach that combines diagnostic technologies, immune-boosting methods, and improved environmental practices to reduce the likelihood of outbreaks. Experimental vaccines, including those utilizing DNA-based and RNA interference (RNAi) methodologies, have demonstrated promise in lowering viral loads and enhancing the survival of shrimp infected with YHV. Additionally, probiotics and nutritional supplements have been explored as tools to strengthen the natural immune response of shrimp, offering a supplementary strategy to enhance traditional biosecurity measures (Sánchez-Barajas et al., 2009; Srisapoome et al., 2018).

Figure 2.6 The clinical signs of yellow head disease. (A) Indicated the YHV-infected shrimp which shows a yellow head, (B) Normal shrimp. (Amarakoon & Wijegoonawardane, 2017).

2.3.1.3 Infectious Myonecrosis (IMN)

Infectious myonecrosis virus (IMNV) poses a serious risk to penaeid shrimp farming, particularly impacting species like *L. vannamei.* Discovered in Brazil in 2002 (Lightner et al., 2004), this viral pathogen has since proliferated to various aquaculture regions, including Southeast Asia, leading to substantial economic damage. IMNV is a double-stranded RNA virus belonging to the *Totiviridae* family, commonly linked to severe muscle necrosis that results in a distinctive whitening of the muscle tissue, especially in the tail. Outbreaks are frequently triggered by environmental stressors, such as overcrowding, temperature fluctuations, and poor water quality, which contribute to mortality rates as high as 40% to 70% in severe cases. IMNV spreads via both horizontal and vertical transmissions. Horizontal transmission occurs through the ingestion of infected tissue or contact with contaminated water, while vertical transmission from broodstock to offspring has also been observed. The virus's ability to survive in aquatic environments and persist within infected tissues complicates efforts to control its spread. Shrimp afflicted by IMNV exhibit symptoms such as decreased feeding, lethargy, and noticeable lesions in muscle tissue. As the disease advances, affected muscle areas become white due to necrosis, which may eventually lead to tissue breakdown and death. Histological analysis often reveals coagulative necrosis in striated muscle fibers accompanied by hemocyte infiltration in damaged tissues (Prasad et al., 2017).

Figure 2.7 The clinical signs of IMN in extensive white necrotic areas of the skeletal muscle (Poulos et al., 2006).

2.3.1.4 Taura Syndrome Virus (TSV)

Taura syndrome virus (TSV) is a major disease that affects the *L. vannamei*, leading to substantial mortality rates and economic setbacks in aquaculture. First identified in Ecuador during the early 1990, the disease has since spread to shrimp farms across the globe. The disease often causes mortalities of 80 to 85% among infected (Lightner et al., 1994). It predominantly affects juvenile shrimp and is marked by rapid death, visible lesions, and reddish discoloration of the exoskeleton, particularly around the tail fan and pleopods. Studies have determined that the causative agent is the Taura syndrome virus (TSV), an RNA virus classified under the *Dicistroviridae* family. TSV spreads through horizontal transmission, including contact between healthy and infected shrimp, exposure to contaminated water, and ingestion of infected material. The virus can also be transmitted vertically from parent shrimp to their offspring, emphasizing the importance of rigorous biosecurity protocols in hatcheries and farming operations. TSV specifically invades tissues associated with the shrimp's cuticle and lymphoid organs, resulting in necrosis and deterioration of the cuticular epithelium. Affected shrimp often display signs such as erratic swimming behavior, reduced activity, and increased susceptibility to predators. Survivors of the infection can become virus carriers, serving as reservoirs for future outbreaks. Diagnosis of Taura syndrome combines clinical evaluation, histological analysis, and advanced molecular techniques like polymerase chain reaction (PCR) to detect TSV's genetic material. Preventive measures include isolating infected populations, using disease-free broodstock, and applying strict sanitation practices to limit the virus's spread. In cases of confirmed infection, affected stocks are often eliminated to curtail further outbreaks. Additionally, breeding programs designed to enhance resistance to TSV have demonstrated potential in mitigating the disease's effects. Continued research into the virus and the immune responses of shrimp holds promise for the development of innovative treatments or vaccines, supporting the long-term viability and productivity of shrimp aquaculture worldwide (Hasson et al., 1995).

Figure 2.8 TSV-infected *P. vannamei*. (A) acute phase has a pale, (B) chronic phase has melanized lesions (Tang et al., 2009).

2.3.2 Bacterial diseases

2.3.2.1 Acute hepatopancreatic necrosis disease (VP_{AHPND})

Acute Hepatopancreatic Necrosis Disease (AHPND) is a devastating bacterial infection that affects shrimp, specifically species like *L. vannamei* and *P. monodon*, which are commonly raised in aquaculture. AHPND is caused by strains of Vp_{AHPND} that carry specific virulence factors known as PirA and PirB toxins. *V. parahaemolyticus* is a rod-shaped, mesophilic, halophilic, and Gram-negative bacterium that often occurs in estuaries and aquatic environments worldwide. Ingesting raw or partially cooked seafood contaminated with *V. parahaemolyticus* can result in gastroenteritis, making it an important human pathoge (Nunan et al., 2014). The disease was initially discovered in 2010 in China and thereafter reports of it were received from major shrimp producing countries in Southeast Asia and Latin America. Outbreaks of AHPND have had significant economic effects on the worldwide shrimp aquaculture industry (REANTASO & Arthur, 2018). By 2010, the rise in the amount of impacted Chinese farms, while 2011 brought the discovery of AHPND in Malaysia and Vietnam (Mooney, 2012). Thailand was exposed to Early mortality syndrome (EMS) in 2012. Similar to previous cases of epizootic shrimp diseases, EMS is seriously reducing productivity in impacted areas and affects Global markets, social welfare, and

employment (Flegel, 2012). The ability of *V. parahaemolyticus* to withstand an extensive range of environmental conditions is one of its significant characteristics. Differences in salinity (0.5–9.5%), pH (7.6–9.0), and temperature (7–43 °C) are all favorable to its growth. Because of its wide tolerance range, *V. parahaemolyticus* may survive and spread over a variety of maritime environments (Drake et al., 2007; Hong et al., 2016).

The virulent pVA1 plasmid (VP_{AHPND}), which carries the genes encoding the binary toxins $PirA^{vp}$ and $PirB^{vp}$, is present in all strains of *V. parahaemolyticus* that cause AHPND. These toxic substances are homologous to the toxins associated with the *Photorhabdus luminescens* insect (Pir). They are important to the pathogenesis of AHPND after they are released into the extracellular environment. It has been shown through reverse gavage tests that the bacterial culture's bacteria-free supernatant is sufficient to cause the characteristic symptoms of AHPND (Tran et al., 2013). Additionally, it has been proven that the reverse gavage injection containing pure recombinant $PirB^{vp}$ toxin is the only way to cause symptoms comparable to AHPND (Lee et al., 2015). The ability of VP_{AHPND} toxins to infiltrate and harm hemocytes suggests that the detrimental effects of AHPND extend beyond the stomach and hepatopancreas to the hemocytes. In the context of shrimp hemocytes, LvAPN1 plays a crucial role. AHPND-inducing *V. parahaemolyticus* invades the shrimp host and releases $PirAB^{vp}$ toxins simultaneously. Subsequently, these toxins interact with the LvAPN1 receptor located on the cell membrane of shrimp hemocytes, potentially initiating $PirAB^{vp}$ toxin oligomerization. This process enhances pore formation and membrane integration, ultimately leading to changes in hemocyte morphology and hemocyte lysis. The VP_{AHPND} toxins caused severe damage to hemocytes, resulting in a decrease in total hemocyte count and cell death. However, silencing LvAPN1 prevented these effects, indicating the involvement of LvAPN1 in the susceptibility of hemocytes to VP_{AHPND} toxins. Immunofluorescence assay revealed that VP_{AHPND} toxins penetrated the cell membrane and localized inside the cell. Nevertheless, when LvAPN1 was silenced, VP_{AHPND} toxins failed to enter the cell and remained localized on the cell membrane. Although these effects were observed in circulating hemocytes, it

is suggested that VP_{AHPND} toxins may similarly damage hemocytes in the hepatopancreas. Therefore, hemocyte infiltration in the late stage of infection may involve damaged hemocytes previously affected by VP_{AHPND} toxins (Fig 3). (Luangtrakul et al., 2021).

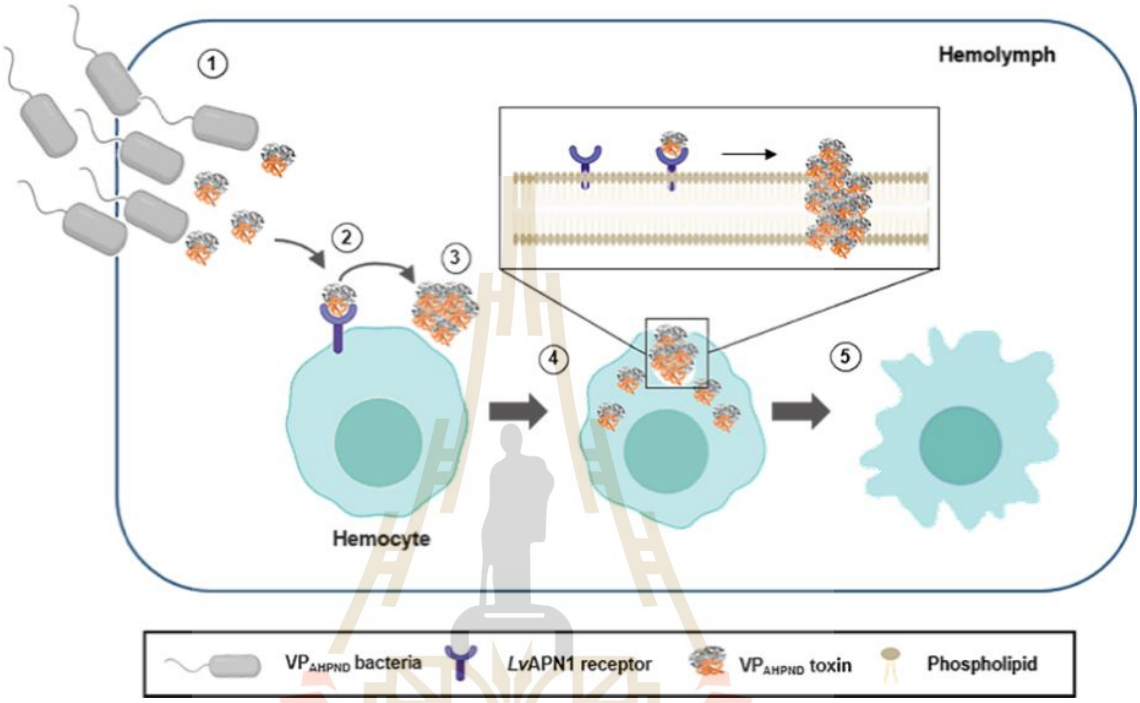


Figure 2.9 Overview of AHPND pathogenesis demonstrates the proposed role of LvAPN1 in hemocyte (Luangtrakul et al., 2021).

2.3.2.2 Early Mortality Syndrome (EMS)

Early Mortality Syndrome (EMS), a serious and emerging disease, presents a significant threat to shrimp aquaculture, particularly affecting the Pacific white shrimp *L. vannamei*. EMS has been linked to a group of bacterial pathogens, particularly Vibrio species like *V. parahaemolyticus* and *V. harveyi*. These bacteria produce toxins that specifically affect hepatopancreas. When these toxins enter the shrimp's body, they induce acute inflammation, leading to necrosis and structural disruption of the hepatopancreatic tissue. Histopathological investigations reveal severe damage in the form of necrosis and cellular degeneration within the hepatopancreas, including the breakdown of tubules and the accumulation of cellular

debris. In advanced stages, these changes can result in a complete collapse of the hepatopancreas. Early identification of these tissue alterations is vital for diagnosing EMS and implementing effective control strategies (De Schryver et al., 2014). Effective management of EMS centers around minimizing bacterial infections and maintaining water quality in shrimp farms. Researchers suggest that Vibrio species may be introduced into shrimp ponds via contaminated feed, water, or broodstock, highlighting the importance of robust biosecurity measures in preventing outbreaks (Zorriehzahra & Banaederakhshan, 2015). Pond management practices, such as using probiotics, reducing organic matter, and applying disinfectants, can help control microbial populations in the aquatic environment, ultimately lowering the chances of Vibrio outbreaks. Additionally, ongoing research into selective breeding programs to develop EMS-resistant shrimp is a promising area of development, though such measures are still in the early stages of implementation. While antibiotics have traditionally been used to manage bacterial infections in shrimp farming, concerns about antimicrobial resistance have raised significant issues, particularly regarding the potential impact on public health. This concern has spurred interest in alternative therapies such as immunostimulants, probiotics, and phage therapy, which aim to boost the shrimp's immune system and restore a balanced pond microbiota. Studies indicate that probiotics, including *Bacillus subtilis* and various Lactobacillus strains, may help inhibit pathogenic Vibrio species while enhancing the shrimp's resistance to infection. The rise of EMS outbreaks also underscores the need for a new approach in microbial management within shrimp farming. Over the past few years, there has been increasing focus on the microbiome of shrimp and its role in disease resistance. By fostering a balanced microbial community both in the shrimp gut and the surrounding environment, shrimp farmers can potentially reduce the need for external antimicrobial treatments. This microbial management approach not only supports shrimp health but also enhances their resistance to diseases like EMS, contributing to the overall sustainability and efficiency of shrimp farming (Manan et al., 2015).

2.3.3 Fungal diseases

2.3.3.1 *Enterocytozoon hepatopenaei* (EHP)

In many countries in Southeast Asia, including China, Vietnam, Thailand, Indonesia, India, and Malaysia, cultured shrimp *L. vannamei* are experiencing an emergence of *Enterocytozoon hepatopenaei* (EHP), a hepatopancreatic microsporidian. EHP infections are opportunistic infections by *Vibrio* spp. (Tourtip et al., 2009). Despite not causing widespread mortality, EHP has been observed to prompt growth retardation and variations in size among affected shrimp. Such consequences have resulted in significant economic losses within shrimp farming, amplifying the threat posed by the disease to sustainable shrimp aquaculture (Sathish Kumar et al., 2022).

The microsporidian parasite *E. hepatopenaei* (EHP) specifically targets shrimp, showing a marked preference for the hepatopancreas of penaeid shrimp species such as the *L. vannamei* and black tiger shrimp (*P. monodon*). Among the notable characteristics of *E. hepatopenaei* is its microscopic size, typically falling within the range of 1 to 2 micrometers in diameter. EHP predominantly infects hepatopancreas tissue in shrimp, initiating pathological changes within the organ. Infected shrimp may exhibit various symptoms, including reduced growth rates, abnormal molting, and a pale hepatopancreas. The precise transmission mechanism of EHP remains only partially understood, with suggestions pointing towards horizontal transmission via ingestion of infected tissues or vertical transmission through infected broodstock (Lightner, 1996). According to prior studies, the intensification of EHP infection frequently aligns with the manifestation of white feces. It's imperative to emphasize that white feces do not constitute typical fecal matter; instead, they may consist of a blend involving EHP spores, intestinal mucus, and necrotic tubular epithelial cells. Multiple research endeavors have pinpointed the presence of EHP spores within white feces, highlighting their importance concerning EHP infection (Fig 4) (Munkongwongsiri et al., 2022; H. Wang et al., 2020).

The infection mechanism of *E. hepatopenaei* (EHP) in shrimp commences with shrimp ingesting spores, sourced from either the environment or infected tissues. Once inside the shrimp, these spores undergo activation within the digestive tract, potentially prompted by factors like pH and temperature (Kumar et al.,

2022). After activation, spores release infective meronts that penetrate the epithelial cells of the hepatopancreas. Within these cells, meronts undergo proliferation and differentiation, ultimately giving rise to spores. These mature spores are then discharged into the hepatopancreatic lumen, where they may exit the shrimp through fecal matter or alternate pathways. Consequently, infected shrimp release EHP spores into the aquatic environment, leading to contamination. This contaminated environment perpetuates the infection cycle as other shrimp may ingest contaminated feed or water, thus sustaining the spread of EHP (Lightner, 2011). Moreover, as a consequence of losing specific metabolic genes over its evolutionary path, EHP resorts to directly acquiring energy from the host to fulfill its metabolic needs. This may result in an inadequate supply of energy for the host, impeding the growth and development of the shrimp (Duan et al., 2021). The transcriptome study of WSSV, which may similarly alter shrimp's lipid and glucose metabolic pathways to meet its energy needs by making use of host resources, is like this situation. Nevertheless, given the complex web of metabolic pathways that underlie living things, changes to some metabolites are frequently regulated by one or more genes. To fully comprehend the impact of metabolic pathways on shrimp growth, more research is also required (Cao et al., 2023; Yu et al., 2022).

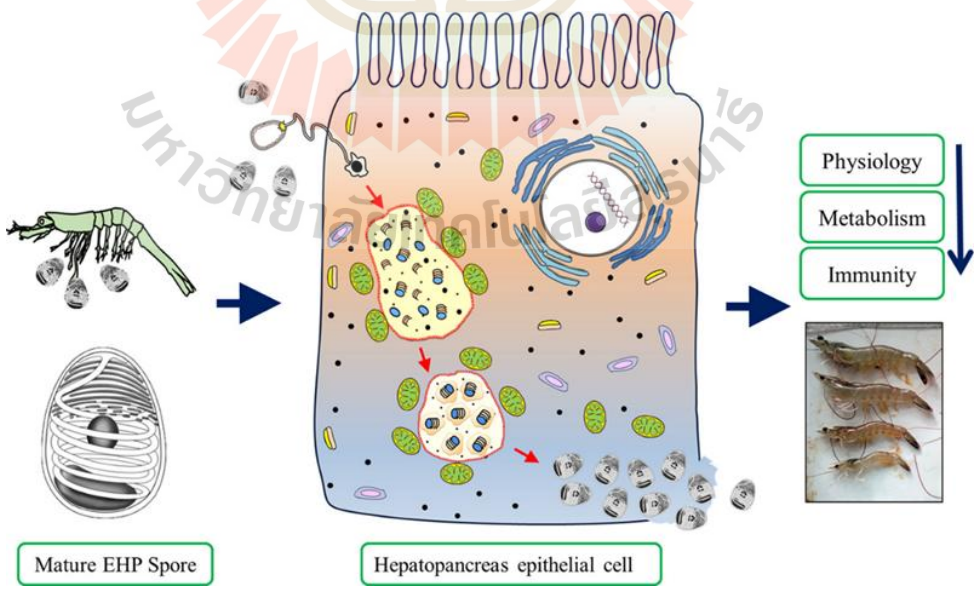


Figure 2.10 Schematics illustrating the transmission routes and effects of EHP infection (Kumar et al., 2022).

