



Faculty of Resource Science and Technology

**Selection of Vaccine Candidates Against *Pseudomonas koreensis* Using
Reverse Vaccinology and a Preliminary Efficacy Trial in *Tor tambroides*
(Empurau)**

Cindy Kho Jia Yung

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Selection of Vaccine Candidates for *Pseudomonas koreensis* Using Reverse
Vaccinology and a Preliminary Efficacy Trial in *Tor tamborides* (Empurau)

Cindy Kho Jia Yung

A thesis submitted

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

Signature

Name: Cindy Kho Jia Yung

Matric No.: 22020047

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Date :21-11-2023

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ABSTRACT

The unregulated and/or incorrect use of antimicrobial agents against diseases of farmed aquatic species poses a considerable threat to the development and growth of a sustainable aquaculture industry. Hence, vaccination has emerged as a pivotal strategy to mitigate this threat. This study marks the first utilization of reverse vaccinology to develop subunit vaccines against *Pseudomonas koreensis* infection in Empurau (*Tor tambroides*). WGS analysis revealed that the genomic size of *P. koreensis* CM-01 is 6,171,880 bp and has a G+C content of 60.5%. The proteome (5,538 proteins) was screened against various filters to prioritize proteins based on features that are associated with virulence, subcellular localization, transmembrane helical structure, antigenicity, essentiality, non-homology with the host proteome, molecular weight, and stability, which led to the identification of eight potential vaccine candidates. These potential vaccine candidates were cloned and expressed, with six achieving successful expression and purification. The antigens were formulated into two distinct vaccine mixtures, Vac A and Vac B, and their protective efficacy was assessed through *in vivo* challenge experiments. Vac A and Vac B demonstrated high protective efficacies of 100% and 81.2%, respectively. Histological analyses revealed reduced tissue damage in vaccinated fish after experimental infection, with Vac A showing no adverse effects, whereas Vac B exhibited mild degenerative changes. Quantitative real-time PCR results showed a significant upregulation of *TNF- α* and downregulation of *IL-1 β* in the kidneys, spleen, gills, and intestine in both Vac A- and Vac B immunized fish after challenged with *P. koreensis*. Additionally, *IL-8* exhibits tissue-specific differential expression, with significant upregulation in the kidney, gills, and intestine, and downregulation in the spleen, particularly notable in Vac A immunized fish. The research

underscores the effectiveness of the reverse vaccinology approach in fish and demonstrates the promising potential of Vac A and Vac B as recombinant subunit vaccines.

Keywords: *Pseudomonas. koreensis*; *Tor tambroides*; Reverse vaccinology; Subunit vaccines; Protective efficacy

Pemilihan Kandidat Vaksin untuk Pseudomonas koreensis Melalui Vaksinologi Terbalik dan Percubaan Keberkesanan Awal dalam Tor tambroides (Empurau)

ABSTRAK

Penggunaan agen antimikrobial yang tidak terkawal dan/atau tidak betul terhadap penyakit-penyakit spesies akuatik yang ditanak membawa ancaman yang signifikan terhadap perkembangan dan pertumbuhan industri akuakultur yang mampan. Oleh itu, vaksinasi telah muncul sebagai strategi penting untuk mengurangkan ancaman ini. Kajian ini menandakan penggunaan pertama vaksinologi terbalik untuk membangunkan vaksin subunit terhadap jangkitan Pseudomonas koreensis dalam empurau (Tor tambroides). Analisis penjujukan genom keseluruhan (WGS) mendedahkan bahawa saiz genom P. koreensis CM-01 adalah 6,171,880 bp dan mempunyai kandungan G+C sebanyak 60.5%. Proteom (5,538 protein) telah disaring terhadap pelbagai penapis untuk mengutamakan protein berdasarkan ciri-ciri yang dikaitkan dengan virulensi, lokalisasi subselular, struktur heliks transmembran, antigenisiti, keesensialan, ketiadaan homologi dengan proteom hos, berat molekul, dan kestabilan, yang membawa kepada pengenalan lapan kandidat vaksin berpotensi. Kandidat vaksin berpotensi ini diklon dan diekspresikan, dengan enam mencapai kejayaan ekspresi dan penulenan. Antigen tersebut dirumuskan kepada dua campuran vaksin yang berbeza, Vac A dan Vac B, dan keberkesanan perlingkungannya dinilai melalui eksperimen cabaran in vivo. Vac A dan Vac B menunjukkan keberkesanan perlindungan yang tinggi masing-masing sebanyak 100% dan 81.2%. Analisis histologi mendedahkan pengurangan kerosakan tisu pada ikan yang divaksin selepas jangkitan percubaan, dengan Vac A tidak menunjukkan kesan buruk, manakala Vac B menunjukkan perubahan degeneratif yang ringan. Keputusan PCR real-time kuantitatif menunjukkan peningkatan yang signifikan dalam ekspresi TNF- α dan penurunan ekspresi IL-1 β dalam

buah pinggang, limpa, insang dan usus dalam kedua-dua ikan yang diimmunisasi Vac A- dan Vac B selepas dicabar dengan P. koreensis. Selain itu, IL-8 menunjukkan ekspresi pembezaan khusus tisu, dengan peningkatan yang signifikan di buah pinggang, insang, dan usus dan penurunan di limpa, terutamanya ketara dalam ikan yang diimmunisasi dengan Vac A. Kajian ini menekankan keberkesanan pendekatan vaccinology terbalik dalam ikan dan menunjukkan potensi menjanjikan Vac A dan Vac B sebagai vaksin subunit rekombinan.

Kata Kunci: Pseudomonas koreensis; Tor tambroides; Vaksinologi terbalik; Vaksin subunit; Keberkesanan perlindungan

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AHL	N-acyl derivatives of homoserine lactone
APS	Ammonium persulfate
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic-resistance genes
B cells	B lymphocytes
BUSCO	Benchmarking Universal Single-Copy Orthologs
CaCl ₂	Calcium chloride
CCW	Counterclockwise
CFU	Colony forming unit
CheY ^P	Chemotaxis signaling molecule phospho-CheY
CpG	Cytosine-phosphorothioate-guanine
CW	Clockwise
cyclic-di-GMP	Cyclic dimeric guanosine monophosphate
DEG	Database of Essential Genes
DNA	Deoxyribonucleic acid
DOF	Department of Fisheries Malaysia
dpc	Days post challenge
dpi	Days post immunization
DVS	Department of Veterinary Services
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization

fap	Flagellin-associated protein
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Gross domestic product
HCN	Hydrogen cyanide
HEPA	High-efficiency particulate absorbing
IAA	Indole-3-acetic acid
IFN	Interferon
Ig	Immunoglobulin
IL-1 β	Interleukin-1 β
IL-8	Interleukin-8
IPTG	Isopropyl β - d-1-thiogalactopyranoside
IUCN	International Union for the Conservation of Nature
Lao PDR	Lao People's Democratic Republic
LB	Luria-Bertani
LKIM	Fisheries Development Authority of Malaysia
LPNS	List of Prokaryotic Names with Standing
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAFI	Ministry of Agricultural and Food Industry
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MLSA	Multilocus sequence analysis
MT	Metric tonnes

NaH ₂ PO ₄	Monosodium phosphate
NAP	National Agrofood Policy
NCBI	National Center for Biotechnology Information
NCCs	Non-specific cytotoxic cells
NETs	Neutrophil extracellular traps
NO	Nitric oxide
OD600	Optical density at 600 nm
PBL	Peripheral blood leukocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PGPR	Plant growth-promoting rhizobacteria
PUFA	Polyunsaturated fatty acid
PVCs	Potential vaccine candidates
RND	Resistance-Nodulation-Division
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPS	Relative percentage of survival
rRNA	Ribosomal ribonucleic acid
S.O.C. Medium	Super optimal medium with catabolic repressor medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
T cells	T lymphocytes
Tc cells	Cytotoxic T-cells
TCS	Two-component signal

TGF- β	Transforming growth factor- β
Th cells	Helper T cells
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
Tris-HCl	Tris Hydrochloride
USDA	United States Department of Agriculture
VFDB	Virulence Factor Database
WC-MALDI-TOF MS	Whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry

CHAPTER 1

INTRODUCTION

Aquaculture involves the cultivation of aquatic organisms such as fish, crustaceans, mollusks, and even aquatic plants in a regulated fashion to allow the independence of wild catches. It is emerging as an important economic agribusiness, worldwide. According to Food and Agriculture Organization (FAO), global aquaculture production hit a record-breaking 122.6 million metric tons (MT) in 2020, with China alone contributing 57% of the global aquaculture volume (FAO, 2022). The increasing demand for aquaculture production stems from the fact that the wild harvesting of numerous seafood species has already reached or surpassed the maximum sustainable yield (Dulvy et al., 2021). As a result, aquaculture offers a viable alternative, enabling farmers to cultivate these species in controlled environments and thus easing the burden on wild stocks. Consequently, this approach holds the potential to restore depleted wild stocks and aid in the recovery of endangered and threatened species in the future (Osathanunkul & Suwannapoom, 2023). However, the fish that are kept in captivity are more prone to infection as the fish are reared at higher densities than wild individuals, which enables pathogens to spread quickly (Bouwmeester et al., 2021). Consequently, disease outbreaks pose a significant challenge to productivity and lead to substantial economic losses in the aquaculture industry.

Tor tambroides, locally known as “kelah merah” in Peninsular Malaysia or empurau in Sarawak, is a promising freshwater fish species from the Cyprinidae family. The cultivation interest in this species arises from its significant cultural and socioeconomic importance, encompassing its roles as food, ornamental, and sporting fish (Lau et al., 2021). Due to the decline in natural populations and distribution of *T. tambroides*, there has been a

pronounced surge of interest in artificial propagation, for aquaculture production and conservation purposes (Ng & Andin, 2011).

The infectious diseases in fish are often caused either by bacteria, fungi, viruses, and parasites. Among them, bacteria pathogens account for the majority of diseases in fish farming (Dhar, 2014). Diverse *Pseudomonas* spp. are pathogenic to fish, resulting in significant economic losses in the aquaculture sector. Fish pathogenic pseudomonads include *Pseudomonas aeruginosa*, *P. putida*, *P. luteola*, *P. fluorescens*, *P. koreensis*, *P. anguilliseptica*, *P. baetica*, *P. chlororaphis*, *P. plecoglossicida*, and *P. pseudoalcaligenes* (Austin & Austin, 2016). The mortality of diseased cultured *Tor tambroides* was associated with *P. koreensis*. The diseased fish displayed scale loss, exophthalmia, abdominal swellings, and some haemorrhaging at the fins and gills (Kho et al., 2023). *P. koreensis* was also described as the causal agent of eye lesions in golden mahseer (*Tor putitora*) from India (Shahi & Mallik, 2014).

The common practice in treating bacterial infections involves the application of antibiotics. However, the long-term use and misuse of antibiotics in the aquaculture industry have reportedly caused many adverse effects on humans, fish, animals, and the environment. For instance, the unregulated use of antibiotics in aquaculture exerted a selective pressure on aquatic bacterial species, and thus generated reservoirs of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the aquatic environment. From these reservoirs, ARB from the aquatic environment may reach humans directly or the ARGs may disseminate to other bacteria through horizontal gene transfer, eventually reaching human pathogens (Amarasiri et al., 2020).

Vaccination is one of the alternatives that have been recommended to be employed in the management of aquaculture as it has been successful in preventing or reducing the

severity of some infectious diseases in farmed fish, subsequently reducing the use of antibiotics worldwide (Du et al., 2022; Mondal & Thomas, 2022). Types of vaccines for use in fish include traditional inactivated (Gao et al., 2016) and attenuated vaccines (Triet et al., 2019), as well as next-generation vaccines comprising DNA (Xing et al., 2019), recombinant (Liu et al., 2022), vectored (Li et al., 2019), and nanovaccines (Angulo et al., 2020).

Reverse vaccinology, one of the new data-driven approaches, has started to capitalize on the vast amount of omics data available for vaccine design. This approach has demonstrated its efficacy in identifying vaccine candidates for bacterial fish diseases in previous studies (Andreoni et al., 2016; Marana et al., 2017; Chukwu-Osazuwa et al., 2022). Furthermore, it enables the rapid identification of potential vaccine candidates by analyzing genomic data to predict antigens that are likely to induce an immune response. This approach allows for the discovery of antigens that may not have been previously recognized through traditional methods (Sette & Rappuoli, 2010). Recombinant subunit vaccines, composed of purified antigens or antigenic fragments from pathogens, offer enhanced safety profiles due to reduced concerns regarding virulence evolution (Zhao et al., 2022). They also offer the capacity to customize vaccines beyond that permitted by traditional whole attenuated pathogen approaches (Moyle, 2013).

It is hypothesised that vaccination of juvenile *T. tambroides* with the newly developed recombinant subunit vaccine could induce a good immune response and result in mortalities decrement after being challenged with the pathogenic *P. koreensis* strain.

Hence, the objectives of this study were:

1. To perform whole-genome sequencing of the fish pathogenic *P. koreensis* strain;

2. To identify and develop the potential vaccine candidates through the reverse vaccinology approach;
3. To evaluate the immune responses and protective efficacy of the subunit vaccine against *P. koreensis* infection in *T. tambroides*.

The successful development of this vaccine could pave the way forward for better health management and prevention of diseases in this highly valuable fish species.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture in Malaysia

Aquaculture, the production of fish and other aquatic species in controlled environments, has been practiced for centuries in many parts of the world. Malaysia is no exception, with a long tradition of fish farming since 1920 (Jumatli & Ismail, 2021). In 2021, the gross domestic product (GDP) from the fisheries industry was 0.8 % and aquaculture represents about 23% of the total fisheries production in Malaysia (DOF, 2021). The combined production of capture fisheries (inland and marine) and aquaculture in Malaysia in 2021 amounted to 1.75 million MT. This represented a reduction of 2.1% compared to the production levels in 2020. However, it is noteworthy that despite the decline in production, the overall value of the sector experienced a notable growth of 7.8%. Figure 2.1 shows the overall fisheries productivity in Malaysia from 2017 to 2021.

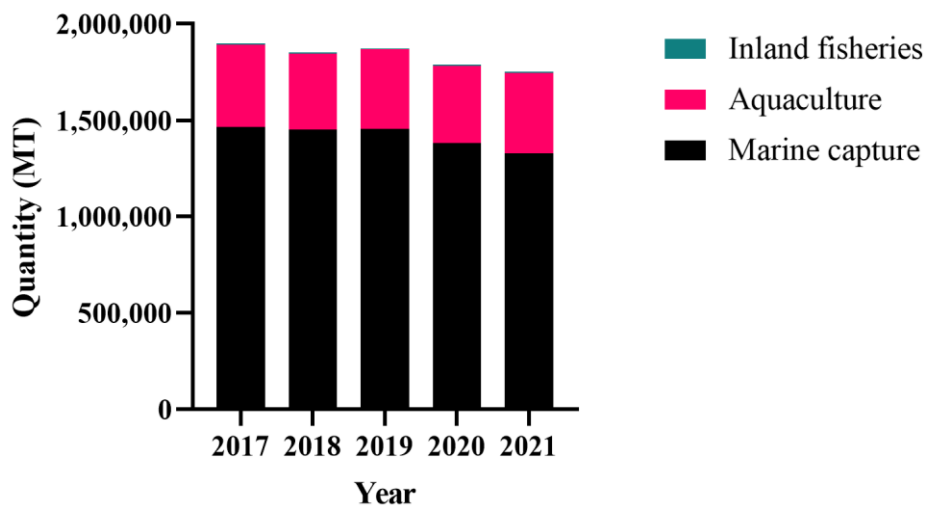


Figure 2.1: Total fisheries production of Malaysia in 2017-2021 by quantity (MT) (DOF, 2017; DOF, 2018; DOF, 2019; DOF, 2020; DOF 2021)

The aquaculture sector in Malaysia is flourishing and holds immense potential, bolstered by ongoing government support aimed at addressing the escalating demand for animal protein (Jumatli & Ismail, 2021). It serves as a critical component in augmenting fish production, alleviating pressure on capture fisheries, and safeguarding against the overexploitation of wild fish populations (Longo et al., 2021). On a global scale, aquaculture presently represents 49% of the total fish production, with Asia alone contributing to over 91.6% of the global aquaculture output in 2020 (FAO, 2022). In the case of Malaysia, aquaculture production has experienced significant growth, surging from less than 80,000 metric tons in 1992 to more than 417,000 MT in 2021, with a total value of RM 3.43 billion (DOF, 2001; DOF, 2021). This progression highlights a substantial increase in the relative importance and contribution of aquaculture within the overall fisheries sector over the years. The major freshwater fish species with the highest production value in the Malaysian aquaculture sector in 2021 were the freshwater catfish (*Tandanus tandanus*) (quantity: 31,957 t; value: RM 228.97 million), red tilapia (*Oreochromis* spp.) (quantity: 30,022 t; value: RM 493.61 million), and river catfish (*Cephalocassis jatia*) (quantity: 21,144 t; value: RM 242.62 million) (DOF, 2021).

The Malaysian government has implemented several policies and programs to promote the development of the aquaculture sector. One of the key policies is the National Agrofood Policy 2021-2030 (NAP 2.0). This policy document recognizes the potential of aquaculture in enhancing food security, alleviating poverty, and fostering economic development. The NAP 2021-2030 also highlights the need to develop a sustainable and competitive aquaculture sector that meets the highest standards of environmental and social responsibility (Ministry of Agricultural and Food Industry (MAFI), 2021). Another important policy is the Fisheries Act 1985, which provides the legal framework for the

management and conservation of fishery resources in Malaysia. The act includes provisions for the regulation of aquaculture activities, such as licensing, and monitoring of aquaculture operations, as well as measures to prevent and control fish diseases and environmental pollution (Nordin et al., 2020). In addition to policies, the government has also implemented programs like Fishermen Community Aquaculture Capacity Enhancement which aims to increase the income and livelihoods of small-scale fishermen through the adoption of modern fishing practices and the promotion of aquaculture as an alternative source of income (Fisheries Development Authority of Malaysia (LKIM), 2022). These initiatives collectively are expected to contribute to the growth and sustainability of the aquaculture industry in Malaysia.

Infections in fish leading to disease outbreaks pose a significant threat to the aquaculture sector as they can cause considerable economic losses due to illness and mortality (Maldonado-Miranda et al., 2022). The current practice of maintaining high densities of fish in aquaculture facilities facilitates the transfer and dissemination of pathogens, often leading to severe outbreaks. Intensive farming methods impose significant stress on farmed aquatic species, compromising their natural immune defenses against various bacterial, viral, and parasitic pathogens. (Irshath et al., 2023). The presence of those pathogens in the environment, as well as their ability to exist asymptotically in some fish, increases the vulnerability of aquaculture facilities to disease outbreaks, hindering the establishment of a reliable and sustainable aquaculture process. The occurrence and severity of fish diseases are influenced by the complex interplay between pathogens, hosts, and environmental factors (Hamed et al., 2018). Stressful conditions, such as overcrowding, temperature fluctuations, and oxygen depletion, can accelerate the transmission of pathogenic bacteria, leading to widespread disease outbreaks. (Irshath et al., 2023). Hence,

multidisciplinary investigations into the characteristics of potential fish pathogens, the biology of fish hosts, and a thorough comprehension of the global environmental factors are imperative. These endeavors are essential for devising appropriate measures to prevent and control the major diseases that hamper fish production in aquaculture (Irshath et al., 2023).

2.2 *Tor tambroides* Overview

Fishes of the genus *Tor*, also known as true mahseers, have great cultural, economic, and religious importance in their native range. It is the iconic genus of large-bodied species of the Cyprinidae family (Pinder et al., 2019). Latest versions and data from the IUCN Red List of Threatened Species TM (Version 2022-2) have shown that the genus *Tor* currently comprises 16 valid species, the same number reported by Pinder et al. (2019), but with some updates on the conservation status (IUCN, 2023). Of the currently valid species, one species is now listed as “critically endangered”, two as “endangered”, one as “least concern”, one as “vulnerable”, three as “near threatened”, and eight as “data deficient” (IUCN, 2023). *T. tambroides* is classified as data deficient due to the lack of information concerning its taxonomy, population status, distribution, and impact of threats (Kottelat et al., 2018).

Despite its classification as data deficient, there has been an increase in the number of studies conducted in recent years to enhance our understanding of this species. *T. tambroides* can be found across a broad range in Southeast Asia, encompassing countries such as Malaysia, Thailand, Indonesia, Myanmar, Lao People's Democratic Republic (Lao PDR), Cambodia, Brunei, and Vietnam (Jaafar et al., 2021). In recent years, the wild population of this fish has declined rapidly, indicating a potential threat to its species status. This decline is primarily attributed to declining water quality, river engineering projects, and

other anthropogenic impacts (e.g., overfishing) which led to the deterioration of their natural habitat (Ingram et al., 2007; Pinder et al., 2019). Its decline is evident in the significant decrease in the catch of *T. tambroides* from inland sources. Furthermore, the decreasing population of this wild species has driven the market value of this fish to as high as RM 1,500 per kilogram (Ismail, 2023), making it one of the most expensive freshwater food fish in Malaysia. The aquaculture production of mahseer in 2021 recorded at 14.84 tonnes (RM 3.31 million) (DOF, 2021). This suggests that there is an economic interest in farming these fish to satisfy market demands and potentially alleviate pressure on the wild population.