2.4 Shrimp immune system

The immune system of shrimp, particularly in species like *L.vannamei*, plays a crucial role in defending against various pathogens and maintaining overall health. Understanding the components and functioning of the shrimp immune system is essential for developing effective strategies to enhance disease resistance and promote sustainable aquaculture practices (Tassanakajon et al., 2018). Innate immunity in shrimp comprises physical barriers, cellular responses, and humoral factors. Physical barriers such as the cuticle and epithelial layers provide the first line of defense against pathogens (Destoumieux-Garzón et al., 2016). Cellular responses involve hemocytes, the circulating immune cells in shrimp, ware crucialhich are involved in phagocytosis and encapsulation, antimicrobial peptides, and the prophenoloxidase system, crucial for melanization and wound healing (Cerenius & Söderhäll, 2004). Additionally, antimicrobial peptides (AMPs) and lectins contribute to the innate immune response by targeting and neutralizing pathogens (Tassanakajon et al., 2018; Tran et al., 2022; Viana et al., 2022). On the other hand, the adaptive immune system, though less understood in invertebrates, involves processes like RNA interference and somatic rearrangement, contributing to specificity in pathogen recognition (Jiang et al., 2018). Environmental factors profoundly influence shrimp immune function, with temperature, salinity, and water quality playing crucial roles in shaping immune responses. Temperature fluctuations, for instance, can modulate the expression of immune-related genes and impact the susceptibility of shrimp to infections (Cheng et al., 2005). Similarly, variations in salinity levels can alter hemocyte activity and compromise immune function in shrimp (Lu-Qing et al., 2005). The immune system of shrimp encompasses a sophisticated network of innate and adaptive immune mechanisms tailored to their aquatic habitat. Further research into the molecular pathways and regulatory mechanisms underlying shrimp immunity is essential for the development of effective disease control strategies and the sustainability of global shrimp aquaculture

2.4.1 Cellular immune response

Shrimp possess an innate immune system comprising cellular and humoral responses that collaboratively defend against microbial invasions. The cellular

component is primarily mediated by hemocytes, which are categorized into hyalinocytes, semi-granulocytes, and granulocytes (Fig. 11), each fulfilling distinct immunological roles (Kumar et al., 2023).

Hyalinocytes are generally small cells with few or no granules. They are primarily involved in phagocytosis, engulfing pathogens as a defense mechanism. Additionally, hyalinocytes may serve as progenitor cells, differentiating them into other hemocyte types. Marker genes such as lysosome membrane protein 2 (LIMP2) and tubulin beta chain (TUBB4B) are highly expressed in hyalinocytes, indicating their role in lysosomal activity and structural functions.

Semi-granulocytes contain moderate numbers of granules and participate in both phagocytosis and the release of immune effectors. They express genes like beta-arrestin-1 (ARRB1) and lysozyme (Lyz1), which are associated with signal transduction and antimicrobial activity, respectively. These cells act as intermediates in the differentiation pathway from hyalinocytes to granulocytes, contributing to both cellular and humoral immune responses.

Granulocytes are characterized by abundant granules and are primarily responsible for the storage and release of immune molecules, such as antimicrobial peptides and components of the prophenoloxidase-activating system. They express genes like phenoloxidase-activating factor 3 (PPAF3) and peroxinectin (Pxt), which are crucial for melanization and pathogen encapsulation. Granulocytes play a significant role in the shrimp's defense mechanisms, including encapsulation and nodule formation (Cui et al., 2022).

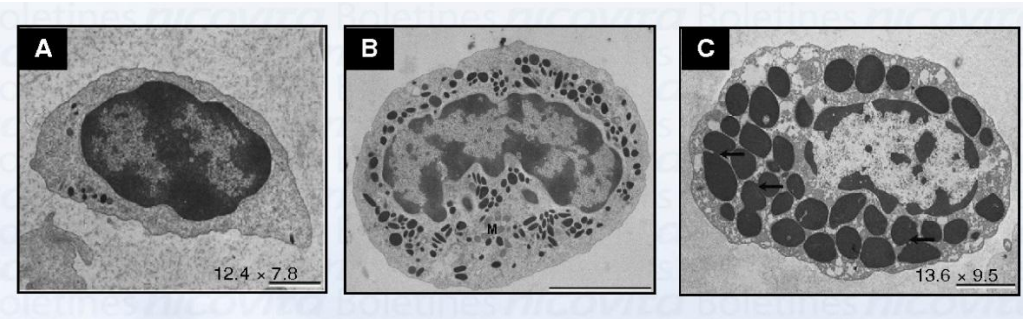


Figure 2.11 Classification of hemocytes: (A) Hyaline, (B) Semi-granular, (C) Granular (Martínez, 2007).

2.4.2 Humoral immune response

The humoral immune response in shrimp refers to the component of their innate immune system that operates through soluble molecules present in hemolymph (the shrimp's equivalent of blood). Unlike vertebrates, shrimp lack an adaptive immune system and rely on a robust innate immune system to combat pathogens. This humoral response is essential for shrimp's survival in pathogen-rich aquatic environments and works in tandem with the cellular immune response, involving hemocytes, for effective immunity.

2.4.2.1 Toll/IMD pathway

The Toll pathway plays a critical role in responding to Gram-positive bacteria and fungi by regulating a diverse set of genes, including antimicrobial peptide

genes, small peptides with unknown functions, and components of melanization and clotting cascades. Intracellular signaling cascades can be initiated through cell-substratum interactions. In *Drosophila*, the canonical components of the Toll pathway include Spätzle, Toll, Pelle, Tube, MYD88, Cactus, Dorsal, and Dorsal-related immunity factor (DIF) (Hoffmann & Reichhart, 2002). TRAF6 has also been identified as a downstream molecule of Pelle in the Toll pathway of *Drosophila* (Aggarwal & Silverman, 2008). Evidence suggests that the Toll pathway is involved in the immune response to viruses in both *Drosophila* (Sabin et al., 2010) and *Aedes aegypti* (Ramirez & Dimopoulos, 2010). In shrimp, homologs of most components in the Toll pathway of *Drosophila* have been identified, including Spätzle, Toll receptor, Pelle, TRAF6, and Dorsal.

The immune deficiency (IMD) pathway parallels the Toll pathway, activating immune responses to pathogen invasion. Some viruses like Sindbis and cricket paralysis also activate the IMD pathway, inducing AMP expression. Shrimp possess a similar IMD pathway regulating AMP expression. Components like *Lv*IKKs, IMD, and *Lv*Relish have been characterized. Silencing shrimp IMD dramatically decreases AMP expression (Li & Xiang, 2013). IMD encodes a death domain-containing protein like Receptor Interacting Protein (RIP) of the tumor necrosis factor receptor (TNF-R) pathway. Over-expression of IMD triggers the transcription/induction of antibacterial

peptide genes even in the absence of infection (Georgel et al., 2001). *Lv*IMD, the first full-length cDNA homolog isolated from *L. vannamei*, encodes a putative protein of 160 amino acids with a death domain at the C-terminal. *Lv*IMD mRNA expression in different tissues is induced by LPS (or Gram-negative bacteria) and WSSV, but not by Gram-positive bacteria or yeast, suggesting its role in the shrimp's immune response to Gram-negative bacteria and viruses. *Lv*IMD can induce the expression of AMP genes, including Drosophila Attacin A and shrimp penaeidin 4 in S2 cells, indicating its involvement in innate signaling to activate AMP gene expression in shrimp (Wang et al., 2009).

The NF-**K**B pathway is a critical regulator of immune functions, inflammation, apoptosis, and cell survival. In shrimp, this signaling pathway is integral to defending against pathogens, as it activates the transcription of genes involved in the immune response, such as cytokines, antimicrobial peptides, and heat shock proteins. Recent studies have demonstrated that the activation of NF-**K**B in response to infections like Vibrio, white spot syndrome virus (WSSV), and other pathogens enhances the shrimp's innate immunity. The molecular mechanisms by which NF-**K**B regulates immune genes, highlight its role in activating transcription factors that drive immune responses. Similarly, identified key upstream signaling molecules, such as I**K**B proteins, which control NF-**K**B activity in response to environmental stressors. These findings underscore the importance of NF-**K**B as a molecular target for improving shrimp health and disease resistance (C. Li et al., 2019).The activation of the NF-**K**B pathway is tightly regulated by various signaling molecules and kinases, which modulate its activity in response to external stimuli. In shrimp, studies have shown that components of the innate immune system, such as pattern recognition receptors (PRRs), trigger NF-**K**B activation upon recognizing pathogen-associated molecular patterns (PAMPs). The signaling cascade involves the phosphorylation and degradation of I**K**B proteins, leading to the translocation of NF-**K**B dimers (p65/p50) into the nucleus, where they initiate the transcription of genes involved in immune defense. A key aspect of the NF-**K**B pathway in shrimp is its cross-talk with other immune pathways, such as MAPK (mitogen-activated protein kinase) and JAK-STAT (Janus

kinase-signal transducer and activator of transcription), which also regulate immune responses (Wang et al., 2023).

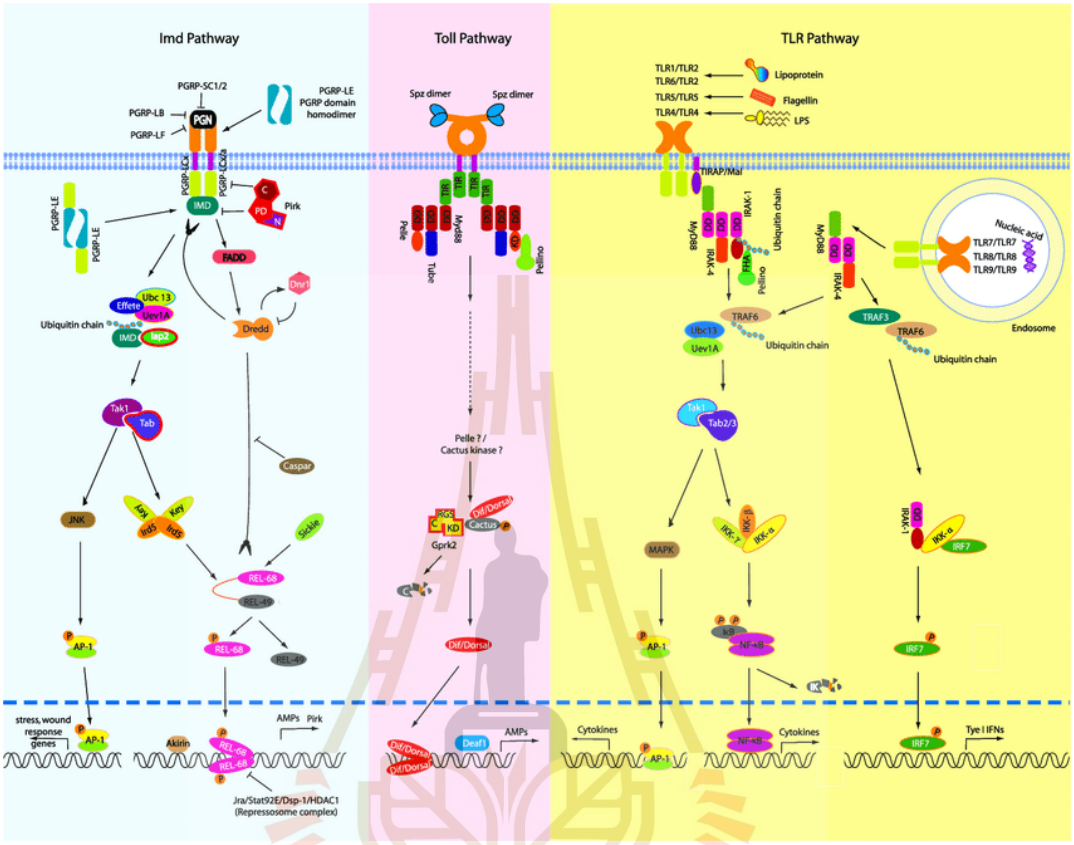


Figure 2.12 Toll/IMD-NF-kB signaling pathways (Valanne et al., 2011).

2.4.2.2 JAK/STAT pathway

The JAK/STAT pathway, known for its evolutionarily conserved antiviral function from arthropods to mammals, was initially identified as an antiviral effector-inducing pathway in mammals (Merkling & van Rij, 2013). This pathway plays a pivotal role in responding to various growth factors and cytokines. Upon binding of type I IFNs like IFN-α and IFN-β to IFN-α receptors, activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) occurs, leading to the recruitment and activation of STAT proteins. Phosphorylation, dimerization, and nuclear translocation of STAT complexes ensue, inducing the expression of numerous IFN-stimulated response element (ISRE)-driven genes, establishing a cellular antiviral state (Ivashkiv & Donlin, 2014).

The JAK/STAT signaling pathway in *Drosophila* plays a crucial role in host antiviral responses. Activation of numerous genes through cytokine signaling via the JAK/STAT pathway has been implicated in combating viral infections in *Drosophila*. Knockdowns and mutations of JAK in *Drosophila* and mosquitoes have shown increased viral infection rates, suggesting an antiviral response mediated by the JAK/STAT pathway, likely due to the STAT-dependent expression of antiviral effectors (Xu & Cherry, 2014). This pathway is also implicated in the antiviral response of other insects and crustaceans such as shrimp (Lin et al., 2012; Okugawa et al., 2013).

In crustaceans, core JAK/STAT pathway components, including domeless, JAK, SOCSs, and STAT, participate in antiviral responses. Shrimp STAT regulates the expression of genes with STAT-binding sites in their promoters. Silencing of JAK or STAT induces WSSV replication, highlighting the positive roles of the JAK/STAT pathway in shrimp antiviral responses. LvJAK and LvSTAT activation, induced by WSSV, demonstrate their involvement in defense against WSSV infection (Fig. 13). Additionally, disruption of the shrimp JAK/STAT pathway by WSSV facilitates viral replication during the early stages of infection (Song et al., 2015).

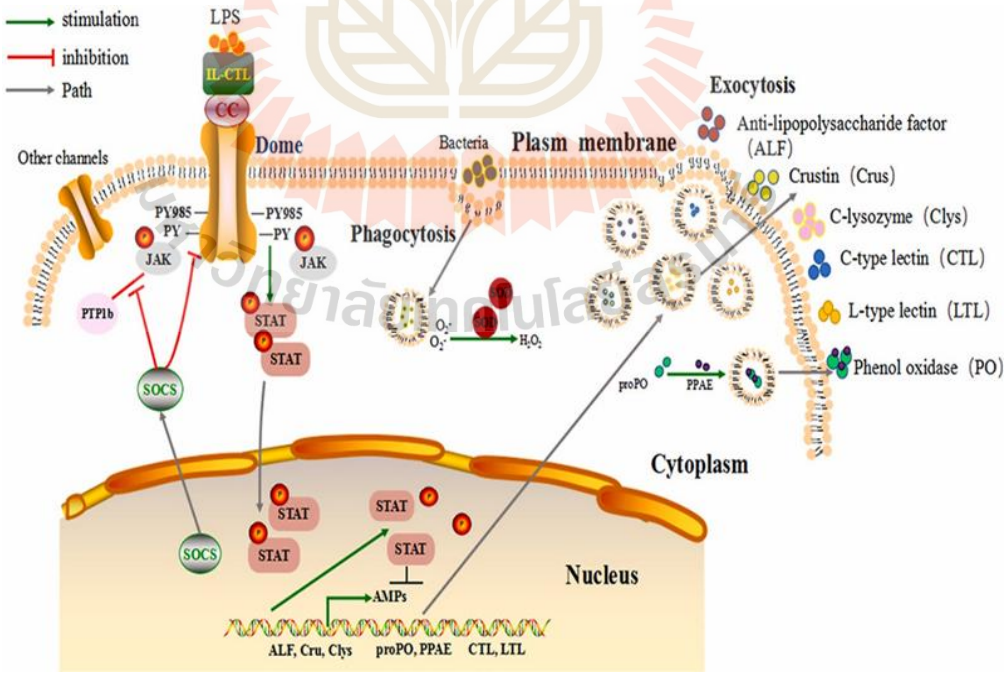


Figure 2.13 Regulation of cellular and humoral immunity by JAK/STAT signaling pathway (Yan et al., 2022).

2.4.2.3 Vago pathway

The Vago pathway in shrimp, a critical component of their immune system, plays a pivotal role in antiviral and antimicrobial responses, resembling the interferon (IFN) system found in vertebrates. This pathway involves the activation of Vago, a cytokine-like protein, which, when triggered by pathogen recognition, induces antiviral immune responses through a JAK/STAT signaling cascade. Vago is function is akin to that of interferons in higher organisms, which stimulate the expression of antiviral genes and other immune effectors. Recent studies have shown that Vago in shrimp is specifically activated during viral infections such as those caused by White Spot Syndrome Virus (WSSV), leading to enhanced antiviral immunity. In particular, shrimp species like *L. vannamei* have been found to rely heavily on the Vago-JAK/STAT pathway to combat such viral threats (H. Li et al., 2020).

The activation of the Vago pathway is complex, involving several immune-related molecules that enhance both innate and adaptive immune responses. Research indicates that different isoforms of Vago can trigger various immune reactions, from direct viral clearance to the stimulation of antimicrobial peptides. These peptides, which include penaeidin and lysozyme, are integral to the shrimp's defense mechanism against a wide array of pathogens, both viral and bacterial (Fig. 4). Furthermore, the Vago pathway appears to be tightly regulated by other immune signaling pathways, such as the NF-**K**B and IRF (Interferon Regulatory Factor) pathways, which together orchestrate a more robust immune response. The coordination between Vago and these pathways ensures that shrimp can mount an effective defense against infections while maintaining immune homeostasis (Koiwai et al., 2021).