In the past, the differentiation of *Tor* species has been based on morphological attributes such as shape, size, fin ray counts, and median length. However, this approach has given rise to considerable confusion and ongoing debates among taxonomists. The features exhibit high intra- and interspecies variability that are affected by localities and environmental factors. Jaafar et al. (2021) have provided a comprehensive overview of distinct morphological characters exhibited by 12 different *Tor* species, which lays the groundwork for a more precise and efficient identification process, contributing to the advancement of our understanding of *Tor* taxonomy and enhancing conservation efforts for these unique aquatic organisms. The body color of *T. tambroides* is silver bronze with dark fins (Figure 2.2). A distinguishing morphological trait unique to *T. tambroides* is the presence of an upper median projection. Other morphological features that *T. tambroides* share closely with other *Tor* species include long lower median lobe (Figure 2.3a), equal caudal fin lobe, sub-terminal mouth position and pointed rostrum hood (Figure 2.3b). Zulfahmi et al. (2018) characterized the morphology of the *T. tambroides* specifically in relation to its axial skeleton. *T. tambroides* has one urostyle bone of the vertebrae, four bones axial vertebrae included in the Weber bone (Weberian apparatus), 16 caudal bones vertebrae,

18 pairs of costal bones, and 19 abdominal vertebrae. The similarity in the number of abdominal vertebrae suggests a resemblance to fish belonging to the Cyprinidae family.

T. tambroides is an omnivorous feeder that scavenges on small fish, plants, algae, insects, and mollusks (Tan, 1980; Kottelat, 2001). *T. tambroides* has a natural dietary preference towards riverine fruits such as engkabang (*Shorea macrophylla*), kemayau (*Dacryodes rostrata*), kepayang (*Pangium edule*), dabai (*Canarium odontophyllum*), and Ara sungai (*Ficus racemosa*) (Redhwan et al., 2022). However, there is little information available on the contribution of these wild fruit phytochemical properties to the savory taste of *T. tambroides*. Nevertheless, several studies suggest that the unique taste of *T. tambroides* could potentially be attributed to its consumption of the Engkabang fruit (Chew et al., 2022). *T. tambroides* has been described as a slow-growing fish species that takes a longer time to achieve larger sizes compared to other fish species. Therefore, in recent years numerous studies were conducted on feed development to enhance the growth performance of *T. tambroides*, with Ramezani-Fard et al. (2012) recommended diets with 23.4% carbohydrates, and Ishak et al. (2016) recommended diets with a n-3 to n-6 polyunsaturated fatty acid (PUFA) ratio of around 3. Additionally, the inclusion of probiotics, specifically *Alcaligenes* sp. AFG22 and *Bacillus* sp. AHG22 in diets has been demonstrated to boost the growth of juvenile *T. tambroides* by up-regulating the growth hormone and insulin-like growth factor 1 genes expressions (Asaduzzaman et al., 2018).

According to Ingram et al. (2007), *T. tambroides* are asynchronous spawners, which spawn multiple times per year with the highest spawning activities occurring during monsoon season. In response to the declining population of wild *Tor* species, many attempts have been made for conservation and aquaculture purposes such as propagating *Tor* species artificially. Ingram et al. (2005) documented the first successful hormonal induction

spawning of *T. tambroides* in captivity. The use of Ovaprim at a dosage of 0.5 mL/kg proved to be the most effective hormone treatment for *T. tambroides*, and the prior administration of Ovaplant (at a dosage of 28-68 mL/kg, 2-7 weeks before inducing spawning) significantly enhanced the success rate of spawning induction. Two years later, Azuadi et al. (2013) observed even better breeding performance in *T. tambroides* females when Ovaplant were implanted into the fishes, followed by injection with Ovatide, resulting in a 78% ovulation rate, surpassing the 55% ovulation rate achieved by Ingram et al. (2005) using Ovaprim in the same species.



Figure 2.2: The image of Empurau (*Tor tambroides*). Adapted from Lau et al. (2021)

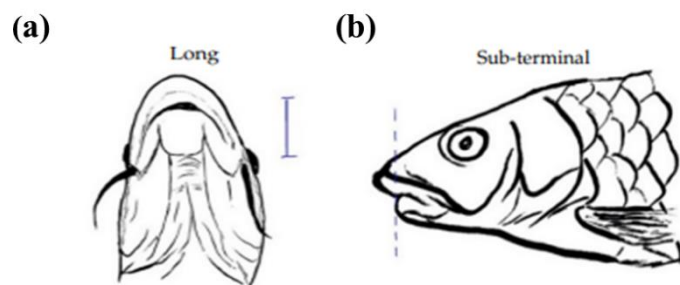


Figure 2.3: (a) Long lower median lobe and (b) Sub-terminal mouth position pointed rostrum hood of *T. tambroides*. Adapted from Jaafar et al. (2021)

Less data are known about diseases. However, *T. tambroides* has been reported to be infected by *Myxobolus tambroides* sp.n. (Székely et al., 2012) and *P. koreensis* CM-01(Kho et al., 2023). *Myxobolus tambroides* sp.n. forms small and round plasmodia that are located

intralamellarly within the gills. The presence of these plasmodia leads to deformations in both the affected gill lamellae and neighboring structures. Furthermore, the 18S rDNA sequence analysis of the parasite revealed no close genetic relationship with any other known *Myxobolus* species in the GenBank database, suggesting that *Myxobolus tambroides* sp. n. is a distinct and previously undescribed pathogenic species affecting *T. tambroides*. Further details on the *P. koreensis* infection in *T. tambroides* were provided in Section 2.3.2.

2.3 *Pseudomonas* spp.

2.3.1 Taxonomy and Classification

Pseudomonas spp. are rod-shaped, Gram-negative, aerobic or facultatively anaerobic bacteria belonging to the family Pseudomonadaceae. Members of this genus are complex, ubiquitous, and versatile bacteria, capable of colonizing a wide range of ecological niches such as soils, water, and organisms (animals, plants, and humans) (Scales et al., 2014; Khan et al., 2022) (Figure 2.4). Their metabolic versatility and adaptability enable them to utilize a vast array of carbon sources, contributing to their ecological success (Nogales et al., 2020). They can function as commensals, but they also have the potential to act as pathogens under certain circumstances.

Pseudomonas stands as one of the most diverse genera and is currently the genus of Gram-negative bacteria with the greatest number of recognized species. The current number of recognized and validly published species is 315, excluding subspecies and synonymous species; according to the List of Prokaryotic Names with Standing (LPNS) in Nomenclature (Parte, 2014).

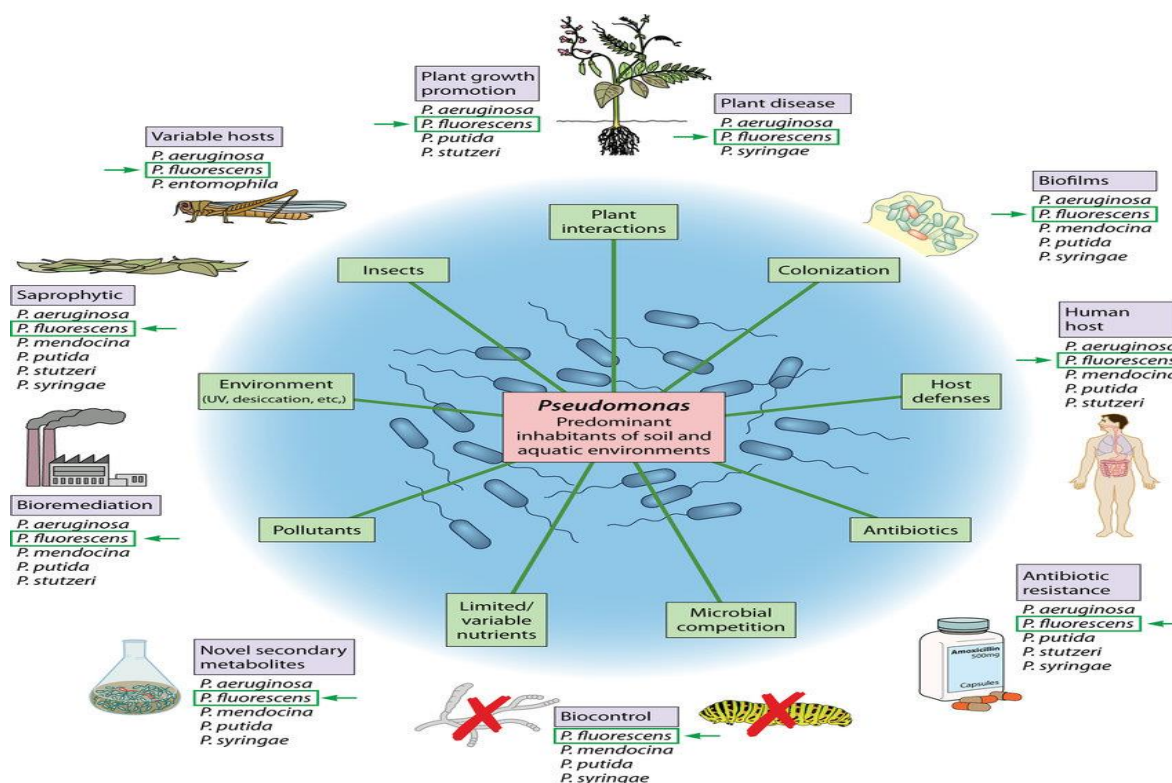


Figure 2.4: The functional and environmental range of *Pseudomonas* spp. Adapted from Scales et al. (2014)

The taxonomy of *Pseudomonas* spp. has undergone significant changes over the years. The genus *Pseudomonas* was first described at the end of the 19th century where the descriptions of genera were solely based upon characteristics of cell morphology (cells with polar organs of flagella) (Duman et al., 2021). A more comprehensive description of the new genus followed in 1966 for which the detail of the physiological, biochemical, and nutritional features of *Pseudomonas* was established (Stanier, 1966). In 1984, the genus was revised and subdivided into five groups (rRNA groups I–V) based on the DNA-rRNA hybridization studies (Palleroni, 1984) (Table 2.1). Later, these five groups turned out to be related to wide varieties of the Proteobacteria; where only the rRNA group I is considered the “true” *Pseudomonas* or “*sensu stricto*”, while rRNA groups II to V have been reclassified to other genera.

Table 2.1: rRNA homology group based on DNA-rRNA hybridization (Palleroni, 1984)

rRNA Group	Subclasses of Proteobacteria	Reclassification into new genus
I	γ (Gamma)	<i>Pseudomonas</i>
II	β (Beta)	<i>Burkholderia</i> or <i>Ralstonia</i>
III	β (Beta)	<i>Acidovorax</i> , <i>Comamonas</i> , or <i>Hydrogenophaga</i>
IV	α (Alpha)	<i>Brevundimonas</i>
V	γ - β (Gamma-Beta)	<i>Stenotrophomonas</i>

While amplicon sequencing of the 16S rRNA gene has emerged as the gold standard for taxonomic profiling of microbial communities, it is worth noting that this gene lacks adequate discriminatory power at the species level for certain genera, such as *Pseudomonas*, with less than 1% nucleotide dissimilarity between many of the species (Lauritsen et al., 2021). Therefore, other protein-encoding housekeeping genes have been used as phylogenetic molecular markers in *Pseudomonas* taxonomic studies, such as *gyrB*, *rpoB*, and *rpoD* genes. Many studies have shown that the concatenated analysis of four housekeeping genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*) effectively resolves the phylogenetic relationships among the *Pseudomonas* species (Mulet et al., 2010; Mulet et al., 2012; Lalucat et al., 2020; Liu et al., 2021). Lalucat and his collaborators demonstrated that the concatenated phylogenetic gene tree for 217 *Pseudomonas* species strains exhibited three main lineages designated “*Pseudomonas fluorescens*”, “*Pseudomonas pertucinogena*” and “*Pseudomonas aeruginosa*” (Lalucat et al., 2020)

Apart from sequence-based techniques, protein-based methods have also proven to be valuable in elucidating the taxonomy of the *Pseudomonas* genus. Mulet et al. (2012) also employed the Whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry (WC-MALDI-TOF MS) for the identification of *Pseudomonas* species. Their findings

indicated that the accuracy of the WC-MALDI-TOF MS was comparable to that obtained through the MLSA approach, with 82.9% of the strains being classified in the same manner by both methods. This result emphasizes the continued significance of the MLSA approach in assessing the phylogeny of the *Pseudomonas* genus, while also highlighting that WC-MALDI-TOF MS can be a viable option for distinguishing and identifying species in ecological and clinical microbial communities.

One of the most intriguing developments in the field of modern-day microbiology is the escalating abundance of publicly accessible whole-genome sequences. The escalating interest lies in leveraging these genome sequences to assess the evolutionary relationships among microbial taxa, given that much additional taxonomic information can be extracted from such data (Hugenholtz et al., 2021). As a result, the use of genomics in taxonomy has become known as ‘phylogenomics’. By considering the entire genomic repertoire of organisms, phylogenomics can reveal previously hidden relationships, resolve taxonomic uncertainties, and provide insights into the processes of speciation and diversification. For instance, several studies have reported cases of rearrangements of *Pseudomonas* species including wrong assignation to species of strains in the genus *Pseudomonas* (Gomila et al., 2015; Girard et al., 2021; Passarelli-Araujo et al., 2022), as well as the transfer of some species to new genera within the Pseudomonadaceae family (Saati-Santamaria et al., 2021; Lalucat et al., 2022; Rudra et al., 2022). A misclassified genome can indeed be a significant concern as it can result in the potential introduction of noise to pangenome studies, reduced strain typing accuracy, propagate labeling errors to subsequent studies (Passarelli-Araujo et al., 2022), and lead to mistaken conclusions.

Despite a considerable reduction in the cost of whole genome sequencing, it continues to pose limitations on the rapid and accurate cataloging of collections of isolates.

As technology continues to advance, we can expect the cost of whole genome sequencing to further decrease, making it more accessible for broader applications in *Pseudomonas* taxonomy. It is likely that the phylogenetic relationships between *Pseudomonas* strains will be clarified as genomic sequences of more strains become available in the future.

2.3.2 *Pseudomonas koreensis*

The *Pseudomonas fluorescens* group is the most complex and includes eight subgroups: *P. fluorescens*, *P. gessardii*, *P. jessenii*, *P. koreensis*, *P. mandelii*, *P. corrugata*, *P. chlororaphis*, and *P. protegens*. Given the evident diversity and complexity seen within the *P. fluorescens* group, which encompasses a substantial count of 76 species and an exceptionally high number of subgroups into which it has been divided (Lalucat et al., 2020), this group is also referred to as “*P. fluorescens* complex”.

P. koreensis is positioned as one of the numerous species that collectively form the *P. koreensis* subgroup, which is situated within the larger framework of the *P. fluorescens* complex. Colonies of *P. koreensis* appear round, opaque, white, and medium-sized on LB agar (Figure 2.5a). Gram staining showed *P. koreensis* as typical rod-shaped appearance (pink in colour), and cells were single in arrangement (Figure 2.5b). *P. koreensis* strain also displayed an intact, clear, and relatively smooth surface (Figure 2.5c). Kho et al. (2023) have reported the biochemical properties of *P. koreensis* using API 20E, and the results were as follows: *P. koreensis* exhibited affirmative responses for citrate utilization, arginine dihydrolase activity, gelatinase production, acetyl methyl carbinol formation, and oxidase activity; Conversely, it displayed negative outcomes for the remaining sixteen diagnostic tests, including β -galactosidase activity, lysine decarboxylase activity, ornithine decarboxylase activity, hydrogen sulfide (H₂S) generation, urease production, tryptophan

deaminase activity, indole formation, as well as glucose, mannose, inositol, sorbitol, rhamnose, and sucrose assimilation.

P. koreensis was initially documented and isolated from Korean agricultural soil (Kwon et al., 2003). Since then, it has been isolated from diverse ecological niches, including gold mining soil (Babalola et al., 2019), yak milk (Kaur et al., 2019), and horse chestnut trees (Crovadore et al., 2020). Ever since its identification as a new species, *P. koreensis* has been recognized for its capabilities as plant growth-promoting rhizobacteria (PGPR) bacteria as it possesses the valuable features characteristic of PGPR microorganisms: plant growth promotion, yield enhancement, and biological control of pathogens (Figure 2.6). In particular, PGPR *P. koreensis* promotes plant development through phosphate solubilization (Li et al., 2017; Gu et al., 2020; Jaborova, 2022); siderophore production (Ghazy & El-Nahrawy, 2020); phytohormone production such as indole-3-acetic acid (IAA) (Jaborova, 2022), gibberellins (Kang et al., 2019), and ethylene (Li et al., 2017); and engaging in biological nitrogen fixation (Singh et al., 2023; Li et al., 2017). On the other hand, it produces volatile compounds such as HCN (Li et al., 2017), and hydrolytic enzymes such as protease and cellulase (Gu et al., 2020), to inhibit the growth of some phytopathogenic fungal species.

While a significant portion of research on *P. koreensis* is indeed focused on its association with plants, it is worth noting that this bacterium has also been identified as a pathogen in freshwater fish. In 2014, *P. koreensis* was described as the causative agent of eye lesions in golden mahseer (*T. putitora*) from India. Besides corneal opacity, the affected fish also exhibited clinical signs such as hemorrhages in the periphery of the eye and internally pale liver (Shahi & Mallik, 2013). Experimental infection of *T. putitora* fingerlings by intraperitoneal injection of 0.1 mL of 7.6×10^6 colony forming unit (CFU) mL⁻¹ of bacterial suspension has resulted in mortalities of 55 % within a 48-hour period. Notably,

despite the observed mortalities, no indications of eye pathology were evident. In the context of histopathological analysis, notable changes were visible in the liver, characterized by the severe disassociation and detachment of hepatocytes, along with the presence of blood cells and mononuclear cells. The authors also highlighted that no prior cases of *P. koreensis* isolation from a diseased fish had been documented previously, posing difficulties in tracing the origin of the infections. Nevertheless, the authors observed that *P. koreensis* expressed beta-hemolysin when exposed to *T. putitora* erythrocytes, implying a possible involvement of hemolysin in its pathogenicity.

The progress in DNA sequencing technologies has brought about a remarkable proliferation in the quantity of complete and draft genome assemblies originating from clinical and environmental isolates of *Pseudomonas* spp. However, it is noteworthy that the NCBI Genome database houses a relatively sparse collection of genomic data pertaining to *P. koreensis*. As of April 2023, only 32 entries of *P. koreensis* genomes (4 as complete genomes, 1 chromosome-level assembly, 8 scaffold-level assemblies, and 19 contig-level assemblies) were deposited in the database, accounting for 0.10% of the total number of *Pseudomonas* genome assemblies. Consequently, there is a clear need for a significant increase in whole genome sequencing efforts targeting *P. koreensis* species as WGS can reveal the complete genetic makeup, shedding light on functional genes, pathways, and potential interactions that contribute to the species' behavior, environmental interactions, and other possible applications. Additionally, it holds the potential to uncover virulence factors and other genetic determinants (particularly if the strain exhibits pathogenic characteristics), thus enhancing our comprehension of its role in infections and diseases.

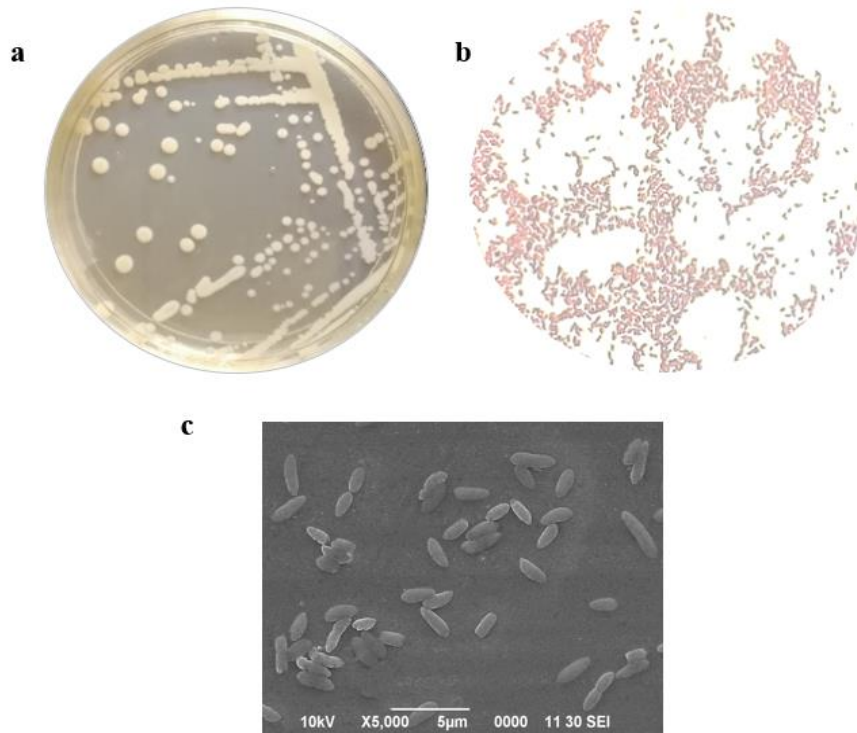


Figure 2.5: Morphological characterization of *P. koreensis*. (a) Bacterial colonies after culturing for 24 h on LB agar (b) Gram staining properties of the *P. koreensis* (c) Scanning electron microscope observation of the bacterial cells with the magnification at 5000×. Adapted from Kho et al. (2023)