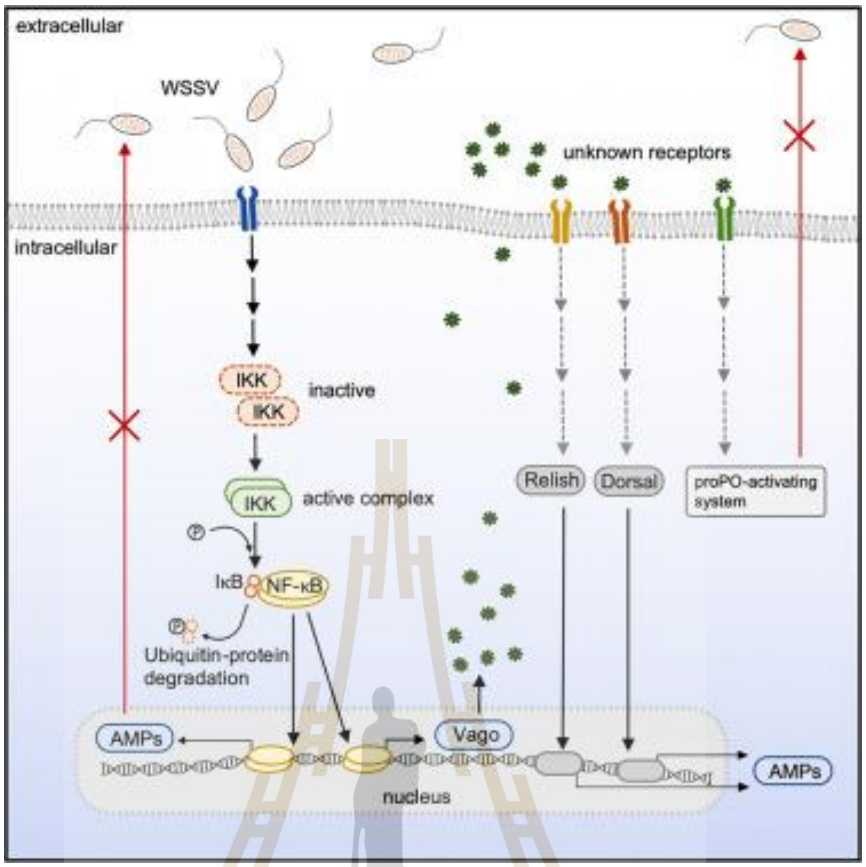


Figure 2.14 Proposed model mechanism of this study for Vago regulation via IKK-NF-KB cascade as a possible alternative pathway to the IRF. WSSV induced the expression and release of Vago through activation of IKK-Vago in the cytokine-like system. The released Vago further activated the immune cascade in the neighboring cells involving Dorsal and Relish, leading to AMP expression (Nanakorn et al., 2024).

2.4.2.4 Prophenoloxidase (PPO)

Hemolymph coagulation is a crucial part of the humoral immune response in crustaceans. Its primary function is to form a stable clot to seal wounds, preventing the loss of body fluids and maintaining hemostasis. Additionally, the coagulation reaction in invertebrates, along with the prophenoloxidase-activating system (proPO system) and the production of antimicrobial peptides, is a key component of the humoral innate immune response (Perdomo-Morales et al., 2019). Prophenoloxidase (PPO) is a crucial enzyme in the immune system of the white shrimp,

L. vannamei, playing a significant role in defense against pathogens. Typically present in an inactive zymogen form (proPPO), it is activated by serine proteases upon encountering pathogen-associated molecular patterns (PAMPs) (Fig. 15). Once activated, PPO catalyzes the oxidation of phenols to quinones, leading to melanin formation, which encapsulates and neutralizes pathogens. This process also produces reactive oxygen species (ROS) with antimicrobial properties (Amparyup et al., 2013). The expression of proPPO is regulated by environmental stressors and pathogen presence, with upregulation in response to infections. Advances in genomics have identified multiple proPPO isoforms, highlighting their genetic diversity and complex regulation. Understanding PPO mechanisms and regulation has significant aquaculture implications, suggesting strategies like immunostimulant administration or genetic selection to enhance shrimp health and disease resistance, thereby improving productivity and sustainability in shrimp farming (Amparyup et al., 2009) (Lai et al., 2005).

Activation of the proPO system is regulated by a multistep pathway. Recognition of microbial PAMPs (elicitors) by PRPs initiates a cascade of serine proteinases (SPs), ultimately leading to the proteolytic cleavage of the proPO zymogen into the active PO enzyme. This cascade is triggered upon detecting microbial PAMPs, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) from Gram-positive bacteria, and β-1,3-glucans from fungi. The interaction between PAMPs and PRPs activates the SP cascade, culminating in the activation of PO, which results in the production and precise deposition of polymeric melanin at infection sites or around foreign microorganisms (Amparyup et al., 2013).

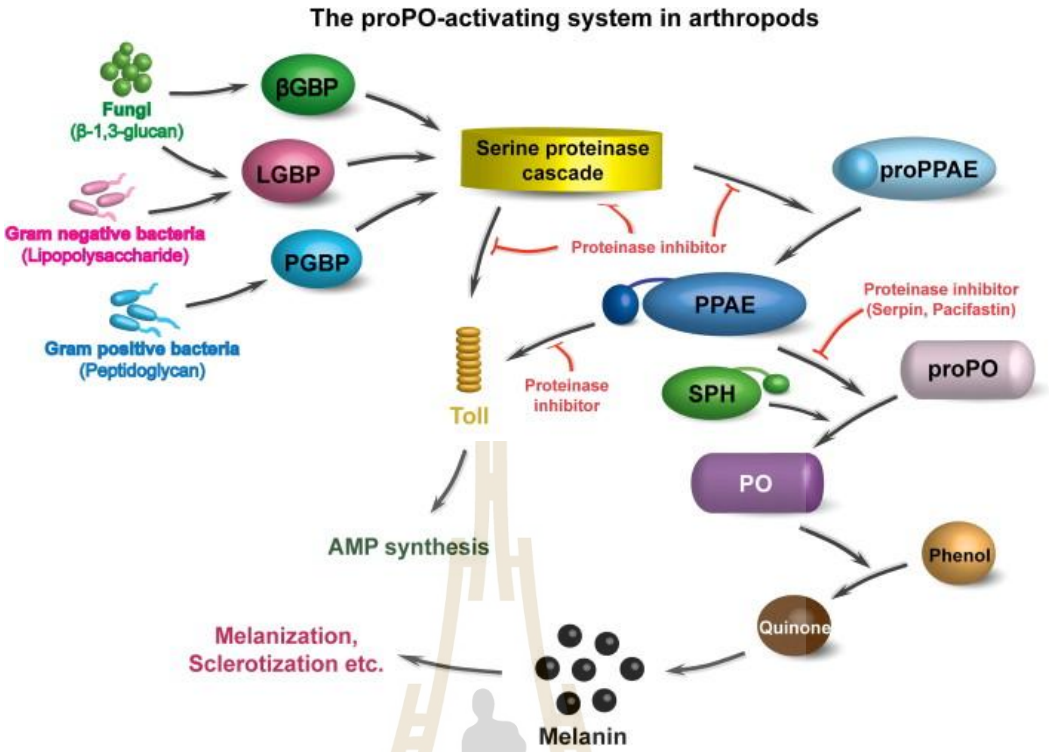


Figure 2.15 The proPO activity system (Amparyup et al., 2013)

2.4.2.5 Antioxidant

Antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT) are indispensable for maintaining cellular health in shrimp, safeguarding them against oxidative stress induced by environmental factors like pollutants, pathogens, and oxygen level fluctuations. These enzymes act as guardians by neutralizing harmful free radicals and reactive oxygen species (ROS), which can otherwise lead to cellular damage and compromise shrimp health.

SOD plays a pivotal role in converting the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2), while CAT further decomposes H_2O_2 into water and molecular oxygen. Together, they constitute a robust defense mechanism against oxidative stress within shrimp tissues. Numerous studies have delved into the presence and activity of SOD and CAT in various shrimp species (Li et al., 2008). For example, observed significant variations in SOD and CAT activities among different shrimp species, suggesting species-specific adaptations to oxidative stress. Similarly, demonstrated that exposure to environmental stressors, such as temperature

fluctuations, prompted an upregulation of SOD and CAT expression in shrimp, underscoring their crucial role in counteracting stress-induced oxidative damage (Zheng et al., 2019).

Recognizing the significance of SOD and CAT in shrimp aquaculture, efforts have been directed towards enhancing their activity through dietary supplementation or selective breeding illustrated the effectiveness of dietary antioxidants in augmenting SOD and CAT activity in shrimp, thereby bolstering their resilience to stressors and promoting growth. Despite their vital roles, the activity of SOD and CAT can be influenced by various factors including diet, environmental conditions, and genetic predispositions. Thus, further research is imperative to comprehensively understand the regulation of these enzymes in shrimp and to optimize strategies for enhancing their activity in aquaculture settings (Liu et al., 2007; Shi et al., 2023).

2.5 Probiotic

The Greek language "probiotic" translates to "for life," encapsulating substances or organisms that promote the host's health. In 2002, the WHO and FAO defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." Probiotic bacteria exhibit numerous positive effects, such as enhancing the nutritional value of food products, reducing serum cholesterol levels, boosting immunity, preventing gut infections, mitigating antibiotic-associated diarrhea, reducing symptoms of lactose intolerance, lowering the risk of colon cancer and depending on the type of probiotic strain, improving the digestion of gliadin against celiac in gluten-containing foods (Oelschlaeger, 2010; Zendeboodi et al., 2020) .

Probiotics are naturally occurring microorganisms found in the gastrointestinal tract of living organisms. They contribute to intestinal health, making the gut stronger. The use of probiotics is considered an effective alternative to antibiotics in aquaculture (Y.-C. Wang et al., 2019). However, there are limitations to current probiotics as they may not effectively prevent diseases such as *V. parahaemolyticus* and WSSV. Therefore, there is a need to search for and develop new probiotic strains to enhance

shrimp health and reduce infection rates (Rattanakhansang et al., 2013). Due to their ability to stimulate non-specific immunity and help maintain the balance of beneficial microorganisms in shrimp ponds, probiotics have been used in aquaculture (Kumar et al., 2022). Examples of probiotics used in aquatic animals include *Lactobacillus*, *Enterococcus*, *Bacillus*, *Aeromonas*, *Alteromonas*, *Arthrobacter*, *Bifidobacterium*, *Clostridium*, *Microbacterium*, *Paenibacillus*, *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Rhodosporidium*, *Roseobacter*, *Streptomyces*, and *Vibrio* (Ringø, 2020). However, the use of probiotics in shrimp farming still faces challenges regarding the specificity of probiotic strains used in different environmental conditions. Since shrimp raised in different environments are exposed to different microbial populations, using probiotics isolated from other organisms or products may not effectively control indigenous microorganisms in shrimp farming areas (Decamp et al., 2008). In a previous study, it was found that a mixture of lactic acid bacteria strains could increase the survival rate against WSSV infection (Peraza-Gómez et al., 2009). Additionally, feeding shrimp with a mixture of *Pediococcus pentosaceus* and *Staphylococcus hemolyticus* as probiotics was found to reduce susceptibility to both WSSV and infectious hypodermal and *hematopoietic necrosis virus* (IHHNV) infections (Leyva-Madrigal et al., 2011). However, cultivating lactic acid bacteria can be challenging for farmers due to slow growth and specific environmental requirements, which may increase the risk of contamination with other pathogens.

Studies have investigated the potential of *B. velezensis* as a probiotic for shrimp. discovered that *B. velezensis* CPA1-1 effectively suppressed the growth of *V. cholerae* non-O1, a shrimp pathogen, while also enhancing the shrimp's immune response (Zhu et al., 2021).

2.6 *Bacillus velezensis* S141

Bacillus velezensis S141 is a plant growth-promoting rhizobacterium (PGPR) isolated from soybean rhizosphere soil in Thailand and is closely related to *B. subtilis* GB03 based on 16S rRNA gene sequencing. *B. velezensis* S141 has shown the capability to increase soybean growth, nodulation, and nitrogen fixation efficiency when co-inoculated with *Bradyrhizobium diazoefficiens* USDA110. Furthermore, *B. velezensis*

S141 was found to possess genes involved in indole-3-acetic acid (IAA). These findings suggest that *B. velezensis* S141 has multiple mechanisms for auxin production, contributing to its plant growth-promoting abilities (Sibponkrung et al., 2017). The study revealed that *B. velezensis* S141 shows promise as a beneficial bacterium in enhancing plant-AMF symbiosis by promoting fungal growth, nutrient uptake, and gene expression associated with symbiotic interactions (Kiddee et al., 2024).

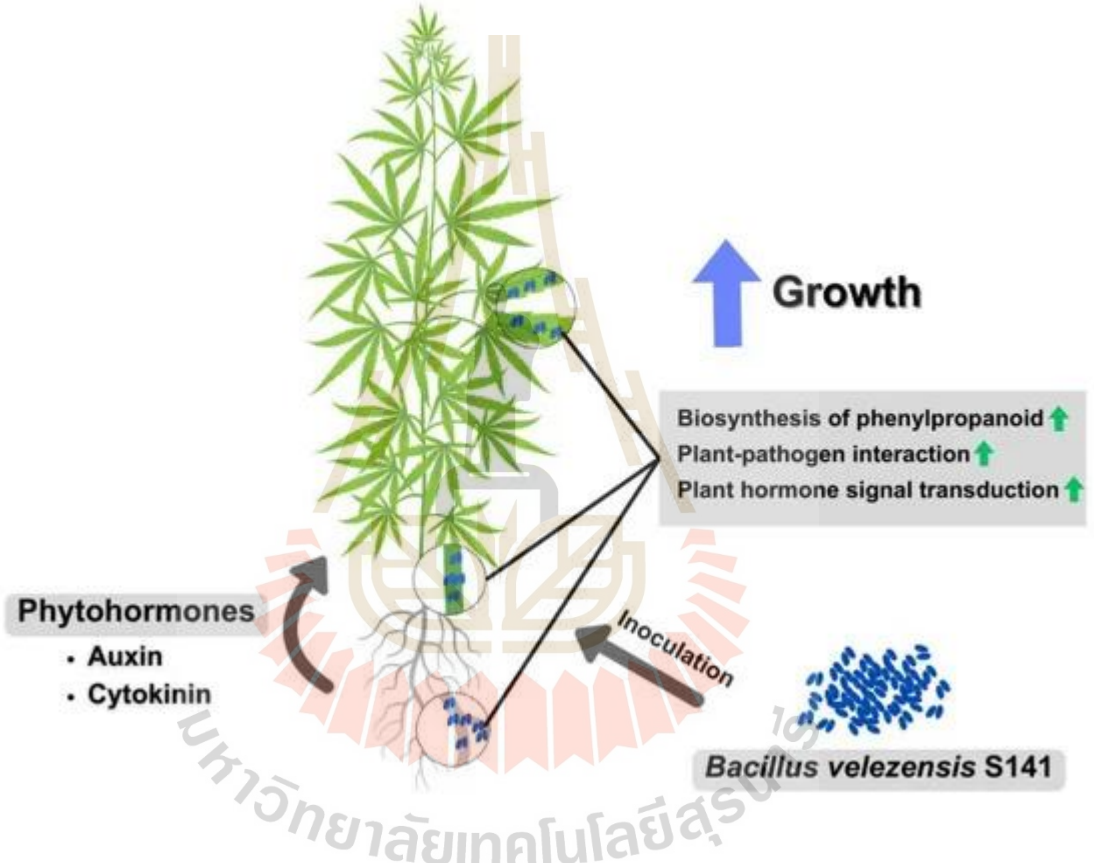


Figure 2.16 Schematic overview of mechanisms of cannabis growth promotion by *Bacillus velezensis* S141. S141, an endophytic cannabis bacterium, promotes cannabis growth by producing phytohormones and triggering genes involved in the biosynthesis of phenylpropanoid, plant–pathogen interaction, and plant hormone signal transduction pathways (Aunkam et al., 2024).

Bacillus velezensis S141 was known to enhance the growth of cannabis (*Cannabis sativ*). The research demonstrates that this strain acts as a plant growth-

promoting rhizobacterium (PGPR) by producing bioactive compounds, modulating nutrient availability, and enhancing stress resilience in plants. *B. velezensis* S141 was found to secrete a suite of phytohormones, including indole-3-acetic acid (IAA), which directly stimulates root elongation and branching, thereby increasing the plant's ability to uptake water and nutrients. The bacterium also contributes to improved nutrient acquisition by solubilizing phosphate and fixing atmospheric nitrogen, ensuring essential macronutrient availability for cannabis growth. Additionally, the strain synthesizes secondary metabolites such as lipopeptides and siderophores, which inhibit the growth of phytopathogens in the rhizosphere, thereby protecting the plants from diseases. Genomic analysis further revealed the presence of genes associated with antibiotic production, quorum sensing, and stress resistance, underscoring its multifunctional role in promoting plant health. Moreover, the study highlights the bacterium's role in mitigating abiotic stresses, including drought and salinity, through mechanisms such as the synthesis of osmoprotectants and the induction of systemic resistance in plants. They also evaluated the symbiotic interaction between *B. velezensis* S141 and cannabis plants in a hydroponic system, where the bacterium significantly enhanced biomass accumulation, cannabinoid content, and overall physiological performance. This was attributed to the bacterium's ability to modulate gene expression in cannabis, particularly genes involved in primary metabolism, stress responses, and secondary metabolite biosynthesis. The findings suggest *that B. velezensis* S141 has significant potential as a bioinoculant for sustainable cannabis cultivation, reducing the reliance on chemical fertilizers and pesticides while improving crop yield and quality (Fig. 16). These insights contribute to the growing body of knowledge on the beneficial roles of PGPRs in horticulture and highlight the importance of harnessing microbial resources to enhance agricultural productivity. The study concludes with a recommendation for further field trials and exploration of the synergistic effects of combining *B. velezensis* S141 with other beneficial microorganisms to optimize its application in diverse cannabis cultivation systems (Aunkam et al., 2024). In addition, *B. velezensis* S141 exhibits potent antifungal activity against *Cercospora canescens* through the production of multiple enzymatic hydrolases and secondary metabolites (Songwattana et al., 2023).

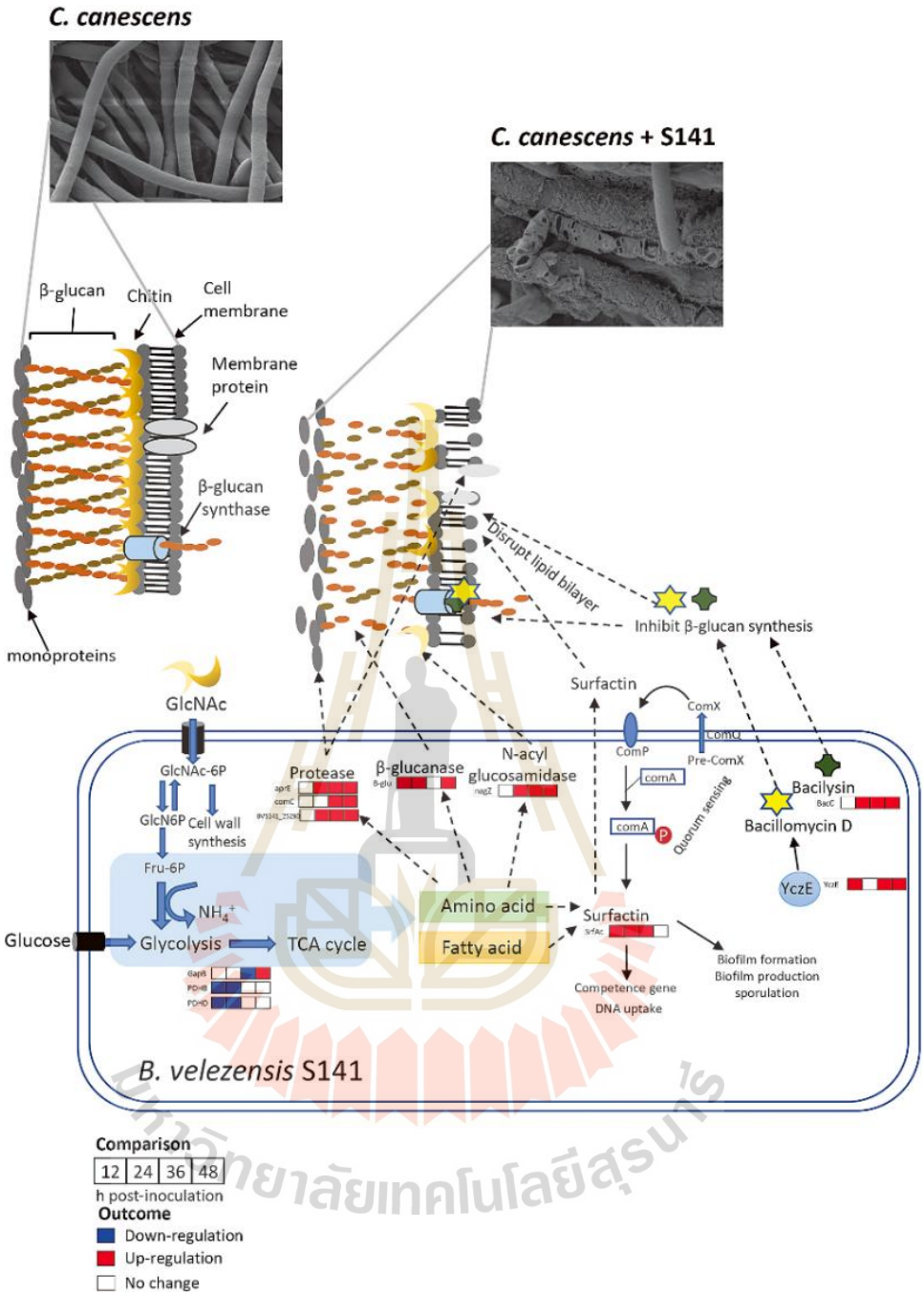


Figure 2.17 Schematic overview of the antifungal activity of *Bacillus velezensis* S1 4 1 against *Cercospora canescens* (Songwattana et al., 2023).

CHAPTER III

SUPPLEMENTATION OF *Bacillus velezensis* S141 IN FEED AS A PROBIOTIC ENHANCES GROWTH PERFORMANCE, PATHOGENIC RESISTANCES, AND IMMUNE SYSTEM IN SHRIMP

3.1 Abstract

In *Litopenaeus vannamei* aquaculture, infectious diseases pose significant challenges, leading to the exploration of alternative strategies for pathogen combat. This study examines the efficacy of *Bacillus velezensis* S141, supplemented in feed at various levels 10^2, 10^4, and 10^6 CFU/g, on immune responses, growth performance, and disease resistances against the White Spot Syndrome Virus (WSSV), Vibrio parahaemolyticus (VP_{AHPND}), and *Enterocytozoon hepatopenaei* (EHP). The results indicated significant improvements in the weight gain (WG), average daily gain (ADG), and specific growth rate (SGR) of shrimp supplemented with *B. velezensis* S141 compared to the control group. Moreover, shrimp supplemented with *B. velezensis* S141 had significantly higher survival rates during post-WSSV infection. Co-injection with *B. velezensis* S141 secretion and WSSV significantly improved survival rates and reduced WSSV copy numbers in the gill, indicating enhanced resistance to the WSSV infection. Administering *B. velezensis* S141 reduced cumulative mortality during the VP_{AHPND} challenge and EHP copy number compared to the control group. Furthermore, the gene expression analyses of immune-related genes in the Toll/IMD pathway (Toll, IMD, LYZ1, LYZ-C, PEN4, ALF1, Relish, NF-kappa, RPX, and DOME), JAK/STAT pathway (STAT and GILT), Vago pathway (Vago4, Vago5), CathC, and α2M showed significant upregulation in the group administered feed containing 10^6 CFU/g of *B. velezensis* S141 compared to the control group in the gills. This study emphasizes the potential of *B. velezensis* S141 as a probiotic supplement in enhancing immune responses and disease resistance against WSSV, EHP, and VP_{AHPND} in *L. vannamei*, providing promising prospects for sustainable shrimp aquaculture practices.

3.2 Introduction

Litopenaeus vannamei, commonly referred to as the Pacific white shrimp, plays a crucial role in global shrimp farming, serving as a significant source of income. However, the industry faces increasing challenges due to the prevalence of infectious diseases, which continue to escalate with the emergence of new (Chiu et al., 2007; Neiland et al., 2001). To counteract these infections, shrimp farmers often rely on antibiotics. While effective in treating diseases, excessive antibiotic usage has raised serious concerns regarding environmental contamination and the potential development of antibiotic-resistant pathogens, which could pose risks to human health in the future (Dawood et al., 2020).

Disease outbreaks have consistently had a devastating impact on shrimp farming (Amaya et al., 2007; Jin et al., 2018). Among the most severe threats is the White Spot Syndrome Virus (WSSV), a large, enveloped double-stranded DNA virus classified under the *Whispovirus* genus in the *Nimaviridae* family. This virus is highly lethal, with mortality rates in shrimp reaching 100% within 3 to 5 days post-infection infection (Pradeep et al., 2012; Sánchez-Martínez et al., 2007). Another major pathogen, *Vibrio parahaemolyticus*, is responsible for acute hepatopancreatic necrosis disease (AHPND), producing pore-forming toxins known as Photorhabdus insect-related toxins (Pir). These toxins, composed of PirA and PirB proteins, cause hepatopancreas cell destruction, leading to acute pancreatitis and liver disease, often resulting in 100% mortality within 48 hours (De Schryver et al., 2014; Prachumwat et al., 2019). Additionally, *Enterocytozoon hepatopenaei* (EHP), a microsporidian pathogen, infects the hepatopancreas and contributes to stunted shrimp growth in aquaculture systems (Chaijarasphong et al., 2021).

To address these challenges, probiotics and prebiotics are being explored as promising alternatives to enhance host immunity, reduce disease susceptibility, and improve the overall health of aquatic organisms (Zhu et al., 2021). In aquaculture, probiotics have garnered attention for their potential in disease diseases (Ninawe & Selvin, 2009). For example, *Bacillus coagulans* ATCC 7050 (BC) supplementation in shrimp diets has demonstrated improvements in growth, intestinal health, immune response, and resistance to *V. parahaemolyticus* infections (Amoah et al., 2019).

Similarly, *Bacillus* PC465 at a concentration of $1x10^9$ CFU/g has been reported to enhance growth, gut microbiota composition, digestive health, and resistance to WSSV infections in *L. vannamei* (Chai et al., 2016)In addition, *Rhodotorula paludigena* CM33 supplementation in shrimp feed has shown promising effects in promoting growth, resistance to bacterial infections, and enhanced antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT), which are key indicators of oxidative stress (Sriphuttha et al., 2023; Yang et al., 2015). Thus, probiotics offer a sustainable alternative to antibiotics, improving shrimp health and reducing the environmental impact associated with antibiotic overuse.

Bacillus velezensis has been extensively studied for its biocontrol properties in plants, where it inhibits bacterial and fungal pathogens, promoting plant health in the rhizosphere (Gu et al., 2017). Additionally, its probiotic potential in shrimp farming has been recognized. Research indicates that *B. velezensis* CPA1-1 can inhibit *Vibrio cholerae* non-O1175, which causes disease in *Macrobrachium nipponense*, while simultaneously stimulating the shrimp immune system (Zhu et al., 2021). In *L. vannamei*, *B. velezensis* BV007 supplementation in shrimp feed has demonstrated improvements in growth performance and resistance to *V. parahaemolyticus* infections (Chen et al., 2021). Furthermore, *B. velezensis* S141, previously studied for its antifungal properties against plant pathogens due to its secretion of bioactive molecules molecules (Sibponkrung et al., 2017; Songwattana et al., 2023), has not yet been evaluated for its effects on shrimp immune responses or its ability to combat pathogens such as WSSV and EHP. Despite the potential of *B. velezensis* S141 to produce antimicrobial compounds beneficial to shrimp, no research has been conducted on its application as a probiotic in shrimp farming.

This study aims to assess the benefits of incorporating *B. velezensis* S141 into the diet of *L. vannamei*, evaluating its effectiveness in delaying disease onset and reducing cumulative mortality caused by WSSV, VP_{AHPND}, and EHP. The research underscores the role of probiotics in shrimp aquaculture, emphasizing their potential as sustainable dietary supplements that can minimize antibiotic dependence.

3.3 Materials and Methods

3.3.1 Ethics statement

The animal study was conducted in compliance with animal use protocol number U1-06656-2560 and received approval from the Suranaree University of Technology Animal Care and Use Committee

3.3.2 Animals

Healthy *L. vannamei* shrimp, weighing between 0.3–0.5 g, were procured from a shrimp farm situated in Chachoengsao Province, Thailand. The shrimp were reared in seawater that had a salinity level of 20 ppt, in a plastic tank with a volume of 60 L (1 shrimp per 2 L of water), at a temperature fluctuation of 30 ± 2°C. The experimental setup was divided into four groups, each subjected to a distinct feed formulation.

3.3.3 Experimental diet preparation

B. velezensis S141 was cultured on nutrient broth (NB), consisting of 10 g/L meat extract, 10 g/L peptone, and 1.5% NaCl, at 180 rpm for 18 h at 37°C. After cultivation, the cells were collected via centrifugation at 4°C, where the cells were collected and resuspended in 0.85% NaCl. These cells were then incorporated into shrimp feed (9092L, Charoen Pokphand Foods, Thailand, comprising 36% protein, 4% fat, 5% ash, and 12% moisture) in three different formulations. Cell densities of 1×10^2, 1×10^4, and 1×10^6 CFU/g were used, coated with 1% (w/w) alpha starch of feed, oven-dried at 60°C, and stored at 4°C. In contrast, a control group was given regular shrimp pellets. Following this, the amount of feed given to each group was monitored, with the feed quantity at 10% of the average body weight of the shrimp per day. Each group of 100 shrimps was placed in four tanks, each containing 300 L of water with a salinity of 20 ppt, to assess their growth performance. The feeding regime extended over 6 weeks (42 days), during which feedings occurred twice a day at 9.00 AM and 6.00 PM. After 42 days, weight gain (WG, g), average daily gain (ADG, g), specific growth rate (SGR, % day^{-1}), and survival rate (SR, %) were determined (Sriphuttha et al., 2023) as follows:

$$\text{Weight gain (WG, g)} = \frac{\text{final weight} - \text{initial weight}}{\text{day}}$$

$$\text{Average daily gain (ADG, g)} = \frac{\text{final weight} - \text{initial weight}}{\text{experimental duration (day)}}$$

$$\text{Specific growth rate (SGR, \%day}^{-1}\text{)} = \frac{(\ln(\text{final weight}) - \ln(\text{initial weight}))}{\text{day}} \times 100$$

$$\text{Survival rate (SR, \%)} = \frac{\text{Final amount of shrimp}}{\text{Initial amount of shrimp}} \times 100$$

Hepatopancreas, stomach, and intestine tissues were collected for genomic DNA (gDNA) extraction and purification using the Tissue Genomic DNA Extraction Mini Kit (Favorgen) to quantify S141 copy numbers with specific primer S141-F (5′-TGATTGCCGGCACAGAAAATAACAGG-3′) and S141-R (5′-GGTTTCCGGTACCACGTC TGTC-3′) by qRT-PCR.

3.3.4 Challenges of White Spot Syndrome Virus (WSSV) and *Bacillus velezensis* S141 secretion challenge

The seed culture was made in the following manner: In a 5-L fermenter, *R. paludigena* CM33 was inoculated into a crude glycerol medium and grown there for 48 hours at 30 °C and 0.7 vvm. The same conditions were employed for cultivation in a 50-L fermenter as well. 10% (v/v) of the seed culture was added to the fermentation medium in order to carry out batch fermentation. With an airflow rate of 0.7 vvm, the batch fermentation process continued for 7 days at 30°C. For the repeated fermentation process, after the overall glycerol concentration decreased, approximately 70% of the fermentation broth was withdrawn, and an equal volume of fresh medium was added. Finally, for the fed-batch fermentation with pulse addition, 10% (v/v) of cultures were inoculated into a 250 L medium and cultivated at 30 °C with a 0.7 vvm air flow level. The fed-batch culture took place in four stages. During the first stage, the fermenter received 25 L of medium, resulting in a total glycerol content of 40 g/L. After three days, the residual total glycerol concentration fell below 5 g/L, prompting the addition. Subsequently, 25 L of medium was added in each of the second to fourth stages to maintain a constant total glycerol concentration of 40 g/L. Daily, 25 mL samples of the fermentation broth were taken throughout the

experiment to measure the levels of carotenoids, OD 600 nm, biomass, and glycerol. A spectrophotometer was used to measure the carotenoid levels (Ribeiro et al., 2019). 10N NaOH was used to keep the pH at 5.5 +/- 0.5. As required, foam generation was managed using silicone antifoam (Kemaus, Australia).

3.3.5 *Vibrio parahaemolyticus* (VP_{AHPND}) challenge

The bacteria were cultured in Tryptic Soy Broth (TSB) supplemented with 1.5% NaCl at 30°C and a rotation speed of 220 rpm. The bacterial concentrations were ascertained by measuring the optical density (OD) at a wavelength of 600 nm (OD_{600}). It is important to note that an OD_{600} of 2.0 corresponds to 1×10^8 CFU/ml [24]. Bacterial inoculants were added to tanks containing 15 shrimps with a final concentration of 1×10^6 CFU/ml (LD_{50} = 24 h). Shrimp mortalities were recorded cumulatively every 12 h. To confirm VP_{AHPND} colonization, hepatopancreases were collected at 24 h post-infection (hpi) for genomic DNA (gDNA) extraction and purification with the Tissue Genomic DNA Extraction Mini Kit (Favorgen). The quantification of VP_{AHPND} copy numbers followed a protocol established previously (Limkul et al., 2023). According to a study by Li et al. (2020) (Y. Li et al., 2020), the antimicrobial activity of *B. velezensis* S141 secretion was assessed using the agar well diffusion technique. A 2% agar plate was coated with a *V. parahaemolyticus* culture, after adjusting the bacterial density to an OD_{600} of approximately 0.1 using a sterile physiological solution (Valgas et al., 2007). A well of 0.7 cm was filled with 100 µL of *B. velezensis* S141 culture. Post this, the plate was incubated at 30°C for 16 h. The diameters of the clear lysis zones were measured using ImageJ.

3.3.6 *Enterocytozoon hepatopenaei* (EHP) challenge

The pathogenicity of *E. hepatopenaei* was investigated. Shrimp infected with EHP (whole animal) were homogenized using a buffer solution containing 0.2 M NaCl, 0.02 M Tris-HCl, and 0.02 M EDTA at a 1:10 ratio. The resulting mixture underwent three freeze-thaw cycles and was subsequently centrifuged at 5,000 rpm for 5 min. The resulting supernatant was used as an inoculant (0.2%) for groups of 15 shrimp each (Karthikeyan & Sudhakaran, 2019). Based on published PCR-specific primers developed for EHP, the 510F/R primer set was chosen for EHP PCR detection (Gao et al., 2023), and for detecting the EHP-PTP2 gene, the PTP2W F/R primer was

used (Table 2) (L. Wang et al., 2020). After 72 h, hepatopancreas tissue samples were collected for gDNA extraction using the Tissue Genomic DNA Extraction Mini Kit (Favorgen). The EHP copy number was quantified via qPCR using 50 ng of genomic DNA extracted from infected shrimp as a template.

3.3.7 Quantitative real-time PCR (qPCR) analysis of immune gene expression

Total RNA was extracted and purified from gill, hepatopancreas, and hemocyte samples using the Tissue Total RNA Purification Mini Kit (Favorgen). The quality and quantity of the RNA were evaluated via agarose gel electrophoresis stained with RedSafe Nucleic Acid Staining Solution (iNtRON) and a Nanodrop 2000 Spectrophotometer (Thermo Scientific), respectively. Subsequently, 1 µg of total RNA was used for complementary DNA (cDNA) synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). For quantitative real-time RT-PCR (qRT-PCR) analysis, the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and 2x Taq THUNDERBIRD Next SYBR qPCR (TOYOBO) were employed, along with specific primer pairs (Table 1). Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, normalized to elongation factor-1 alpha (*EF-1α*) (Livak & Schmittgen, 2001).

Table 3.1 The list primers used in this study.