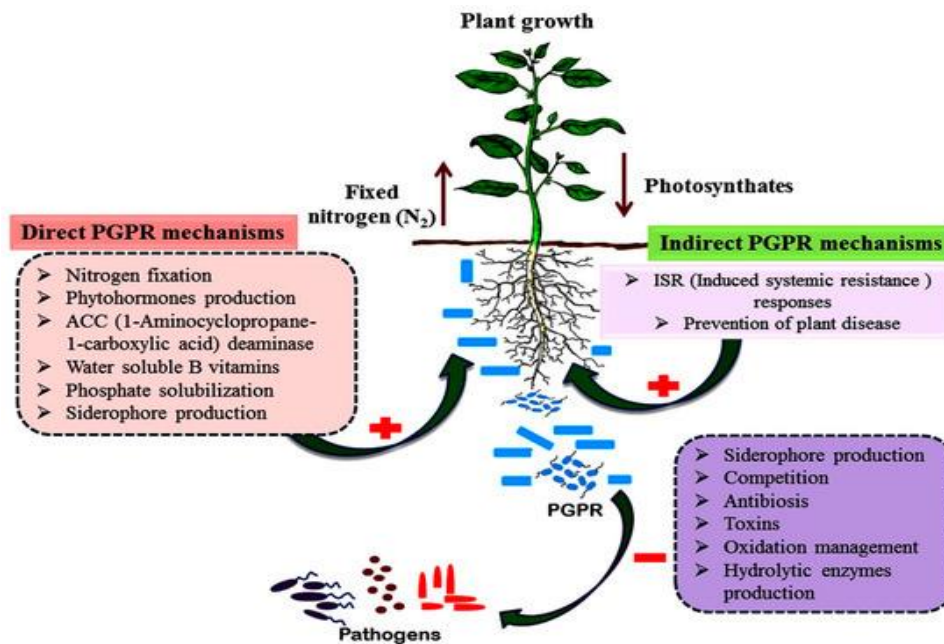


Figure 2.6: Schematic diagram represents the mechanism of PGPR. Adapted from Chandran et al. (2021)

2.3.3 Virulence factors

A bacterial pathogen is commonly characterized as a bacterium that possesses the capability to colonize, invade, and damage the host and thus cause illness. The ability to cause disease is called pathogenicity. Virulence factors, on the other hand, refer to the properties (often represented by gene products) that empower a microorganism to establish itself either on or within a host of a specific species and enhance its potential to cause disease (Relman & Falkow, 2015).

Pseudomonas species are known for their diverse virulence factors (Figure 2.7), which contribute to their ability to cause a wide range of infections in both plants and animals. In general, the virulence factors of *Pseudomonas* can be broadly categorized into bacterial surface structures, secretory, or bacterial cell-to-cell interaction. In this part, the main virulence factors that contribute to *Pseudomonas* pathogenesis will be presented.

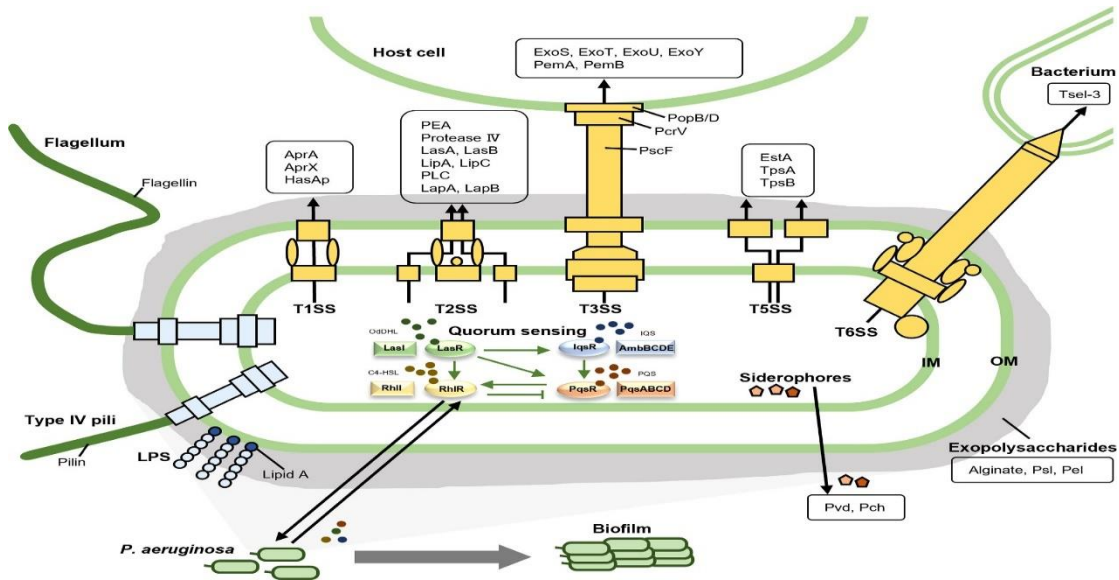


Figure 2.7: The virulence factors of *P. aeruginosa*. Adapted from Liao et al. (2022)

2.3.3.1 Bacterial surface structures

The bacterial surface structures include surface appendages like type IV pili, flagella, outer membrane components such as lipopolysaccharide (LPS), and six types of protein secretion systems (T1SS to T6SS) (Liao et al., 2022).

Type IV pili and flagella are involved in motility in *Pseudomonas* spp. These two organelles promote invasion and colonization of the host by acting as adhesins, as well as influencing biofilm formation by regulating cyclic-di-GMP levels (Azuama et al., 2020). A *fliC* mutant or a *pilA* mutant, which does not produce a flagellum and pili, has been shown to exhibit attenuated virulence in the host (He et al., 2012; Marko et al., 2018). The structural component of flagella, flagellin, can trigger the activation of the host immune response via toll-like receptor 5 (TLR5), and is highly immunogenic (Hajam et al., 2017). For instance, vaccination with flagellin or flagellin-associated protein (fap) has successfully elicited protective immunity in several fish species (Wangkahart et al., 2019).

LPS, another important classical structural component of the outer membrane of Gram-negative bacteria, plays an important role in the pathogenicity of *Pseudomonas*. It is composed of two major components, hydrophobic lipid A and hydrophilic polysaccharide (O-region). The former is associated with toxicity while the latter is associated with immunogenicity (Sampath et al., 2018). LPS can trigger the innate immune response via Toll-like 4 receptors (TLR4), expressed by many immune cells such as macrophages, dendritic cells, and monocytes (Ciesielska et al., 2021). Furthermore, the presence of O antigen also protects bacteria from phagocytosis (Sampath et al., 2018).

Currently, *Pseudomonas* spp. exhibit six distinct secretion systems, designated from T1SS to T6SS, and these secretion systems can be categorized into two main groups based on the secretion routes of transport proteins: one-step secretion system (T1SS, T3SS, T4SS,

and T6SS) and two-step secretion system (T2SS and T5SS). The former directly secretes proteins from bacterial cytosol to the surface, while the latter requires a brief periplasmic stay of the secreted proteins on the export way before being released to the external environment (Qin et al., 2022). Notably, T3SS holds particular importance in the pathogenesis of *Pseudomonas* spp. It serves as a complex machinery that significantly hampers the host's defense mechanisms through the injection of exotoxins such as ExoU, ExoS, ExoT, and ExoY. These exotoxins have various effects on host cells, including cell death, disruption of the cytoskeleton, inhibition of phagocytosis, and modulation of cellular signaling (Jouault et al., 2022).

2.3.3.2 Secreted virulence factors

Secreted virulence factors in *Pseudomonas* spp. include exopolysaccharides (EPS), siderophores, protease, toxins, and many others.

The EPS produced by *Pseudomonas* spp. has been shown to serve as crucial adhesins that mediate initial bacterial attachment to host tissues. This adhesive property is of paramount importance for the establishment of infections, as EPS-coated bacterial cells can effectively adhere to surfaces, resist shear forces, and evade host clearance mechanisms. Moreover, EPS also contributes significantly to the formation of biofilms. The EPS matrix serves as a barrier that shields bacterial cells within the biofilm from immune responses, antibiotics, and adverse environmental conditions (Singh et al., 2021).

Iron, a vital nutrient for bacterial growth and metabolism, is often limited in host tissues and other ecological niches due to the host's mechanisms of iron sequestration. Siderophores are key tools utilized by *Pseudomonas* spp. to overcome this iron scarcity by

efficiently scavenging iron from the environment (Bonneau et al., 2020). These molecules exhibit exceptional iron-binding affinities, enabling *Pseudomonas* spp. to compete effectively for iron even in conditions of low availability. This competitive advantage contributes significantly to their colonization and persistence within host organisms.

Proteases are enzymes that degrade host extracellular matrix proteins, such as collagen and elastin, facilitating bacterial penetration into tissues and promoting the establishment of infections. *P. fluorescens* TSS (a pathogenic fish isolate of *P. fluorescens*), for instance, secretes AprX, an extracellular protease, that is associated with the degradation of host proteins that are present on the cell surfaces and in mucus (Zhang et al., 2009). In addition, the aprX mutant strain, TAM, exhibited a significantly reduced dissemination and survival in host blood and tissues. These findings, coupled with the observation of the high toxicity of purified recombinant AprX on cultured Japanese flounder gill cells, provide substantial evidence supporting the role of AprX as a virulence factor that contributes to bacterial infection (Zhang et al., 2009).

Besides the exotoxins mentioned earlier, *Pseudomonas* spp. also produces other extracellular toxins that can contribute to its pathogenicity. Exotoxin A, produced by the virulent *Pseudomonas* spp., is encoded by the *tox*A gene in their chromosome. It plays a decisive role in inhibiting the protein biosynthesis process of host cell therefore, facilitating bacterial invasion and dissemination, thus accelerating of diseases progression (Algammal et al., 2020; Altaa & Al-Dabbagh, 2022).

2.3.3.3 Bacterial cell-to-cell interaction

Pseudomonas species employ a sophisticated mechanism known as quorum sensing for intercellular communication, enabling synchronized metabolic and physiological responses to specific environmental conditions. This communication relies on small signaling molecules called autoinducers, which are continuously produced and secreted in very small quantities (Liao et al., 2022). As the bacterial population proliferates and autoinducers accumulate in the surroundings, their concentration eventually surpasses a critical threshold, commonly referred to as the "quorum." This threshold initiates a signaling cascade that regulates gene expression, leading to, e.g., virulence (such as regulating the release of multiple virulence factors or biofilm formation (Liao et al., 2022). The predominant signaling molecules in Gram-negative bacteria are N-acyl derivatives of homoserine lactone (AHLs). A comprehensive summary of AHL quorum sensing systems identified in *Pseudomonas* is presented in Table 2.2 (Venturi, 2006).

Table 2.2: AHL quorum-sensing systems of *Pseudomonas* (Venturi, 2006)

<i>Pseudomonas</i> spp.	Quorum-sensing genes	Signal molecule	Quorum-sensing-regulated function(s)
<i>P. aeruginosa</i>	<i>lasI, lasR</i> <i>rhlI, rhlR</i>	3-oxo-C ₁₂ - AHL C ₄ - AHL	Virulence, rhamnolipids, porycyanin, twitching, swarming, HCN, alkaline protease, lectins, elastase, Xcp secretion, biofilm formation, lipase, exotoxin A, other cellular functions
<i>P. syringae</i>	<i>ahlI, ahlR</i>	3-oxo-C ₆ - AHL	Epiphytic fitness, cell aggregation
<i>P. aureofaciens</i>	<i>phzI, phzR</i> <i>csaI, csaR</i>	C ₆ -AHL currently unknown	Rhizosphere colonization, phenazine antibiotics cell-surface components,
<i>P. chlororaphis</i>	<i>phzI, phzR</i>	C ₆ -AHL	Phenazine-1-carboxamide biosynthesis

<i>P. putida</i>	<i>ppuI, ppuR</i>	3-oxo-C ₁₂ - AHL	Biofilm development
<i>P. fluorescens</i>	<i>mpuI, mpuR</i>	Long acyl-chain-AHL	Mupirocin biosynthesis
<i>P. fluorescens</i>	<i>hdtS</i>	3-OH-C _{14:1} - AHL	Currently unknown

2.4 Fish Immunity

Fish are a diverse group of vertebrates that inhabit various aquatic environments, spanning from freshwater to marine ecosystems. As with all living organisms, fish are constantly exposed to a multitude of pathogens including bacteria, viruses, fungi, and parasites. To thrive in these challenging environments, fish have developed a complex and multifaceted immune system (comprising both innate and adaptive immunity) that provides defense against infections and maintains their overall health (Mokhtar et al., 2023). Figure 2.8 is a schematic representation of the response of a fish following an encounter with a pathogen.

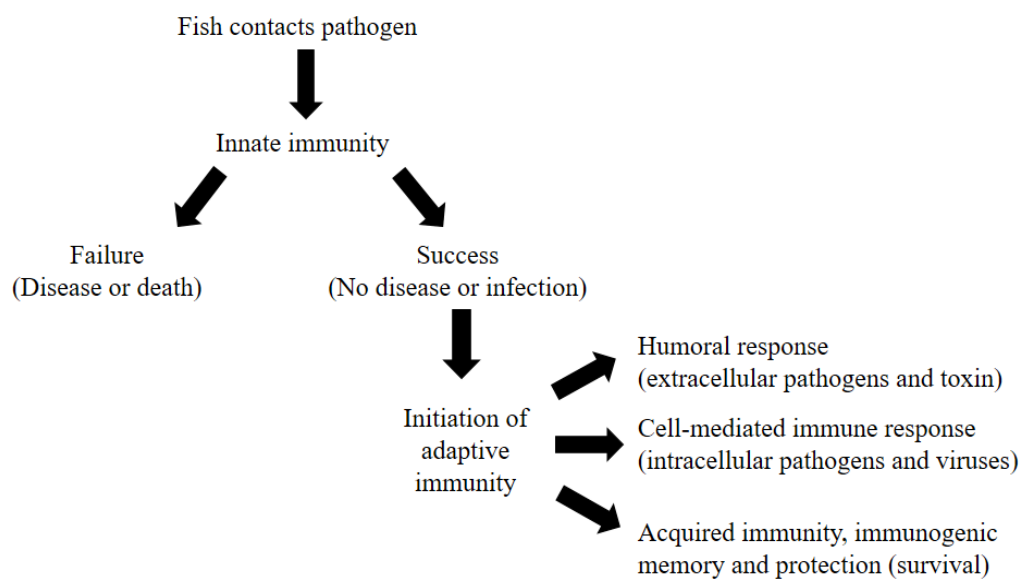


Figure 2.8: Schematic representation of the response of a fish following an encounter with a pathogen (Modified from Kum & Sekkin, 2011)

2.4.1 Innate Immunity

Innate immunity, often referred to as non-specific immunity, is a ubiquitous defense mechanism found in all living organisms and serves as the initial line of protection against various pathogens. In contrast to higher vertebrates, fish exhibit a pronounced reliance on their innate immune system for survival from the early embryonic stage. This reliance is primarily due to the inherent inefficiency of the acquired immune response in fish (attributed to their evolutionary status and poikilothermic nature), resulting in relatively slow lymphocyte proliferation and restricted antibody repertoire, affinity maturation, and memory (Rodrigues et al., 2020; Mokhtar et al., 2023). Consequently, the acquired immune response in fish operates at a comparatively protracted timescale (up to 12 weeks after infection for its activation) (Rodrigues et al., 2020). The innate immune system of fish is composed of three main components: physical or surface barriers, humoral, and cellular factors.

2.4.1.1 Physical or surface barriers

The physical or surface barriers include fish scales, mucous surfaces of skin and gills, and the epidermis, which acts as the first barrier in resisting infection. Fish mucus contains a diverse array of immune substances, particularly lysozyme, pentraxins, lectins, antibacterial peptides, and IgM, which play a crucial role in the efficient trapping and sloughing of pathogens (Dash et al., 2018). In addition to the mucus barrier, the epidermis which is composed of epithelial cells, is populated with macrophages, lymphocytes, and eosinophilic granular cells, which prevents the entry of foreign materials (Mokhtar et al., 2023).

2.4.1.2 Humoral factors

Teleost fish contain a large number of non-specific defense substances, including complement systems, cytokines, chemokines, and many others, which can destroy microorganisms or suppress their growth.

The complement system is a sophisticated network of proteins and protein complexes that operate as a first-line defense against infections in fish. There are three pathways of complement activation: classical, lectin, and alternative, and C3 is the central complement component of all these three activation pathways. The proteolytic activation of C3 enhances the phagocytosis of pathogens, recruits immune cells to the site of infection, promotes inflammation, stimulates B cell proliferation, and ultimately leads to the formation of the membrane attack complex (MAC) for the direct destruction of target cells (Mokhtar et al., 2023). The bactericidal activity of the complement system has been reported in many fish species (Bai et al., 2022).

Cytokines, soluble mediators, are pivotal in regulating the initiation, maintenance, and amplification of immune responses. In recent years, the study of fish cytokines and chemokines has garnered substantial interest, yielding significant progress in their isolation from fish species (Tanekhy & Sakai, 2019). Several critical cytokines have been recognized in teleost fish, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), transforming growth factor- β (TGF- β), and interferon (IFN). Particularly, TNF- α and IL-1 β are widely used in fish studies investigating inflammatory responses against infection (de Figueiredo Soveral et al., 2022). An important chemokine related to the pro-inflammatory process is CXCL-8 (IL-8). It is produced by numerous cell types and was known to recruit neutrophils other immune cells to inflammatory sites (Li et al., 2023).

2.4.1.3 Cellular factors

Several cell types are involved in the innate immune responses of teleost fish including phagocytic cells monocytes/macrophages, neutrophils, and non-specific cytotoxic cells (or NK cells).

Teleost neutrophils are professional phagocytes and the first immune cells that are recruited to the site of infection. They showcase robust antimicrobial responses through diverse intracellular and extracellular mechanisms including the release of granules containing cytotoxic and antimicrobial enzymes, neutrophil extracellular traps (NETs), phagocytosis, and the generation of reactive oxygen species (ROS) and nitric oxide (NO) (Kordon et al., 2018).

Monocytes, found in the bloodstream, undergo a transitory transformation when they migrate to tissues during the inflammatory process, differentiating into macrophages (Lu & Chen, 2019). In teleost fish species, there are two distinctive classifications of macrophages, known as M1 and M2. M1 macrophages produce cytokines such as $\text{TNF}\alpha$ and $\text{IL-1}\beta$, alongside ROS and NO, swiftly eliminating pathogens through processes like phagocytosis, the release of toxic reactive intermediates, restriction of nutrient availability. In contrast, M2 macrophages primarily associate with immunosuppressive responses, trauma, and the secretion of anti-inflammatory cytokines like IL-10 (Grayfer et al., 2018).

Teleost non-specific cytotoxic cells (NCCs) exert their cytotoxic effects by physically contacting target cells, without involving processes such as membrane fusion or fragmentation. Notably, NCC-mediated killing is non-specific, spontaneous, and does not require any apparent induction period. The head kidney of teleost is the primary site where NCCs display their maximal activity (Sahoo et al., 2021). Nevertheless, the spleen and peripheral blood leukocytes (PBL) also exhibit cytolytic abilities, albeit to a somewhat lesser

extent. Notably, the activity of NCCs is influenced by factors such as age, strain, temperature, and stress, and is most pronounced when specific immune responses are less active (Sahoo et al., 2021).

2.4.2 Adaptive Immunity

The adaptive immune system, akin to the innate immune system, encompasses both humoral and cellular components. When the innate immune system proves insufficient in clearing infectious agents, adaptive immunity is summoned, offering a targeted and specific defense mechanism, along with the capacity for long-lasting protection through the formation of immunological memory (Stosik et al., 2021). In contrast to innate immunity, adaptive immune responses pivot on a repertoire of highly diverse antigen-specific receptors expressed on T- and B-cells. Consequently, the adaptive immune system possesses remarkable specificity, enabling it to recognize a multitude of distinct pathogens.

2.4.2.1 Humoral responses

Teleost fish have developed immunological memory, enabling them to respond more rapidly and effectively to pathogens that have been encountered previously (Yamaguchi et al., 2019). Lower vertebrates typically have a more limited diversity of immunoglobulin (Ig) classes compared to mammals (Mashoof & Criscitiello, 2016). For teleost fish, only three immunoglobulins (Igs) isotypes have been discovered: IgM, IgD, and IgZ/T. Nevertheless, it's important to note that not all of these isotypes are expressed in every studied fish species.