Primer name	Sequence (5' to 3')	Annealing temperature (°C)
Toll-F	GGCTGTCCAGTATACGAAGG	60
Toll-R	CCAGGTCATCAGCCTGTTG	
IMD-F	CGGCTCTGCGGTTCACAT	60
IMD-R	CCTCGACCTTGTCTCGTTCCT	
PEN4-F	ATATTTTCTTTCTTTCTTTCCCAGGG	60
PEN4-R	GTCCTCTGTGACAACAATCCCC	
LYZ-F	TACGCGACCGATTACTGGCTAC	55
LYZ-R	AGTCTTTGCTGCGACCACATTC	
TRAF-6-F	ATCTCCTTCTTGCCACACC	60
TRAF-6-R	TCCTCCCATCTTCCTTCCC	
NF-kappa-F	TCGGATCAACTCAGCTCTC	60

Table 3.1 The list primers used in this study. (Continued)

Primer name	Sequence (5' to 3')	Annealing temperature (°C)
NF-kappa-R	GCTGAGTGTCTCCGCTCCT	
Dorsal-F	GATGGAATGATAGAATGGGAAGC	60
Dorsal-R	CACTGGTACTCTTGTCTGGTGGTC	
Relish-F	AATTCCGATTCCGCTACAAGAGT	60
Relish-R	TCATCGACAACAACCTCACACAT	
STAT-F	TATATCCGAATGTGCCTA	60
STAR-R	ATAGTTTGTGGTGTGTTG	
GILT-F	TCCTTCACCTGCCAACA	60
GILT-R	CGAGAGAAGGCAGTTGA	
Vago4-F	AGCTGCTGCCCCATCATCT	60
Vago4-R	ATCCAATTCGTGAACTCGTCGTA	
Vago5-F	GAGGGATTGTGATGCTGCTTG	60
Vago5-R	AAATGCCTGCCTTCTTGTCG	
ALF1-F	GTCCTCCGTGATGAGATTACTCTG	60
ALF1-R	TTACTTCAATGGCAGGATGTGG	
PRX-F	ACGGACAGTTCAAGGAGATC	60
DOME-F	TCAGACAGGAGGTCTCATAC	60
DOME-R	GTACCAGTGTGAAGCCTTAC	
CathC-F	CATGACCAGACTGCGTGAC	60
CathC-R	CATGGAAGCAAAGGCATAGC	
LYZ-C-F	CCCATGTTCCGATCTGATGTC	60
LYZ-C-R	CACTTGCTGTTGTAAGCCACC	
α2M-F	TGCAGGTTCTAGTGTGGTAC	60
α2M -R	CACCCAGGTAGTCGATGAC	
LvPO-F	ACTGACCTGGAAATCTGGCG	60
LvPO-R	TCCTCCTTGTGAGCGTTGTC	
LvPO2-F	CCGTGAACAACTCCGGAAGA	60
LvPO2-R	CTGAGATTCGAGTCGGCCTC	
EHP 510-F	GCCTGAGAGATGGCTCCCACGT	60

Table 3.1 The list primers used in this study. (Continued)

Primer name	Sequence (5' to 3')	Annealing temperature (°C)
EHP 510-R	GCGTACTATCCCCAGAGCCCGA	
EHP-PTP2W -F	GCAGCACTCAAGGAATGGC	60
VP-F	GTGTTGCATAATTTTGTGCA	60
VP-R	TTGTACAGAAACCACGACTA	
EF-1α-F	CGCAAGAGCGACAACTATGA	60
EF-1α-R	TGGCTTCAGGATACCAGTCT	

3.3.8 Statistical analysis

A one-way analysis of variance (ANOVA) at a 5% probability level was used to ascertain significant differences, and Tukey's range test was used to examine mean differences in growth performance, WSSV, VP_{AHPND}, EHP challenge, and gene expression. The data is presented as mean ± standard deviation (SD). Survival percentages are demonstrated as mean ± 1 standard error (SE) for every time point. The log-rank (Mantel-Cox) test was utilized for analyzing the survival rate in each trial group (Bland & Altman, 2004). All statistical analysis was conducted using GraphPad Software (Massachusetts, USA) for Windows.

3.4 Results

3.4.1 Growth performance of *Bacillus velezensis* S141 in shrimp

The effects of *B. velezensis* S141 on shrimp growth performance were analyzed by examining the impact of this bacterium in differing amounts in shrimp feed. After a feeding period of 42 days, a significant increase in weight gain (WG, g) was observed in shrimp fed with 10^2, 10^4, and 10^6 CFU/g, showing gains of 1.97 ± 0.44, 2.52 ± 0.36, and 2.15 ± 0.77 g, respectively. All groups fed with S141 also exhibited average daily gain (ADG, g) of 0.05 ± 0.01, 0.06 ± 0.01, and 0.05 ± 0.02 g, and the specific growth rate (SGR, % day^{-1}) was 4.11 ± 0.49, 4.80 ± 0.44, and 4.09 ± 0.45% day^{-1} (Table 2). However, no significant difference was observed in the survival rate of shrimp across all S141 groups and the control group (Table 2).

Following feeding, the number of S141 copies in the intestines of the 10^2, 10^4, and 10^6 CFU/g groups increased by 4.6×10^4, 1.1×10^6, and 1.3×10^6copies/20 ng DNA, respectively. In the hepatopancreas, populations were higher by 7.5×10^4, 8.1×10^4, and 1.8×10^5 copies/20 ng DNA, respectively, while in the stomach, they were higher by 1.2×10^3, 3.7×10^3, and 6.5×10^3 copies/20 ng DNA, respectively, compared to the control groups (Fig. 18 A–C).

Table 3.2 Growth performance of *L. vannamei* fed with experimental diets containing different concentrations of *B. velezensis* S141 for 42 days.

parameters	Treatments			
	Control	10^2 CFU/g	10^4 CFU/g	10^6 CFU/g
WG (g)	1.06 ± 0.47^a	1.97 ± 0.44^b	2.52 ± 0.36^b	2.15 ± 0.77^b
ADG (g)	0.03 ± 0.01^a	0.05 ± 0.01^b	0.06 ± 0.01^b	0.05 ± 0.02^b
SGR (%day^{-1})	2.92 ± 0.79^a	4.11 ± 0.49^b	4.80 ± 0.44^c	4.09 ± 0.45^b
Survival rate (%)	81.67 ± 4.19^a	81.25 ± 4.25^a	84.25 ± 7.2^a	82.50 ± 2.50^a

The values were provided as mean ± SD (n = 9). Tukey's range test revealed significant differences ($P < 0.05$) between mean values in the same row with different superscripts. The experiment was repeated three times (n.d. = not detected, WG = weight gain, ADG = average daily gain, and SGR = specific growth rate).

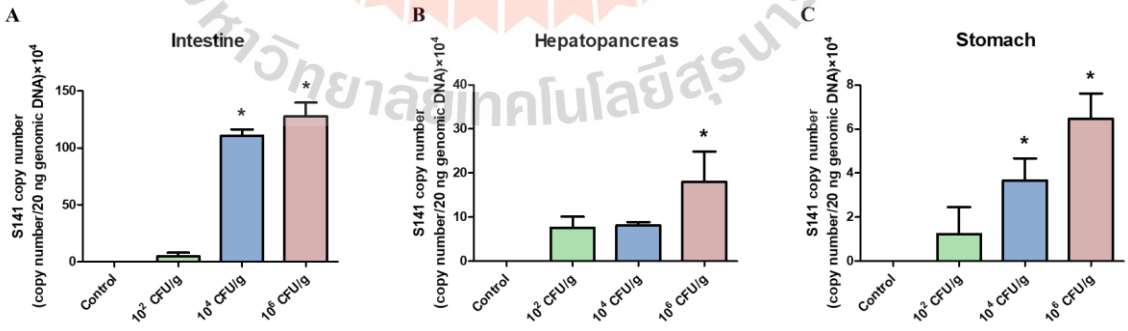


Figure 3.1 Copy number of *B. velezensis* S141 in shrimp.

(A) Number of cells in the intestine, (B) number of cells in the hepatopancreas, and (C) number of cells in the stomach of shrimp fed different

experimental diets containing *B. velezensis* S141 (n = 12 for each group). Asterisks indicate significant differences between the data of each treatment compared to the control group ($P < 0.05$)

3.4.2 Effect of feeding *B. velezensis* S141 on cumulative mortality with WSSV infection, and the impact of co-injection with *B. velezensis* S141 secretion

After 42 days of feeding, 15 shrimps were collected from each tank to assess mortality upon WSSV infection. At 120 h post-infection (hpi), the survival rates of the control and 10^2 CFU/g groups were 0%. In contrast, the 10^4 and 10^6 CFU/g groups exhibited rates of 41.67% and 25.00%, respectively. This demonstrated a significant decrease in mortality rates in response to WSSV challenges for the groups that sufficiently received *B. velezensis* S141, compared to both the control and 10^2 CFU/g groups (Fig. 19A). To further validate the impact of *B. velezensis* S141 supplementation on WSSV resistance, the WSSV copy numbers were measured in the gill using qRT-PCR (Fig. 19B). Furthermore, a reduction in the WSSV numbers in the gill was apparent across all *B. velezensis* S141-supplemented groups. This included the 10^4 CFU/g group (with 3.50×10^6 copies/20 ng DNA) and the 10^6 CFU/g group (with 1.20×10^6 copies/20 ng DNA), which was notably lower when compared to the control (at 13.2×10^6 copies/20 ng DNA) and the 10^2 CFU/g group (at 1.18×10^7 copies/20 ng DNA). This finding suggests a significant decrease in shrimp mortality following WSSV infection.

To evaluate the direct influence of S141 on WSSV, shrimps were co-injected with *B. velezensis* S141 secretion and WSSV. Mortality was noted every 12 h, up to a total duration of 120 hpi. In the case of the control groups, the survival rate was reported as 0% at 108 h post-infection (hpi), while at 120 hpi for the 10-fold, 2-fold, and undilute groups, the rates were 13.33%, 37.50%, and 23.08% respectively. These rates were significantly higher than the control group (Fig. 19C).

A decrease in WSSV numbers in the gill was observed in all co-injected *B. velezensis* S141 10-fold, 2-fold, and undilute groups, with reductions reported as 3-fold, 8-fold, and 10-fold, respectively when compared to the control group (Fig. 19C). The experiment including the co-injection of S141 secretion with WSSV, as well as the

challenged with WSSV injection following 42 days of feeding with *B. velezensis* S141, showed reduced mortality and a decline in the WSSV copy number. The efficacy of *B. velezensis* S141 in providing increased protection to shrimp against WSSV suggests its functional role in reducing WSSV (Fig. 19D).

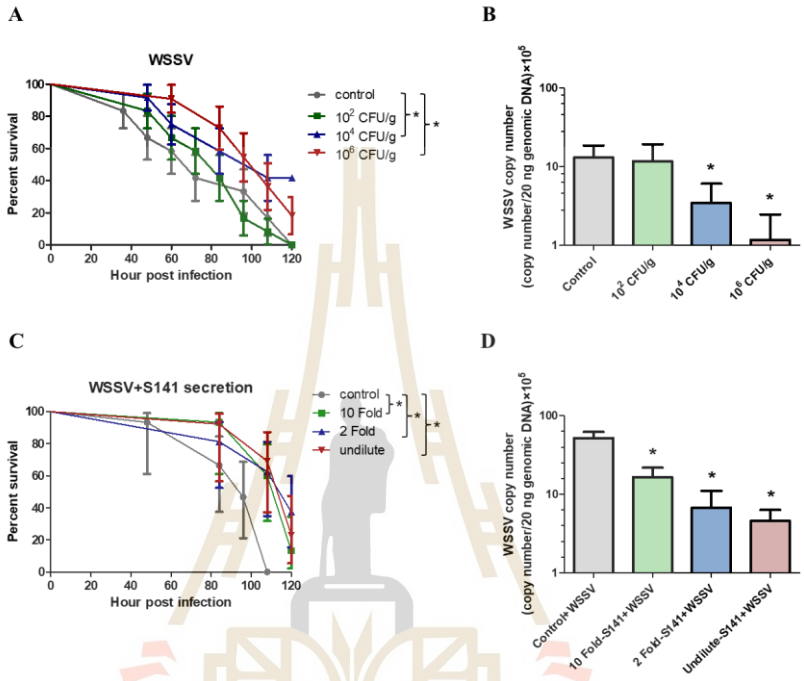


Figure 3.2 The effect of *B. velezensis* S141 on shrimp mortality with WSSV infection and WSSV co-infected shrimp with *B. velezensis* S141.

(A) Cumulative mortality of shrimp resulting from the WSSV challenge after 42 days of feeding with *B. velezensis* S141. Shrimp survival was observed every 12 h for a total of 120 h.; ● = control, ■ = 10^2 CFU/g, ▲ = 10^4 CFU/g, and ▼ = 10^6 CFU/g. The survival percentage was displayed as mean ± 1 standard error (SE) (n = 15) at each time point. (B) Total WSSV in the gill was observed for 24 hpi after 42 days of feeding with *B. velezensis* S141; results are displayed as means ± SD (n = 9). (C) Cumulative mortality of shrimp resulting from the co-injection of S141 secretion with WSSV. Shrimp survival was observed every 12 h for a total of 120 h; ● = control, ■ = 10-fold, ▲ = 2-fold, and ▼ = undiluted. (D) The WSSV co-infected shrimp with *B. velezensis* S141 secretion were observed for 120 hpi. The shrimp were divided into

four groups of 15 each. The survival percentage is displayed as mean ± 1 SE (n = 15) at each time point. All experiments were performed in triplicate. Asterisks indicate significant differences between the data of each treatment ($P < 0.05$).

3.4.3 The impact of *B. velezensis* S141 feeding on cumulative mortality with VP_{AHPND} infection

To determine the impact of *B. velezensis* S141 on the cumulative mortality of shrimp infected with VP_{AHPND}, shrimps were fed a diet supplemented with S141 for 42 days before being exposed to VP_{AHPND}. At 42 h post-infection, the survival rates of the control, the 10^2 CFU/g, and the 10^4 CFU/g groups were 5%, 22%, and 45%, respectively. The control and 10^2 CFU/g group reported 100% mortality first at 82 h. In the 10^6 CFU/g group, there was a 60% survival rate observed at 120 h (Fig. 20A).

To validate the impact of *B. velezensis* S141 supplementation on VP_{AHPND} resistance, the copy numbers of *V. parahaemolyticus* in the hepatopancreas were noted to be 5, 25, and 166-fold lower in the 10^2, 10^4, and 10^6 CFU/g *B. velezensis* S141 groups, respectively, as compared to the control group at 24 h post-infection (Fig. 20B).

Given the significant reduction in shrimp mortality upon VP_{AHPND} infection, colonies of *B. velezensis* S141 secretion were inoculated on a double-layer agar plate with VP_{AHPND} cells and incubated for 1–2 days at 30°C. Following a day of incubation, *B. velezensis* S141 secretion produced a clear zone on the plate (Fig. 20C), indicating its inhibitory effect. The *B. velezensis* S141 colonies displayed a consistent form and border. Nevertheless, *B. velezensis* S141 demonstrated an inhibitory effect on VP_{AHPND}, showcasing its ability to effectively counteract VP_{AHPND}.

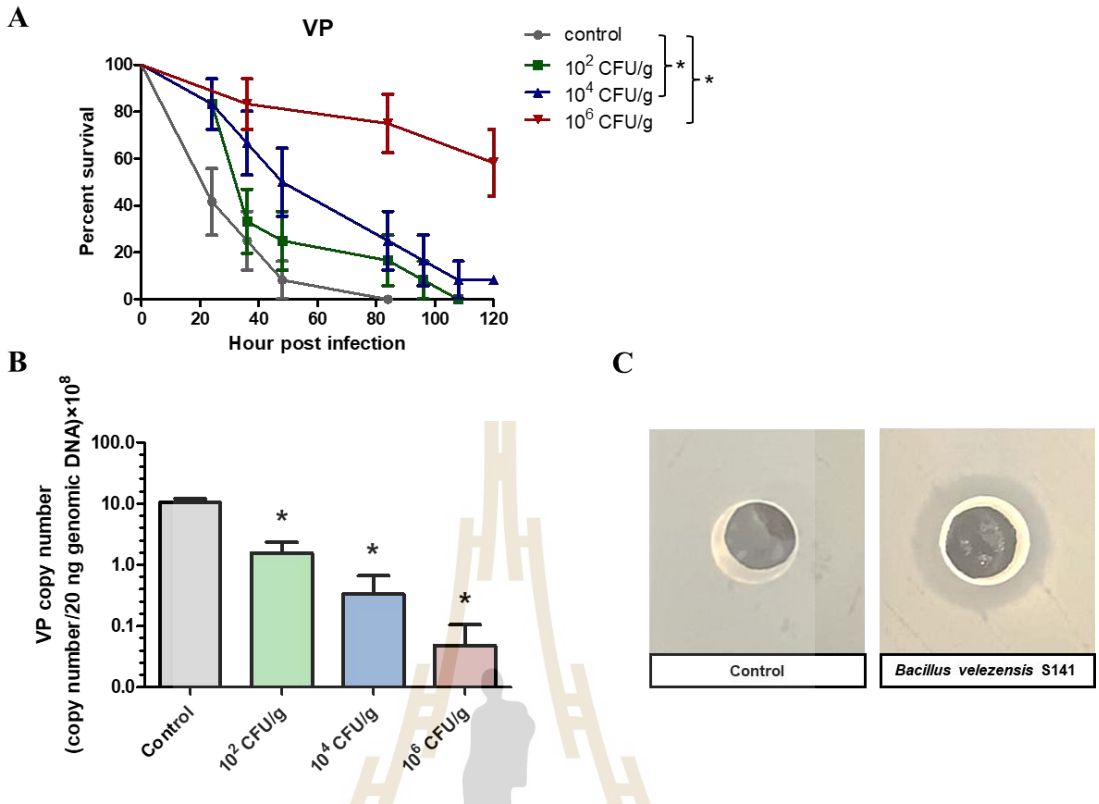


Figure 3.3 The effect of *B. valezensis* S141 on shrimp mortality with VP_{AHPND} injection.

(A) Cumulative mortality of shrimp resulting from VP_{AHPND} injection after 42 days of feeding with *B. velezensis* S141: control (●), 10^2 CFU/g (■), 10^4 CFU/g (▲), and 10^6 CFU/g (▼). Shrimp survival was observed every 12 h for a total of 120 h. The survival percentage was displayed as mean ± 1 SE (n = 15) at each time point. (B) Total VP_{AHPND} in hepatopancreas was elucidated by qRT-PCR, and results are displayed as means ± SD (n = 9). All experiments were performed in triplicate. Asterisks indicate significant differences between the data of each treatment ($P < 0.05$). (C) Agar diffusion antimicrobial assay of *B. velezensis* S141 secretion against VP_{AHPND} and control (nutrient broth).

3.4.4 The effect of *B. velezensis* S141 on cumulative mortality with EHP

To assess the impact of *B. velezensis* S141 on decreasing EHP copy numbers post-EHP infection, shrimps were given an S141-supplemented diet for 42 days, followed by EHP infection. The results indicated a decrease in EHP

concentrations in the hepatopancreases of infected shrimps (Fig. 21A–B). Every level of *B. velezensis* S141 correlated with EHP-specific gene targets (EHP-510 primer and EHP-PTP2W primer) exhibited considerable decreases of 80-fold and 20-fold, correspondingly, in comparison to the control group post-EHP infection (Fig. 21A–B). Owing to the remarkable reduction in EHP copy numbers post-infection, both the control and all *B. velezensis* S141 groups were chosen for further experiments to evaluate immune-related gene expression.