IgM is the predominant immunoglobulin class in teleost fish and is the main player in systemic immune responses (Tongsri et al., 2020). It plays a pivotal role in diverse immunological processes, including complement system activation which results in the opsonization of pathogens and cell lysis, and the facilitation of cellular cytotoxicity through antibody-dependent cellular cytotoxicity (ADCC) (Mendoza & Magadán, 2022).

In contrast to mammals, which typically exhibit limited C δ domains (normally two or three) in IgD, teleost fish manifest substantial variability in the number of C δ domains (7 to 17 in zebrafish) (Mashoof & Criscitiello et al., 2016). This structural plasticity is an evolutionary hallmark among species, endowing fish with a diverse repertoire of IgD molecules that contribute to immune adaptation (synthesize more flexible H-chain products.) (Yu et al., 2020).

IgZ/T emerges as the unique immunoglobulin class specific to teleost fish. In teleost fish, IgZ/T, assumes a prominent role in safeguarding gut mucosal immunity. Extensive research in trout has underscored the critical role of IgT in protecting mucosal territories, as evidenced by its local abundance, with a gut mucus-to-serum IgT ratio approximately 63 times higher (Zhang et al., 2010). Furthermore, fish that successfully endured an infection caused by *Ceratomyxa shasta* exhibited heightened levels of IgT exclusively within the mucosal lining of the gut, with no corresponding increase observed in their serum (Zhang et al., 2010). It is noteworthy that some teleost fish species lack IgT (Mirete-Bachiller et al., 2021), and hence, IgM and IgD may play a role, to some extent, in defending against mucosal pathogens, as they are also detectable in the fish mucosal secretions (Yu et al., 2020; Herranz-Jusado et al., 2023).

2.4.2.2 Cell-mediated responses

T cells are classified into two major subtypes: CD8⁺ cytotoxic T-cells (Tc) and CD4⁺ helper T cells (Th). Tc cells are primarily responsible for eliminating infected host cells, while Th cells play a regulatory role in modulating the responses of other leukocytes. Like other vertebrates, fish acquire immunocompetency during ontogeny (Uribe et al., 2011). Notably, in ballan wrasse (*Labrus bergylta*), the early emergence of CD4-1⁺ and CD8β⁺ cells in larval development have been observed (Etayo et al., 2023). In addition, Vinh et al. (2023) investigated the immune responses triggered by an immersion heat-killed *Streptococcus iniae* vaccine in Asian seabass (*Lates calcarifer*) at 35- and 42 days post-hatching (dph). Encouragingly, the results demonstrated immune genes such as MHC I, MHC II, CD4, and CD8 were upregulated at seven dpi in the V35 group. Collectively, these studies suggest the feasibility of vaccinating very young fish to stimulate cellular immunity.

2.5 Application of Recombinant Subunit Vaccine for Bacterial Diseases in Aquaculture

Vaccines are now considered a leading solution in preventing disease outbreaks in the aquaculture industry (Ridzuan et al., 2021). As of now, the United States has approved eight commercial vaccines for use (United States Department of Agriculture (USDA), 2023), while Malaysia has given the green light to four, all of which have been developed using conventional methods that involve the cultivation of specific targeted pathogens (Department of Veterinary Services (DVS), 2022). It is noteworthy that other vaccine forms, such as recombinant and DNA have not yet been authorized for application in the aquaculture sector. Though some of the conventional vaccines have been very successful in the control of some major bacterial diseases in fish, they present some concerns, such as the

possibility of reversion to its pathogenic state, the residual virulence in targeted species might spread to non-target species, and protracted and resource-intensive nature of conventional vaccine development processes (Dadar, 2017).

Over the past three decades, there has been a trend in developing subunit vaccines due to their distinct advantages. These vaccines consist of one or a small number of immunogenic components of pathogens that are produced using heterologous expression systems, ensuring a well-defined composition (Wang et al., 2016). Unlike some other vaccine types, subunit vaccines offer a significant advantage in terms of safety, as they exclusively comprise recombinant proteins or synthetic peptides, with no involvement of infectious pathogens. In addition, subunit vaccines can be scaled up in a more cost-effective manner (de Sá Magalhães & Keshavarz-Moore, 2021). Table 2.3 gives an overview of several experimental trials in the development of subunit vaccines for protection against challenging fish pathogens. Promising results suggest their potential to reduce disease incidence and economic losses in aquaculture.

While subunit vaccines possess many desirable qualities, their capacity to provoke a robust immune response is often less potent than that of killed or live whole-cell vaccines. This limitation arises from the restricted array of components within subunit vaccines that are capable of stimulating the host immune system. Hence, the incorporation of a large immunogenic carrier called an adjuvant is important to permit a prolonged antigen release, resulting in continued stimulation of local inflammation, and the activation of lymphocyte proliferation (Raman & Kumar, 2022). Different types of adjuvants have been used in fish vaccines, such as Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), chitosan oligosaccharide, aluminum hydroxide, and CpG oligonucleotides (Abotaleb et al., 2023), yielding encouraging outcomes.

Table 2.3: Central literature on subunit vaccination and selected background information on pathogens

Name of pathogen	Vaccination route	Stage of trial	Fish species	Finding	References
<i>A. hydrophila</i>	Intraperitoneal injection	Lab trial	<i>Megalobrama amblycephala</i>	• RPS of the vaccinated groups ranged from 58% to 82.3%. • Serum IgM levels increased significantly in the vaccinated groups compared to that of the control group. • Vaccinated fish did not show obvious histological alterations after experimental infection with <i>A. hydrophila</i>.	Zhang et al. (2023)
<i>A. salmonicida</i>	Intraperitoneal injection	Lab trial	<i>Oncorhynchus mykiss</i>	• RPS of rOmpC immunized fish after infection was 81.6%. • The expression of MHCII, TCR, CD4 and CD8 were all upregulated in rOmpC- immunized fish after infection.	Diao et al. (2020)
<i>S. agalactiae</i>	Oral	Field trial	<i>Oreochromis</i> sp.	• IgM levels increased significantly in the serum, mucus, and gut lavage of vaccinated fish. • Aggregation of lymphocytes and development of GALT was observed in vaccinated fish.	Nor (2015)
<i>S. parauberis</i>	Intraperitoneal injection	Lab trial	<i>Paralichthys olivaceus</i>	• RPS of SimA immunized fish after infection was 50%. • The expression of MHCII, IL-8, TNF-α, TLR2, CD4-1 were all upregulated in SimA- immunized fish after infection.	Kim et al. (2022)
<i>V. alginolyticus</i>	Intraperitoneal injection	Lab trial	♀ <i>Epinephelus fuscoguttatus</i> × ♂ <i>Epinephelus lanceolatus</i>	• Vaccinated fish had significantly higher levels of IgM, superoxide dismutase, catalase, and lysozyme. • RPS of Lrp+COS (Chitosan oligosaccharide) immunized fish was 72%.	Wan & Ding (2023)
<i>Vibrio alginolyticus</i> ; <i>V. harveyi</i>	Intraperitoneal injection	Lab trial	<i>Lates calcarifer</i>	• The blood and gut lavage antibody of fish vaccinated with r-OmpK were increased significantly. • The expression of IL-10, IL-8, IL-1β were all upregulated in the spleen, intestine, and kidney of OmpK- immunized fish. • Fish vaccinated with r-OmpK had a 90% survival rate against <i>V. harveyi</i> and 100% against <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i>.	Silvaraj et al. (2020)

2.6 Reverse Vaccinology

Reverse vaccinology was first employed as a groundbreaking approach in countering *Neisseria meningitidis* serogroup B, following a series of unsuccessful attempts utilizing traditional vaccine development approaches. This pioneering effort involved the whole genome sequencing of *N. meningitidis* serogroup B, leading to the identification of a vast reservoir of more than 600 potential antigens (Masignani et al., 2019). The principal distinction between conventional vaccinology and reverse vaccinology can be seen in Figure 2.9. Conventional vaccinology necessitates direct manipulation of the entire pathogen or its constituent parts, involving antigen discovery through empirical techniques, such as pathogen culture and component isolation, often requiring prior pathogen knowledge. In contrast, reverse vaccinology uses genomics and computational tools to predict antigens, starting with the pathogen's genome sequence and screening its encoded proteins in a data-driven, *in silico* approach.

Despite the resounding success of reverse vaccinology in the realm of vaccine development, its potential remains underexplored when it comes to the discovery of novel antigens for fish vaccinology. Nevertheless, this innovative approach has been effectively employed to identify potential vaccine candidates for various fish pathogens. It also enables the simultaneous analysis of targets in multiple genomes, potentially providing broader coverage and being effective against a wider range of pathogen variants. For instance, it has been applied for marine species like Lumpfish and Atlantic Salmon, in combating five gram-negative bacterial pathogens: *Moritella viscosa*, *Aeromonas salmonicida*, *Piscirickettsia salmonis*, *Vibrio anguillarum*, and *Yersinia ruckeri*, and (Chukwu-Osazuwa et al, 2022). Notably, five antigens were found to be conserved across all the species containing 13 exposed epitopes capable of interacting with CD4⁺ T and B cells. These epitopes could serve

as a foundation for developing a polyvalent anti-bacterial vaccine for finfish (Chukwu-Osazuwa et al., 2022).

In addition, reverse vaccinology has the potential to identify a larger number of protein targets compared to the conventional approach, which often focuses on the most abundant and readily antigenic antigens during disease. This broader exploration of potential targets can enhance the chances of identifying effective vaccine candidates. Ellul et al. (2021) performed an *in silico* study and functional analysis for 70 coding regions in *Pasteurella atlantica*, ultimately, 17 target genes were recognized as the most promising gene targets for prioritized consideration in future vaccine development efforts aimed at addressing potential pasteurellosis outbreaks.

Reverse vaccinology not only serves as a predictive tool for identifying vaccine targets in fish pathogens but also demonstrates promising results in *in vivo* studies. Marana et al. (2017) identified 14 potential vaccine targets through reverse vaccinology and formulated them into three different subunit vaccine combinations. Fish immunized with those three vaccines exhibited markedly reduced mortalities (17–30%) compared to the control saline group (56%). Furthermore, the enzyme-linked immunosorbent assay (ELISA) analysis indicated a significant elevation in antibody levels against several protein antigens detected in immunized fish.

The studies collectively emphasize that reverse vaccinology holds great importance in the aquaculture sector. There is still untapped potential in its application to discover new antigens related to other prominent Gram-negative pathogens affecting freshwater aquaculture, representing a promising avenue for future exploration.