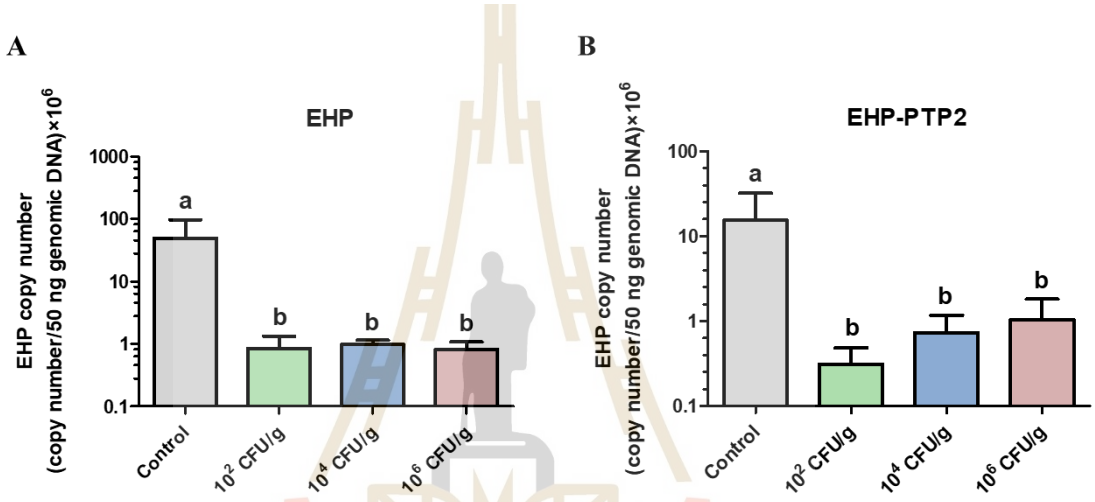


Figure 3.4 The effect of *B. velezensis* S141 with EHP.

Total EHP in the hepatopancreas was quantified using qRT-PCR, and the EHP copy number was monitored after 72 h. (A) Total EHP in the hepatopancreas was measured using qRT-PCR with the EHP 510F/R primer. (B) Total EHP in the hepatopancreas was measured using qRT-PCR with the EHP PTP2WF/R primer. Results are presented as means ± SD (n = 9). Triplicate experiments were performed. Significant differences between treatments are indicated by letters ($P < 0.05$).

3.4.5 Modulation of immune-related genes from dietary *B. velezensis* S141 feeding

To evaluate the impact of supplementary dietary *B. velezensis* S141 on gene expression, shrimp were given a diet supplemented with *B. velezensis* S141 for 42 days. This supplementation significantly influenced the expression levels of

immune-related genes within the shrimp's hepatopancreas, gills, and hemocytes. In the gills' 10^6 CFU/g group, expression levels of genes within the Toll/IMD pathway (Toll, IMD, LYZ, LYZ-C, Relish, ALF1, PEN4, Dorsal, and NF-**K**B), the JAK/STAT pathway (STAT and GILT), the Vago pathway (Vago4 and Vago5), the TLR pathway (PRX and DOME), plus CathC and **α**2M, were significantly upregulated by 4- to 147-fold in comparison to the control group. Additionally, TRAF6 was notably upregulated in the 10^2 and 10^4 CFU/g groups, both by 6-fold, as compared to the control, as seen in Fig. 22A.

In the hepatopancreas 10^6 CFU/g group, the genes' expression levels associated with the Toll/IMD pathway (such as Toll, IMD, LYZ, LYZ-C, Relish, and ALF1), the Vago pathway (like Vago5), the NF-**K**B pathway (including NF-**K**B and TRAF6), the Toll pathway (for example, Dorsal), the TLR pathway (e.g., PRX and DOME), and CathC were significantly upregulated by 1 to 4-fold (Fig. 22B). Similar to this, in all the *B. velezensis* S141 hemocyte groups, the genes in the Vago pathway (like Vago4), Toll, Relish, and PRX were considerably upregulated from 1 to 27-fold. However, expression levels of genes such as IMD, PEN4, STAT, GILT, Vago5, NF-**K**B, PRX, and **α**2M were not significantly increased, while LYZ, ALF1, Dorsal, DOME, and LYZ-C were downregulated by 0.5 to 1-fold when compared to the other groups (Fig. 22C). Despite this, no significant differences were found in the expression levels of PPO1 and PPO2 across all the experimental models, and they were downregulated in both the hepatopancreas and hemocyte (Fig. 22A–C). In addition, presents gene expression graphs demonstrating the effects of various genes, beyond the data shown in Fig. 23A–C.

Several studies have reported that *Bacillus* spp. can influence gene expression, with *B. velezensis* S141 in particular demonstrating enhanced gene expression. Therefore, when *B. velezensis* S141 is administered through probiotic-enriched feed, it has beneficial effects on the immune system of shrimp. The group receiving a concentration of 10^6 CFU/g displayed the highest expression profiles of immune-related genes.

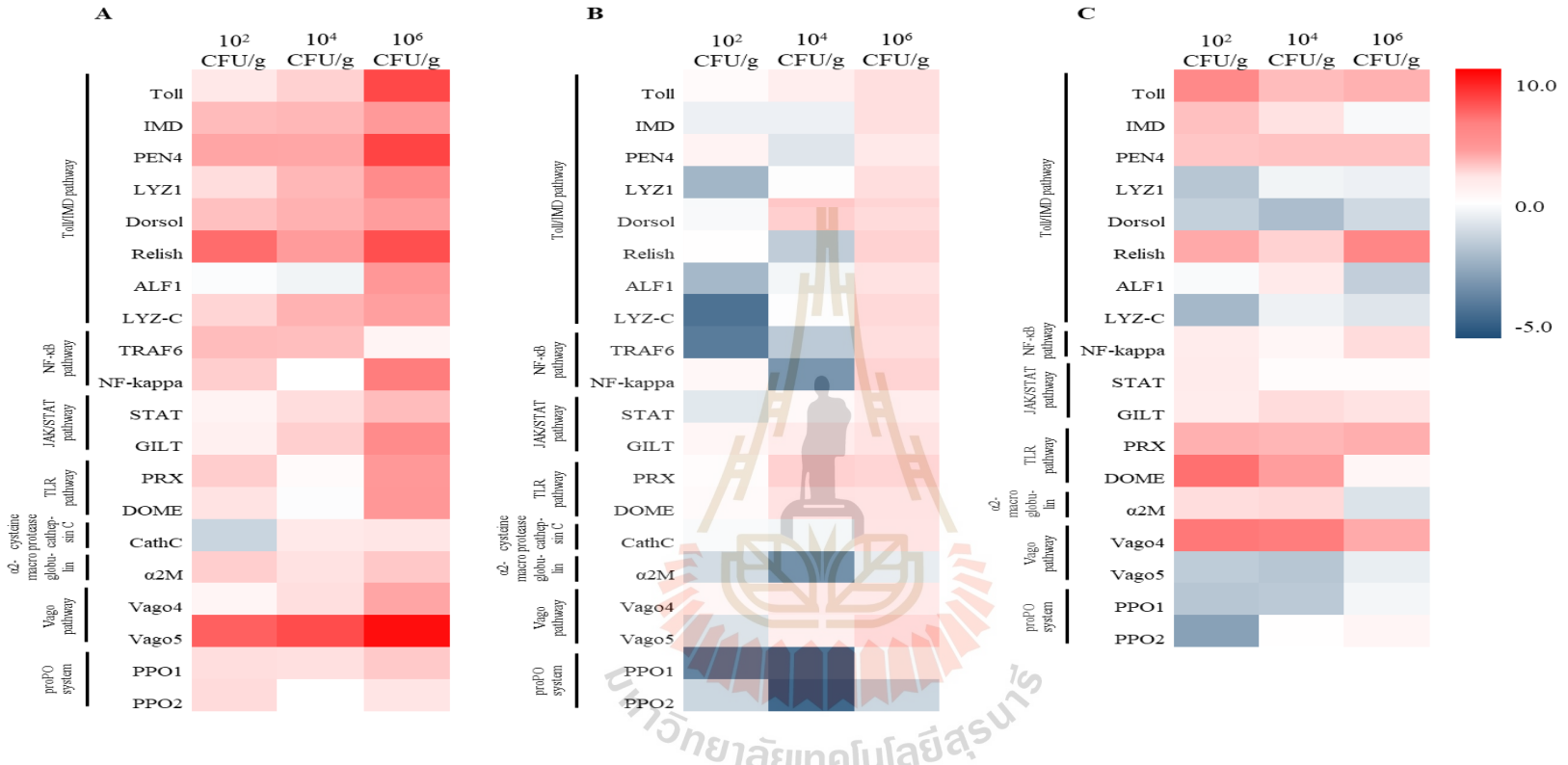
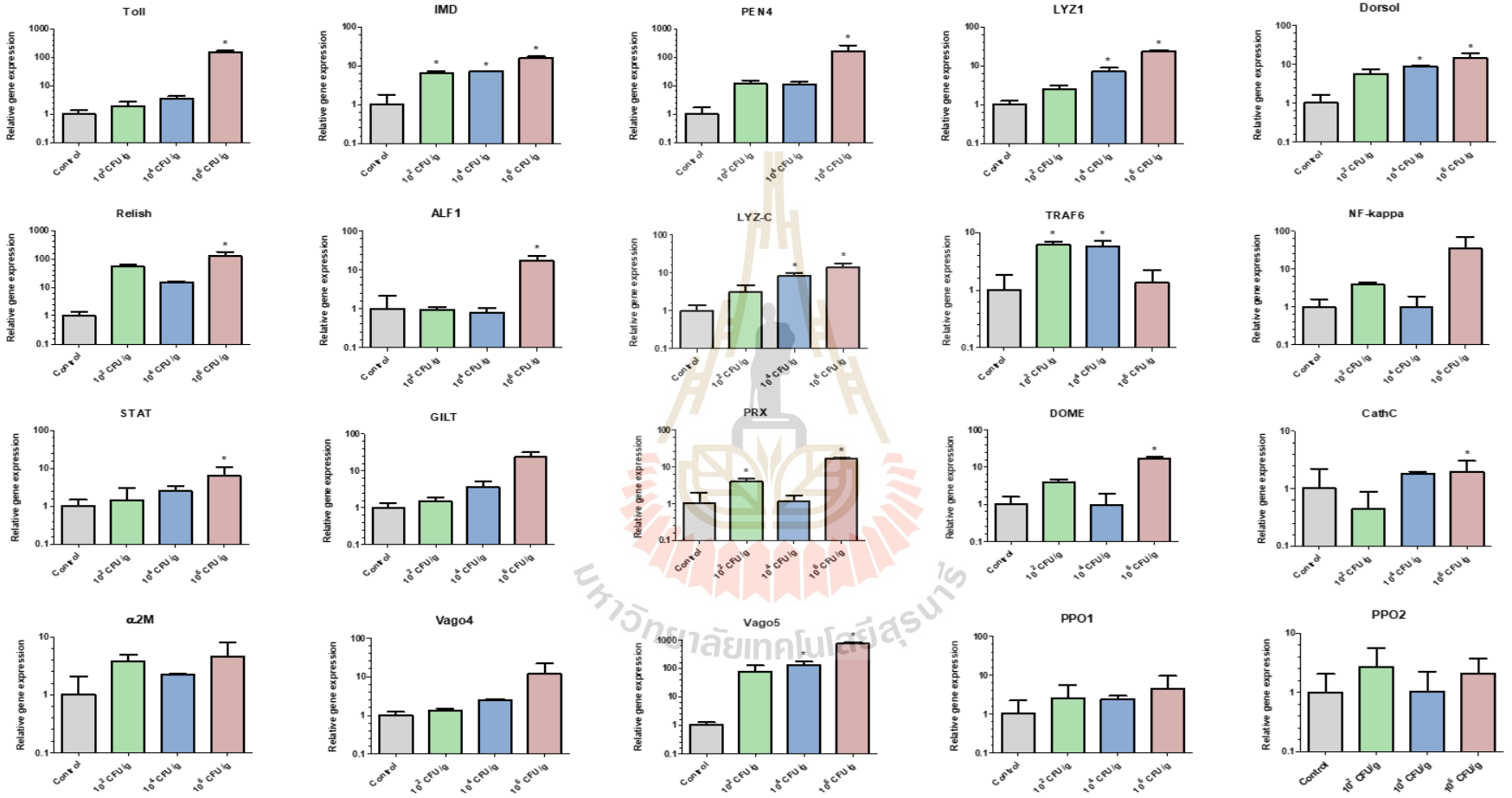


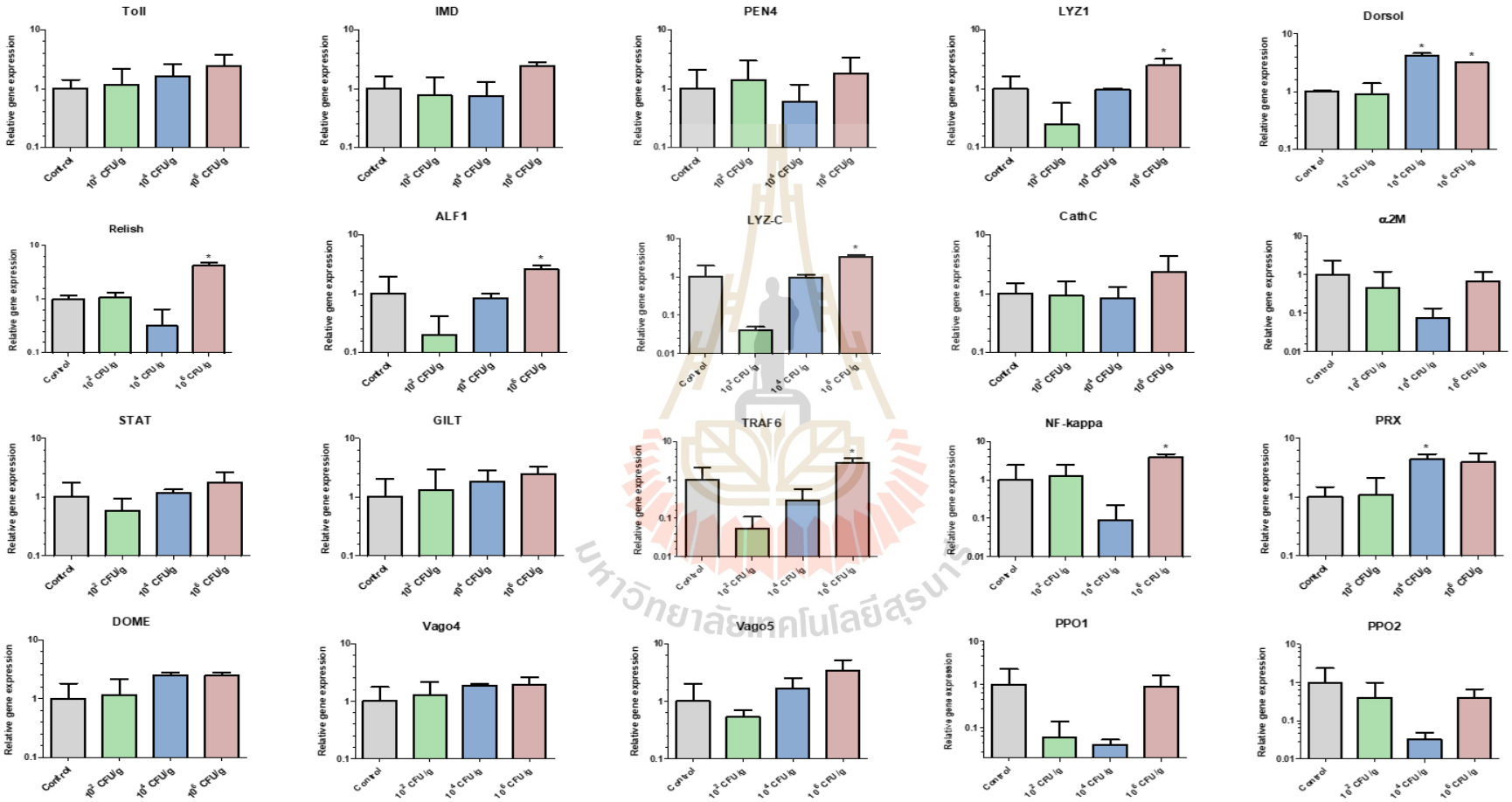
Figure 3.5 The effects of different experimental diets of *B. velezensis* S141 on gene expression in (A) gill, (B) hepatopancreas, and (C) hemocyte.

The expression profiles of Toll, IMD, PEN4, LYZ1, Dorsal, Relish, ALF, LYZ-C, STAT, GILT, TRAF6, NF-kappa, PRX, DOME, CathC, α2M, Vago4, Vago5, PPO1, and PPO2 were normalized against EF-1α and compared to control group by qRT-PCR. Triplicate replications (n = 9) were performed. The upregulation was shown in red whereas the down-regulation was shown in blue.

A



B



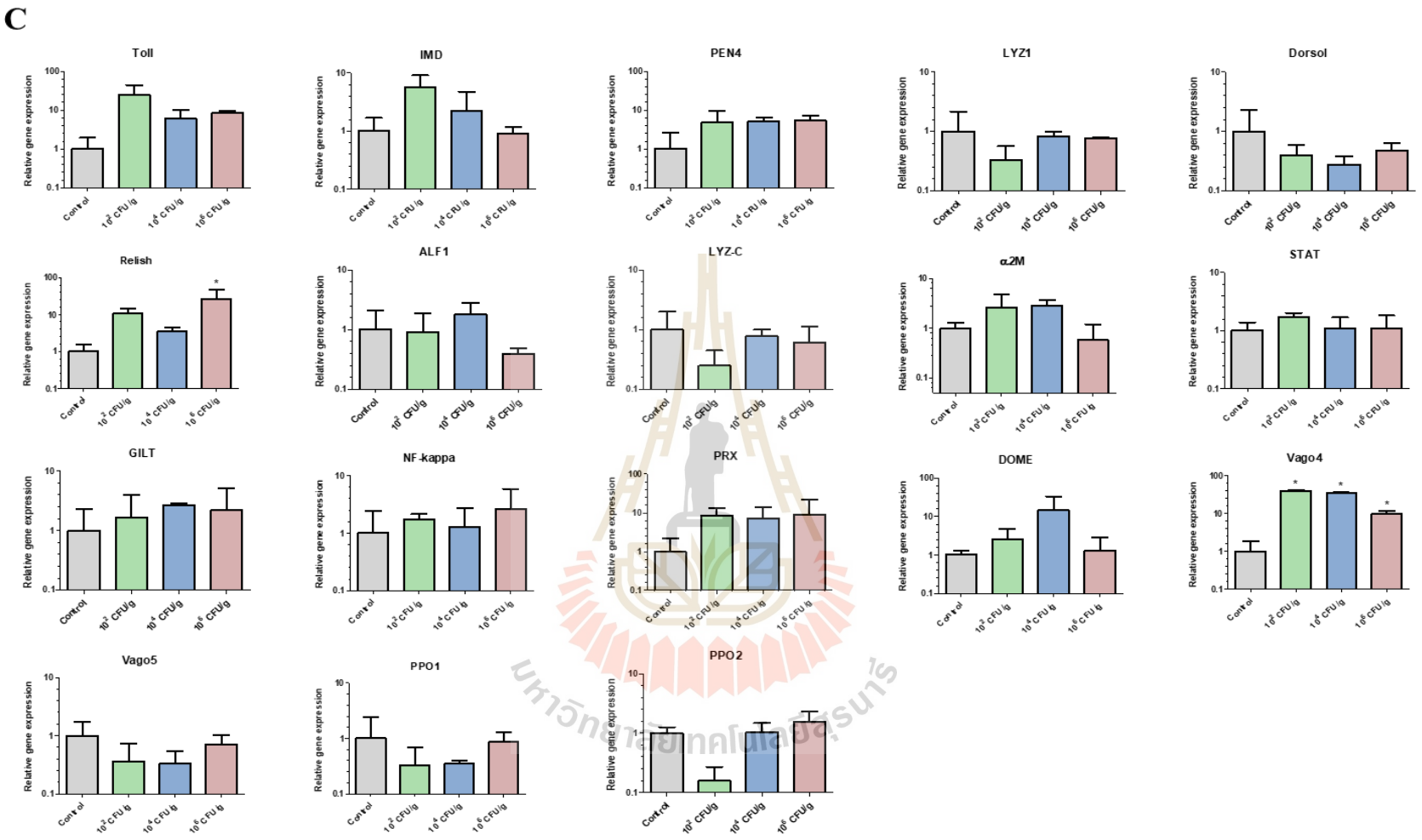


Figure 3.6 The effects of different experimental diets of *B. velezensis* S141 on gene expression in (A) gill, (B) hepatopancreas, and (C) hemolymph.

The expression profiles of Toll, IMD, PEN4, Lysozyme, TRAF6, NF-kappa, Dorsal, Relish, STAT, GILT, Vago4, Vago5, ALF, PRX, DOME, CathC, C-type lysozyme, α2M, PO, PO2 were normalized against EF-1α by qRT-PCR. Triplicate replications (n = 9) were performed, and results are displayed as means ± SD. Asterisks indicate significant differences between the data of each treatment ($P < 0.05$).

3.5 Discussion

Aquatic probiotics can be classified into two types based on their route of administration. The first involves the integration of probiotic bacteria with a dietary supplement to augment the number of beneficial bacteria in the gut. The second type involves adding probiotics directly to the water, which facilitates the absorption of nutrients and suppresses the growth of pathogens. Both types of probiotics have been employed in fish and shrimp farming (Ghosh et al., 2008; Nageswara & Babu, 2006). It has been demonstrated that probiotic bacteria in aquaculture significantly influence shrimp health and growth by modifying the microbial composition of the water. A solution featuring various *Bacillus* spp. as probiotics has been developed for enhancing shrimp productivity (Rajasulochana & Gummadi, 2022). Previous studies suggested that freeze-dried *B. subtilis* and *B. licheniformis* used as probiotics were effective in enhancing the performance of juvenile shrimp. Parameters like survival rate, feed conversion ratio (FCR), ADG, and SGR all showed improvement following probiotic administration (Sadat Hoseini Madani et al., 2018). Similarly, earlier research indicated that adding 0.3 g/kg of the *B. velezensis* T23 fermentation product to shrimp feed augmented growth performance (Yang et al., 2024). Furthermore, *B. velezensis* JW has been widely incorporated into aquafeeds due to its ability to improve growth performance (Yi et al., 2018). In addition, *B. velezensis* represents a promising probiotic that can be safely included in the diet of *L. vannamei* at 1×10^9 CFU/g, which significantly enhances growth, survival rates, and proximate body composition (Abdelsamad et al., 2024). Similarly, our study demonstrated that the supplementation of *B. velezensis* S141 for 42 days also led to enhanced growth performance (WG, ADG, and SGR) (Table 2). These findings suggest that the inclusion of *B. velezensis* S141 in the shrimp diet can improve growth performance.

To combat the detrimental effects of White Spot Disease, induced by WSSV, on *L. vannamei* shrimp farming, enhancing immune responses via the use of probiotics, prebiotics, and symbiotics is expected to be a promising approach for disease prevention (Widanarni et al., 2020). The inclusion of lactic acid bacteria (LAB) in the diet of juvenile shrimp has been found to enhance immunity in cultured shrimp and decrease the incidence of WSSV in these populations (Partida-Arangure et al., 2013). Three strains, identified as *B. subtilis* KA1, *B. licheniformis* KA2, and *B. subtilis* KA3, have shown promising results. In a pilot-scale test against WSSV, the survival rate of shrimp treated with *B. subtilis* KA1 and *B. subtilis* KA3 was 84%, compared to 0% in the WSSV control group after 26 days (Sekar et al., 2019). Moreover, a 30-day feeding trial with *Bacillus* PC465 and subsequently, a 20-day WSSV infection phase revealed that shrimp-fed diets with varying probiotic doses (0, 10^7, and 10^9 CFU/g), had improved survival rates following WSSV infection. The higher dose had a greater effect (Chai et al., 2016). Similarly, our study observed a reduction in cumulative mortality rates among shrimp supplemented with *B. velezensis* S141 during WSSV infection, highlighting the probiotic's ability to enhance disease resistance. The significant increase in survival rates in groups supplemented with *B. velezensis* S141 suggests a protective effect against WSSV-induced mortality and reduced viral loads. This aligns with previous studies demonstrating the efficacy of probiotics in enhancing disease resistance in shrimp, particularly against viral infections like WSSV. Moreover, *B. velezensis* S141 supplementation significantly increased shrimp survival rates and reduced viral loads during WSSV infection (Fig. 19A–B), further highlighting its potential to enhance disease resistance.

Probiotics can reduce the occurrence of bacterial infections in shrimp, thereby decreasing the likelihood of infectious diseases (Prabawati et al., 2022). Supplementing with *B. subtilis* E20 decreased shrimp mortality after injection with *V. alginolyticus* (Adilah et al., 2022). Similarly, *B. subtilis* P2.24 increased shrimp survival by 40% following a *V. parahaemolyticus* injection (Aribah & Wahyudi, 2022). Moreover, *B. subtilis* K3, which is srfAA+ and bacA+, effectively inhibited VP_{AHPND} in Pacific white shrimp, achieving 80% survival. It also reduced shrimp mortality across various salinities (75–95% survival) (Proespraiwong et al., 2023). After ingesting *B. subtilis*, the

shrimp were exposed to *V. parahaemolyticus* through an immersion method at a concentration of 2 × 10^5 CFU/ml for 193 h. No *V. parahaemolyticus* was detected in shrimp fed on the *B. subtilis* diet, while no hepatopancreas samples could be taken from the control group due to 100% mortality (Chorong et al., 2019). Our research has shown that a feed with 10^6 CFU/g of *B. velezensis* S141 improves the survival rate of shrimp by 60% after exposure to VP_{AHPND} throughout 120 h. These findings suggest that 10^6 CFU/g could be used as a feed supplement to enhance shrimp resistance to VP_{AHPND} infection.

Hepatopancreatic microsporidiosis, caused by EHP, has emerged as a devastating disease in global shrimp production, leading to significant economic losses. Over the past decade, extensive research on EHP has provided invaluable insights into productivity losses associated with the parasite (Patil et al., 2021; Thitamadee et al., 2016). Key among these is understanding that EHP is a microsporidian parasite. Microsporidia are fungi-like, intracellular parasites that infect both vertebrates and invertebrates (Weiss, 2001). A substantial number of microsporidian species infect aquatic hosts, including crustaceans and fish (Stentiford et al., 2013). Interestingly, as the severity of EHP infection increased, the diversity of bacteria in the gut of *L. vannamei* decreased (Shen et al., 2022). Earlier studies showed that the supplementation of 75 mg/L of albendazole at 24 h intervals effectively controlled EHP in *P. vannamei* (Subash et al., 2023). Moreover, after 21 days of dietary supplementation with 60 ppm 5-ALA, EHP-infected *L. vannamei* showed reduced mortality and increased biomass in the affected shrimp (Kongplong et al., 2023). However, there have been no reports on the use of probiotics to reduce EHP numbers. Our study revealed that EHP infection significantly decreased after supplementation with *B. velezensis* S141 (Fig. 21A – B). Thus, *B. velezensis* S141 appears to have a positive effect in reducing EHP infection in *L. vannamei*.

During the pathogenic invasion, shrimp utilize the innate immune system to manage infections through various pathways such as the prophenoloxidate (proPO) system, Toll/IMD-, Toll-, JAK/STAT-, Vago-, TLR- and NF-**K**B pathways. In these pathways, the major components and downstream products are signal transducer and activator of transcription (STAT), gamma-interferon-inducible lysosomal thiol

reductase (GILT), Vago-like protein 4 (Vago4), Vago-like protein 5 (Vago5), Toll-like receptor (Toll) immune deficiency (IMD), penaiedin 4 (PEN4), lysozyme 1 (LYZ1), c-type lysozyme (LYZ-C), anti-lipopolysaccharide factors (ALF), phenoloxidase 1 (PPO1), phenoloxidase 2 (PPO2), Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kappa), and tumor necrosis factor receptor-associated factor 6 (TRAF6), Dorsal, Relish, Peroxiredoxins (PRX), Domeless (DOME), α2-macroglobulin (α2-M) and cysteine protease cathepsin C (CathC) (Kiruthiga et al., 2012; Laohawutthichai et al., 2023; Sriphuttha et al., 2023; Tassanakajon et al., 2018). Consequently, these genes are considered crucial targets for assessing the health status of shrimp.

In this study, the gills of the 10^6 CFU/g group exhibited significantly upregulated transcriptional levels of immune-related genes, including Toll, IMD, PEN4, LYZ1, NF-kappa, Dorsal, Relish, STAT, GILT, Vago4, Vago5, ALF1, PRX, DOME, CathC, LYZ-C, and α2-M. Conversely, TRAF6 was significantly upregulated in the 10^2 and 10^4 CFU/g groups (Fig. 22A). Additionally, it was observed that the genes in the 10^6 CFU/g group of the hepatopancreas exhibited similar effects to those in the gills (Fig. 22B).

These findings align with previous research showing that the expression of lysozyme and anti-lipopolysaccharide factor genes was augmented by the probiotic *B. subtilis* AQAHBS001 (Kewcharoen & Srisapoome, 2019). Moreover, shrimp administered *B. subtilis* E20 showed elevated transcriptional levels of lysozyme (Liu et al., 2010). Other data include TLR5, TGF-β1, and pro-inflammatory cytokines (IL-8 and IL-1β) that were expressed in the intestinal and head kidney of *Epinephelus coioides* following probiotic *B. clausii DE5* treatment (Wang et al., 2018).

Moreover, probiotic supplementation exerted a profound influence on the immunological resilience and disease resistance of shrimp. In this study, the Toll/IMD signaling pathway was markedly upregulated in the 10^6 CFU/g group following 42 days of dietary inclusion. Previous investigations into *Clostridium butyricum* supplementation in *L. vannamei* revealed that after a 42-day feeding regimen, the diet significantly elevated the expression of lysozyme, Toll, Immune Deficiency (IMD), Relish, and TOR genes, thereby fortifying the shrimp's resistance against *V. parahaemolyticus* (H. Li et al., 2019).

Furthermore, the 10^6 CFU/g group elicited a significant upregulation of the JAK/STAT pathway. Correspondingly, a previous study demonstrated that supplementation with 5% *Rhodotorula paludigena* CM33 led to a marked elevation in immune-related genes in *L. vannamei*, notably lysozyme, IKKβ, IKKε, STAT, and GILT, along with a substantial reduction in cumulative mortality following the VP_{AHPND} challenge (Sriphuttha et al., 2023). Additionally, the incorporation of the combined probiotics *Leuconostoc mesenteroides* B4 and *B. pumilus* D5 into the diet significantly enhanced the survival rate following challenges with *V. parahaemolyticus* while also promoting the upregulation of LYZ and α2-macroglobulin genes (Huang et al., 2024).

The aforementioned results indicate that probiotics play a crucial role in aquaculture, particularly *Bacillus* sp. This research suggests that *B. velezensis* S141 has enormous potential to aid sustainable shrimp farming by enhancing growth, strengthening immunological responses, and providing protection against diseases, particularly bacterial infections.

Our study revealed downregulated expression of myeloid differentiation primary response protein PPO1 and PPO2 in the entire group (Fig. 22A–C). Furthermore, gene expression graphs illustrating the effects of various genes are presented in Fig. 23, augmenting the data shown in Figures 22A–C. This suggests that *B. velezensis* S141 supplementation may not directly affect melanization and encapsulation responses in shrimp. The proPO activation and the evasion of elimination by the host immune defense occur through the modulation of genes encoding the proPO system or its activators and regulators (Goncalves et al., 2014). However, the precise connections between the proPO system and other immunological components in the shrimp immune response remain ambiguous (Ji et al., 2009). Previous research has demonstrated that reducing the expression of the HSP70 gene with a mix of *Bacillus* sp. lessens the intensity of cellular stress in sea bream larvae, thereby enhancing the fish's tolerance to rearing conditions (Avella et al., 2010). Moreover, similar results were observed in shrimp fed with *Lactobacillus pentosus* HC-2 in the midgut of *L. vannamei*. Treatment with stripped surface proteins of HC-2 using lithium chloride (LiCl) neither induced the upregulation of these genes nor decreased heat shock protein expression (Du et al., 2019). Similarly, introducing *R.*

sphaeroides did not significantly activate the immune response in the *P. vannamei* gut, leading to the down-regulation of several immune-related genes. The down-regulation can also reduce susceptibility to infections, such as WSSV and *Vibrio* sp., which is manifest in a decreased viral infection rate in *L. vannamei* whose peritrophic gene expression was reduced (Yang et al., 2020). Besides, in the intestinal tissues of *P. vannamei*, previous research has shown that *R. sphaeroides* increases the number of advantageous gut microorganisms, which caused a down-regulation of immune-related genes and an upregulation of growth- and metabolism-related genes (Song et al., 2024). The reduced expression of these genes might imply mitigation of stresses arising from probiotic supplementation; however, the implications and outcomes of these patterns remain unclear and warrant further exploration.

Consequently, the findings of this research underscore the immense potential of *B. velezensis* S141 as a game-changer in shrimp farming. Its use significantly improves immune functionality, growth performance, and resistance against prevalent pathogens such as WSSV, VP_{AHPND}, and EHP in *L. vannamei*. *B. velezensis* S141, through significant upregulation of vital immune-related genes and significant improvements in survival rates, exhibits a strong ability to bolster shrimp health, diminish dependency on antibiotics, and manage disease outbreaks. These results not only broaden our comprehension of probiotic efficacy in aquaculture but also offer significant economic prospects for the industry by increasing productivity, minimizing losses, and fostering sustainable practices. The incorporation of *B. velezensis* S141 as a dietary supplement could revolutionize shrimp farming, clearing the path for innovative, environmentally-friendly strategies that bolster long-term economic growth and stability in global aquaculture markets.

3.6 Conclusion

This study highlights the potential of *B. velezensis* S141 as a promising probiotic supplement for the aquaculture industry, particularly for improving disease resistance and immune response in *L. vannamei* shrimp. Supplementation with *B. velezensis* S141 significantly enhanced survival rates and lowered bacterial, viral, and fungal loads in shrimp challenged with WSSV, VP_{AHPND}, and EHP. Furthermore, an analysis of

immune-related gene expression showed the upregulation of genes tied to various immune pathways in shrimp supplemented with *B. velezensis* S141, indicating a heightened immune response. These results underscore *B. velezensis* S141 as a sustainable alternative to antibiotics in shrimp farming, offering a wise approach to disease management and enhancing overall shrimp health.

CHAPTER IV
PRODUCTION AND PURIFICATION RECOMBINANT SVP28 PROTEIN FOR ANTIBODY GENERATION

4.1 Abstract

sVP28 is a part of the VP28 gene found in a circular RNA derived from White Spot Syndrome Virus (WSSV), which is one of pathogens that affects the aquaculture of shrimp globally. sVP28 is found to play an important role in the shrimp immune response. According to previous studies, VP28 protein is one of WSSV capsid proteins being utilized to produce antibodies able to detect the infection of WSSV in shrimp. However, no report has established the antibody against the sVP28 protein. Therefore, this study wanted to produce and purify recombinant sVP28 protein to generate antibodies that are specific to the sVP28 protein. The protein was overexpressed using bacteria, which was then purified using the protein purification from polyacrylamide gel instead and the highly purified protein was attainable via this technique. The purified protein can, hence, be used to generate antibodies, which are able to thoroughly examine the role of the sVP28 protein, as well as develop new methods to prevent and treat diseases caused by WSSV, which could reduce its impact on the shrimp farming industry.

4.2 Introduction

White Spot Syndrome Virus (WSSV) is a significant pathogen in the global aquaculture industry, particularly affecting shrimp farming (Lightner, 2011). The economic impact of WSSV outbreaks can be devastating, with shrimp mortality rates reaching up to 100% within 3 to 5 days post-infection. WSSV is a large, enveloped double-stranded DNA virus associated with the genus *Whispovirus* within the virus family *Nimaviridae*. It has a wide host range among crustaceans (Pradeep et al., 2012; Sánchez-Paz, 2010). One of the viral proteins, VP28, is a major structural protein that has been extensively studied for its role in the virus's infection mechanism and its

potential as a target for diagnostic tools and vaccines (van Hulten et al., 2001). Previous studies have demonstrated that VP28 can induce protective immunity in shrimp. The recombinant VP28 expressed in insect cells conferred significant protection against WSSV in shrimp (Kumar et al., 2008). Furthermore, silencing of the VP28 gene using RNA interference (RNAi) has shown promise in reducing viral loads and improving survival rates in infected shrimp (Westenberg et al., 2005). Antibodies against the VP28 protein can effectively detect WSSV infections in shrimp, facilitating early diagnosis and management of the disease (Chaivisuthangkura et al., 2004; Hou et al., 2011). sVP28 is a part of the VP28 gene found in a circular RNA derived from the WSSV, plays an important role in the shrimp's immune response. Despite its potential significance, there has been no report to date of antibodies developed specifically against the sVP28 protein. The availability of sVP28-specific antibodies will facilitate in-depth studies on the role of sVP28 in the shrimp immune response and its interaction with WSSV. Additionally, these antibodies could lead to the development of novel diagnostic methods and therapeutic interventions aimed at reducing the impact of WSSV on shrimp farming.

This research aimed to produce and purify the recombinant sVP28 protein to generate antibodies specifically targeting the sVP28 protein. To achieve this, the protein was overexpressed in bacterial systems. Following overexpression, the protein underwent a purification process using polyacrylamide gel electrophoresis. This method proved effective in isolating the sVP28 protein to a high degree of purity. The successfully purified sVP28 protein can be utilized to produce antibodies. These antibodies will play a crucial role in detailed studies of the sVP28 protein's function. Additionally, they can be instrumental in the development of novel strategies for the prevention and treatment of diseases caused by the White Spot Syndrome Virus (WSSV).

4.3 Materials and Methods

4.3.1 Expression of recombinant sVP28 protein

To produce the sVP28 protein, the expression vector pET21a+/sVP28 was transformed into Escherichia coli BL21 (DE3)-CodonPlus and Escherichia coli Rosetta

(DE3) strain, which served as the host strain for protein synthesis. These transformed cells were then cultured in an LB medium until they reached an optical density (OD600) between 0.4 and 0.6, which is optimal for protein expression. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, the induction was carried out under controlled conditions, maintaining the cultures at 30℃ and shaking at 250 rpm 4 h. After the induction period, the cells were collected by centrifugation to separate them from the culture medium. The harvested cells were then stored at -80℃ to preserve the protein and maintain the cells' integrity for further downstream processing and analysis

4.3.2 Purification of recombinant sVP28 protein

To purify the recombinant protein, cells were harvested and resuspended in binding buffer (1X PBS pH 7.4). The cells were then disrupted using sonication, and the inclusion bodies were harvested and dissolved in binding buffer supplemented with 1XPBS pH 7.4. The recombinant sVP28 proteins were subsequently purified using a Ni-NTA agarose bead column (GoldBio). The protein was eluted using elution buffer containing different concentrations of imidazole (50 mM, 150 mM, and 250 mM). Protein purity was assessed using SDS-PAGE.

4.3.3 Recombinant protein production of sVP28

The purification process of the recombinant sVP28 protein involved initial observation using SDS-PAGE. After the electrophoresis, the protein bands of interest were carefully cut out from the gel using a razor blade. These excised gel pieces were then washed three times, each for 5 minutes of 1XPBS pH 7.4. The gel slices were then finely chopped into small pieces of 2–5 mm. Subsequently, 1X PBS pH 7.4, containing 0.1% SDS, was added to the gel fragments to achieve a buffer-to-gel volume ratio of about 2:1. The mixture was then sonicated for 3 minutes in an ice bath. The sonicated gel fragments were separated from the extraction buffer, the sample was applied to a filter column and centrifuged at 500g for 10 minutes. The purity of the isolated protein was again assessed using SDS-PAGE.

Next, the recombinant sVP28 proteins were purified using a Ni-NTA agarose column (GoldBio). The proteins were subjected to washing and elution steps, first with 50 mM imidazole in PBS pH 7.4 and then with 250 mM imidazole in 1XPBS

pH 7.4, respectively. The fractions containing the recombinant proteins were combined and subjected to dialysis against a solution of 1XPBS pH 7.4. The protein concentration was then determined using the BCA method.

4.3.4 Confirmation and detection of sVP28 protein

To confirm and detect the sVP28 protein, the recombinant sVP28 protein was first separated using SDS-PAGE and subsequently transferred to a membrane. The membrane was then blocked with 5% skim milk in 1X PBS containing 0.1% Tween 20 for 1 hour at room temperature to prevent non-specific binding. Following this, the membrane was incubated for 1 hour at room temperature with a VP28 antibody (diluted 1:10,000), prepared according to the method described by Tsai et al. (2006) (Tsai et al., 2006), The membrane was washed three times with 1X PBS with 0.1% Tween 20. Next, an HRP-conjugated goat anti-rabbit IgG (diluted 1:10,000) from Abbkine was used as the secondary antibody, followed by three additional washes with 1X PBS containing 0.1% Tween 20. The chemiluminescent signal was detected using the Amersham ECL Prime Western blotting detection reagent substrate from Cytiva.

4.4 Results and discussion

4.4.1 Expression and Extraction of recombinant sVP28 protein

To produce recombinant sVP28 protein, we utilized the E. coli expression system with strains including *E. coli* BL21(DE3)-CodonPlus and *E. coli* BL21 Rosetta (DE3). The protein was successfully overexpressed in *E. coli* BL21(DE3)-CodonPlus, demonstrating the expected size of approximately 19.5 kDa (Fig. 24). However, consistent overexpression was not achieved in *E. coli* BL21 Rosetta (DE3). Following expression, the recombinant sVP28 proteins in *E. coli* BL21(DE3)-CodonPlus were subsequently purified using a Ni-NTA agarose bead column (GoldBio).

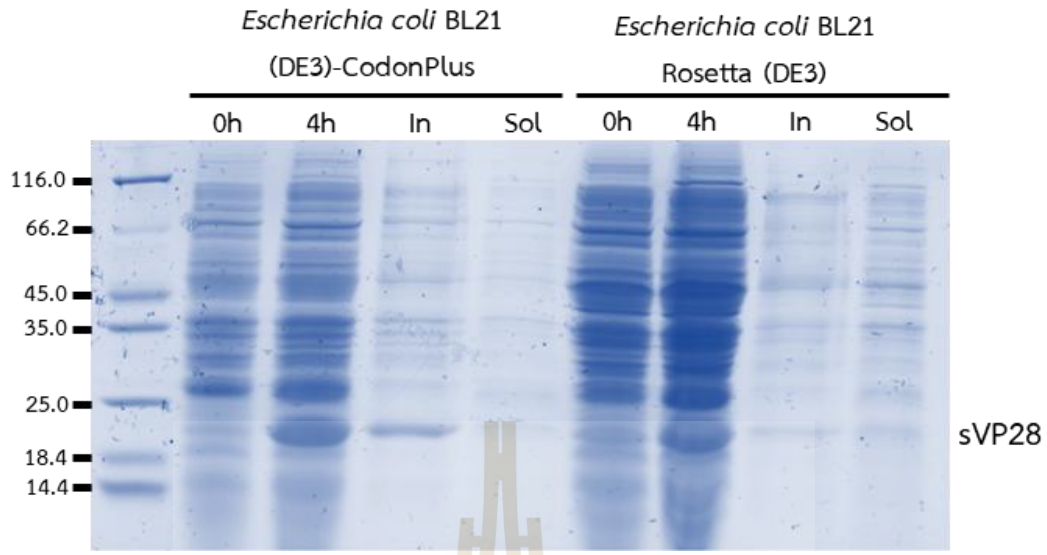


Figure 4.1 The expression of recombinant sVP28 protein. The expected size of approximately 19.5 kDa. (0h: befor adding 1 mM IPTG, 4h: after adding 1 mM IPTG, In: inclusion bodies, Sol: soluble)

4.4.2 Protein Purification from affinity chromatography

For the purification of recombinant sVP28 protein, *E. coli* BL21(DE3)-CodonPlus was employed for protein expression. Initially, the inclusion bodies were purified using a Ni-NTA agarose bead column (GoldBio). The protein was initially eluted using elution buffers containing different concentrations of imidazole (50 mM, 150 mM, and 250 mM) (Fig. 25). However, proteins eluted from the column effectively only at 250 mM imidazole, suggesting the presence of some contaminants. Further purification was carried out using polyacrylamide gels via sonication extraction.

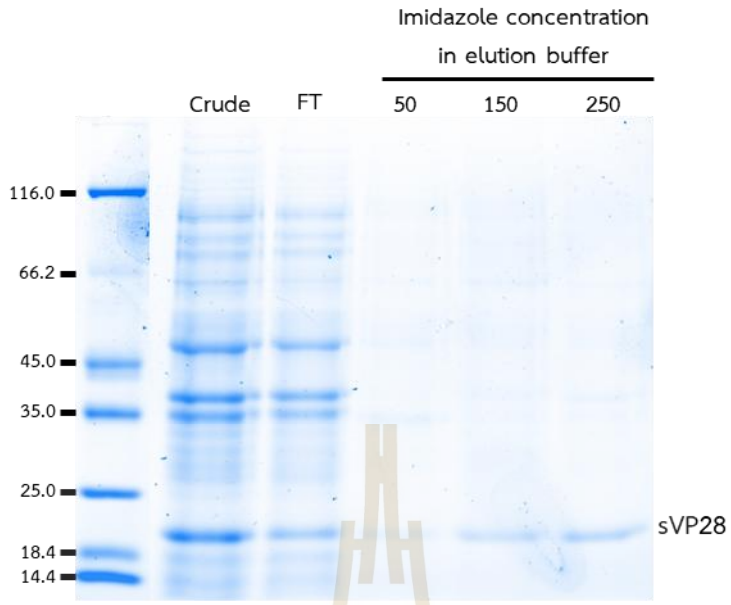


Figure 4.2 Recombinant sVP28 protein using Ni-NTA agarose bead column (GoldBio). The expected size of approximately 19.5 kDa. (F: flow-throug, 50-250: imidazole concentration in elution buffer)

4.4.3 Purification from Polyacrylamide Gels by Sonication Extraction and Confirmation of sVP28 protein

To confirmation and detection for enhanced recombinant sVP28 protein, protein was purified from polyacrylamide gels using sonication extraction, indicating some impurities persisted (Fig. 26A). This necessitated further purification using Ni-NTA agarose bead column (GoldBio) led to (Fig. 26B), where only a characteristic molecular weight band of 19.5 kDa was observed on SDS-PAGE analysis. The Western Blot analysis of recombinant sVP28 protein shows two major immunoreactive bands with appare 19.5 kDa.

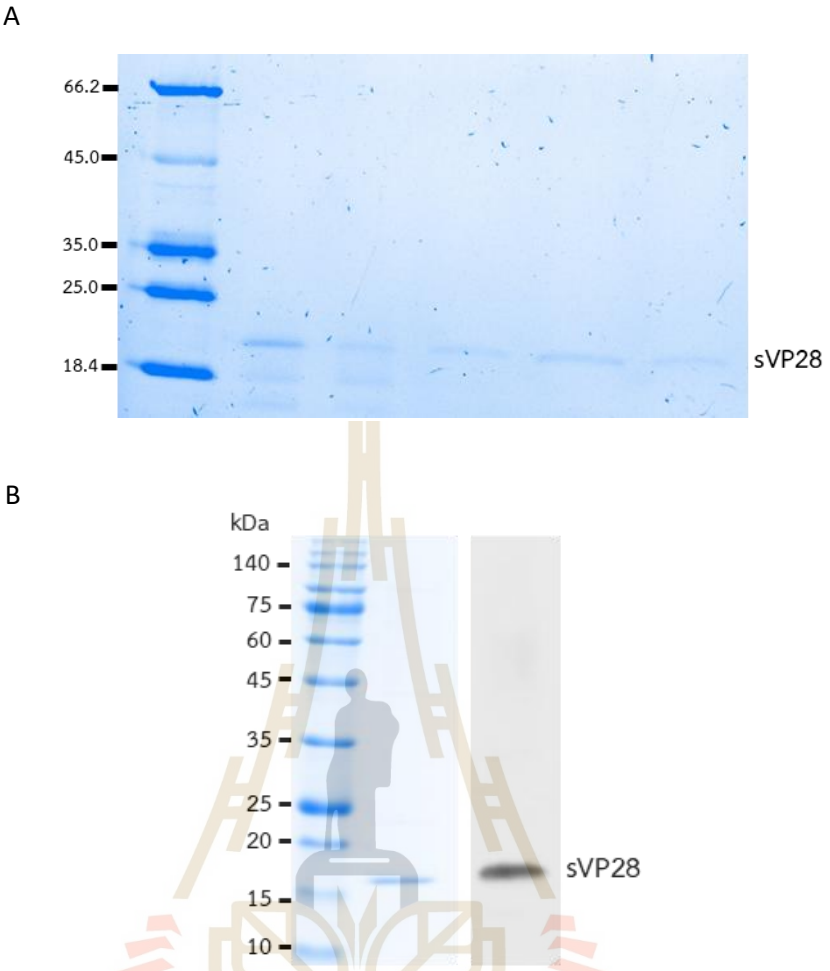


Figure 4.3 Protein purification from Polyacrylamide Gels by Sonication Extraction and Western blot analysis of recombinant sVP28 protein. (A) The purified sVP28 proteins extracted from polyacrylamide gels using sonication were analyzed by SDS-PAGE. (B) Western blot analysis confirmed the presence of a band corresponding to sVP28.

4.5 Discussion

To produce and purify recombinant sVP28 protein from White Spot Syndrome Virus (WSSV) to generate specific antibodies, a significant step towards understanding its role in shrimp immunity and developing diagnostic and therapeutic tools against WSSV. The successful expression and purification of sVP28 were achieved using both affinity chromatography and polyacrylamide gel extraction techniques, confirming its purity and specificity through SDS-PAGE and Western blot analysis.

The expression of sVP28 in *Escherichia coli* strains BL21 (DE3))-CodonPlus and Rosetta (DE3) yielded differing results, with optimal expression observed in *E. coli* BL21 (DE3))-CodonPlus (Fig. 24A). This variation underscores the importance of host strain selection in recombinant protein expression, aligning with previous studies that highlighted the influence of host genetic background on protein yield and quality (Shevchenko et al., 1996).

Affinity chromatography using Ni-NTA agarose beads initially provided purification, although at 250 mM imidazole concentration (Fig. 25), indicating some impurities persisted. This necessitated further purification using polyacrylamide gel extraction via sonication, which improved purity as evidenced by the absence of non-specific bands on SDS-PAGE (Fig. 26A-B). The use of sonication extraction from polyacrylamide gels has been reported as effective in isolating proteins from complex mixtures (Retamal et al., 1999; Shevchenko et al., 1996), supporting its application in this study. Western blot analysis confirmed the presence of sVP28 protein at approximately 19.5 kDa, consistent with the expected molecular weight, validating the purification methods used. The successful generation of antibodies specific to sVP28 opens avenues for exploring its immunological function in shrimp, potentially enhancing diagnostic accuracy and therapeutic efficacy against WSSV infections in aquaculture.

This research contributes to the broader understanding of WSSV pathogenesis and host immune response mechanisms, offering insights into how sVP28 interacts with shrimp immunity. Future studies could focus on elucidating the precise role of sVP28 in WSSV infection dynamics and evaluating its potential as a vaccine candidate or diagnostic marker.

4.6 Conclusion

In conclusion, this study successfully expressed and purified recombinant sVP8 protein from Escherichia coli using both affinity chromatography and polyacrylamide gel extraction techniques. The results indicate that polyacrylamide gel extraction techniques demonstrating its purity and specificity as evidenced by confirmed through SDS-PAGE and Western blot analysis. This achievement lays the groundwork for further research into the role of sVP2 8 in shrimp immune response and its potential applications in diagnostics and therapeutics for WSSV. By generating antibodies specific to sVP2 8, this study opens avenues for developing novel strategies to mitigate the impact of WSSV outbreaks on shrimp farming.

CHAPTER V
CONCLUSIONS

5.1 To investigate the beneficial properties of *B. velezensis* S141 towards the growth performance and survival rate of shrimp fed with the probiotic-formulated feed.

5.1.1 After 42 days of administration, the weight gain (WG), average daily gain (ADG), and specific growth rate (SGR) of the shrimp fed *B. velezensis* S141-supplemented were significantly higher than those of the control group.

5.2 To determine the disease resistance of *B. velezensis* S141-received shrimp against various pathogens including WSSV, VP_{AHPND}, and EHP.

5.2.1 Shrimp fed *B. velezensis* S141 showed increased survival against WSSV, with 41.67% and 25.00% survival in the 10^4 and 10^6 CFU/g groups at 120 hpi. WSSV copy numbers in the gill were significantly reduced, with the 10^6 CFU/g group showing the lowest levels. These results highlight the protective effects of *B. velezensis* S141 against WSSV infection.

5.2.2 The shrimp were challenged with VP_{AHPND} after 4 weeks of feeding, and shrimp mortality was recorded at 12-hour post-infection (hpi). Shrimp fed *B. velezensis* S141 showed increased survival against VP_{AHPND}, with 60% survival in the 10^6 CFU/g group at 120 h. V. parahaemolyticus copy numbers in the hepatopancreas were significantly reduced by up to 166-fold at 24 h post-infection. *B. velezensis* S141 secretions formed inhibition zone against VP_{AHPND}, indicating antimicrobial activity. These findings highlight its potential to enhance shrimp resistance to VP_{AHPND}.

5.2.3 After 42 days of feeds S141-supplemented diet, followed by EHP infection, after 72 h. Our observations indicated a decrease in EHP concentrations in

thee hepatopancreases of infected shrimps. In 10^6 CFU/g follows by 10^4, and 10^6 CFU/g of *B. velezensis* S141 comparison to the control group

5.3 To disclose an insight into the immunogenic perspectives of *B. velezensis* S141 to shrimp's immune system.

5.3.1 The results demonstrate the Immune-related genes, in the Toll/IMD pathway (Toll, IMD, LYZ1, LYZ-C, PEN4, ALF1, Relish), JAK/STAT pathway (STAT and GILT), Vago pathway (Vago4, Vago5), NF-**K**B pathway (NF-kappa), TLR pathway (RPX and DOME), CathC, and **α**2M, were significantly upregulated in the group administered feed containing 10^6 CFU/g of *B. velezensis* S141 compared to the control group in the gills. This study emphasizes the potential of *B. velezensis* S141 as a probiotic supplement to promote immune responses and disease resistance

5.4 To purify the sVP28 protein derived from White Spot Syndrome Virus (WSSV) for generate specific antibodies

5.4.1 This study successfully expressed and purified recombinant sVP8 protein from E. coli using affinity chromatography and polyacrylamide gel extraction. SDS-PAGE and Western blot confirmed its purity and specificity. These findings support further research on sVP28 role in shrimp immunity and its potential in WSSV diagnostics and therapeutics. Antibody generation against sVP28 may help develop new strategies to combat WSSV in shrimp farming.

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