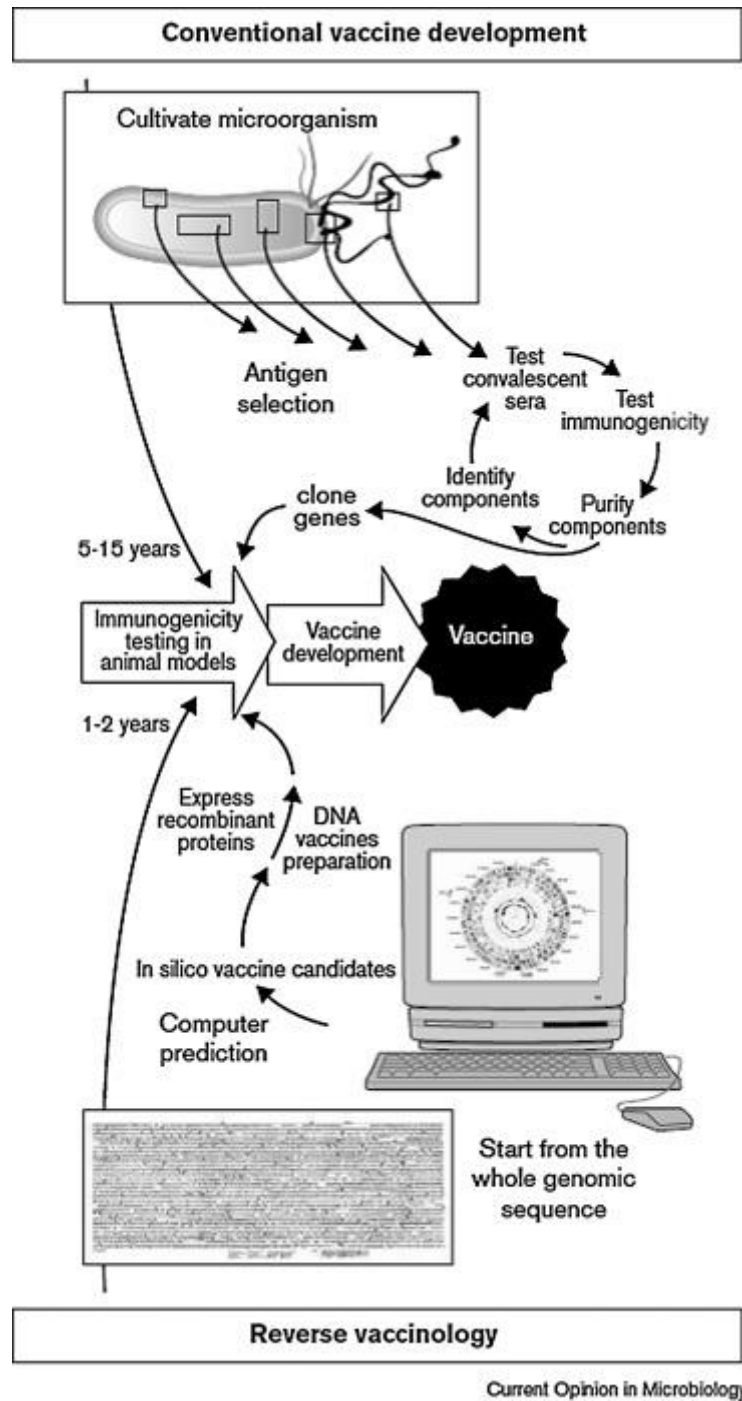


Figure 2.9: A schematic demonstration of the steps involved in vaccine development using the conventional approach and reverse vaccinology. Adapted from Lew-Tabor & Valle (2016)

CHAPTER 3

MATERIALS AND METHODS

3.1 Whole Genome Sequencing of *P. koreensis* strain

3.1.1 Bacterial DNA Extraction

A 1.2 mL aliquot of *P. koreensis* culture was spun at 13,000 rpm for 5 minutes. The supernatant was discarded, and the pellet was then washed with Tris-Cl buffer once. Then 500 µL of lysozyme solution (2 mg/mL) prepared with Tris-Cl buffer was added to the pellet and left to incubate for 1 hour. A 40 µL of 10% SDS was added to the tube and inverted several times. The mixture was centrifuged at 13,000 rpm for 5 minutes and the pellet was discarded. The mixture was then added with one volume of phenol: chloroform: isoamyl alcohol (25:24:1) and inverted several times. The tubes were centrifuged at 13,200 rpm for 5 minutes. The aqueous phase was then collected, and an equal volume of chloroform was mixed gently with the aqueous phase and centrifuged at 13,200 rpm for 3 minutes. The aqueous phase was then collected and transferred to a new Eppendorf tube. DNA was then precipitated by adding sodium acetate (3M at pH 5.2) by 1:10 volume of the aqueous phase. A 2 volume of absolute ethanol was added to the aqueous phase, chilled in the ice bath, and incubated for 5 minutes. The mixture was centrifuged at 13,000 rpm at 4 °C for 5 minutes and absolute ethanol was discarded. The pellet was washed with 1 mL of 75% ethanol and followed by centrifugation at 13,000 rpm for 2 min and the supernatant was discarded. The washing step was repeated until a white pellet can be seen. The pellet was left air-dry and 30 µL of elution buffer was added to elute the DNA. A 1 µL eluted DNA was taken to check for its concentration using Nanodrop DS-11 Series (DeNovix, USA). Only samples with a purity of around 1.8 at 260 nm/280 nm were selected for further processing.

3.1.2 Library Construction and Whole Genome Sequencing

The genome library preparation and whole genome sequencing were outsourced to a private sequencing services provider, Genseq Sdn. Bhd (Malaysia). The genome library construction was performed using the NEBNext® Ultra™ II DNA Library Prep Kit with the corresponding protocol (New England Biolabs, Ipswich, MA, USA) (Figure 3.1). Briefly, 1 µg of genomic DNA was sheared and the fragment ends were repaired by blunting and 5' phosphorylation and A-tailed in preparation for the ligation of the Illumina paired-end oligo adapters: F: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA; R: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT. Subsequently, the ligated DNA products were purified with NEBNext Sample Purification Beads (Beckman Coulter, USA), and further enrichment was achieved by three cycles of PCR with Illumina-compatible primers (i7 and i5). These amplicons were subjected to an additional step of purification with AMPure XP beads (Beckman Coulter, USA). Subsequently, it was sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) with a 2×150 bp run configuration.



Figure 3.1: Workflow demonstrating the use of NEBNext Ultra II DNA Library Prep Kit for Illumina

3.1.3 Data Preparation for Assembly Process

The FASTQ files were adapter-trimmed with Trimmomatic v0.39 (Bolger et al., 2014), and de novo assembled using SPAdes v3.15.4 (Bankevich et al., 2012). In addition, contigs

smaller than 500 bp were discarded. Quality assessment of complete assembly statistics was performed in QUAST v5.02 (Gurevich et al., 2013). The completeness of the final assembly was evaluated using Benchmarking Universal Single-Copy Orthologs (BUSCO) v5.2.2 (Simão et al., 2015) using the pseudomonadales_odb10 lineage dataset. The assembled genome was annotated using National Center for Biotechnology Information (NCBI)'s Prokaryotic Genome Annotation Pipeline 2.0 (PGAP) (Tatusova et al., 2015).

3.2 Screening of Potential Vaccine Candidates Using Reverse Vaccinology

Protein sequences from the annotated genome of *P. koreensis* CM-01 were downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/>) database and extracted in FASTA format for further analysis. In the RV approach, a protein vaccine candidate is identified based on defined desirable attributes including protein subcellular localization, transmembrane topology, antigenicity, virulence, and essentiality.

The list of 5538 proteins was initially screened against the Virulence Factor Database (VFDB) (<http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFalyzer>) (Liu et al., 2019) to identify potential virulence genes and proteins. Next, the subcellular localization of those proteins was identified using PSORTb version 3.0.3 (<https://www.psort.org/psortb/>) (Yu et al., 2010). A transmembrane helices detection was done with TMHMM 2.0 (<https://services.healthtech.dtu.dk/service.php?TMHMM-2.0>) (Krogh et al., 2001). Numbers less than 2 of transmembrane helices were selected. Furthermore, the antigenicity of the proteins was predicted using the VaxiJen tool (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) (Doytchinova & Flower, 2007) with a cut-off value ≥ 0.4 . The proteins were screened against the Database of Essential Genes (DEG) (<http://origin.tubic.org/deg/public/index.php/index>) (identity > 30%, E-value < 10^{-4}) (Luo et

al., 2014) to get information about the essentiality status of the shortlisted proteins as potential vaccine candidates. Ideally, the vaccine targets should not be similar to the host proteome (*Tor tambroides*, taxid: 329116), therefore BLASTp (Altschul et al., 1990) was utilized to filter those *P. koreensis* proteins having >30% identity with fish proteins (E-value <10⁻⁴).

Physicochemical properties such as molecular weight, instability index, aliphatic index, and estimated half-life of the proteins were analyzed using the ProtParam tool (<https://web.expasy.org/protparam/>) (Gasteiger et al., 2005). Only proteins with a molecular weight of less than 110 kDa and instability index <40 were proceeded further. The cutoff value employed in this study corresponds to the established literature cutoff value (Yousaf et al., 2022; Fereshteh et al., 2023).

3.3 Vaccine construction

3.3.1 Bacterial Strains, Plasmid, and Culture Conditions

The pathogenic *P. koreensis* CM-01 strain used in this study was originally isolated from diseased gut *T. tambroides* (Lau et al., 2023). The stored *P. koreensis* CM-01 glycerol stock was streaked out onto a Luria-Bertani (LB) agar plate, and single colonies were randomly isolated for expanding culture at 30 °C for 16 h. The bacteria were then verified again by 16S rRNA gene PCR amplification and amplicon sequencing (to prevent the recurrence of glycerol stock contamination). Then, the correctly identified *P. koreensis* CM-01 was stored for further use. pGEM-T easy (Promega, USA) and pET-32a (+) (Novagen, Germany) were used as cloning and expression vectors, respectively. *E. coli* XL1-Blue (Stratagene, USA) and *E. coli* BL21 (DE3) (Thermo Fisher Scientific Inc., Massachusetts,

USA) were used as cloning and expression hosts, respectively. *E. coli* strains were cultured on LB broth with ampicillin (100 µg/mL) for selection and incubated at 37 °C throughout the study.

3.3.2 Primer Design

The nucleotide sequences of the selected eight proteins were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Specific primer pairs were designed using OligoAnalyzer™ Tool (<https://sg.idtdna.com/pages/tools/oligoanalyzer>) (Owczarzy et al., 2008) to amplify the entire sequence of the selected genes. Specifically, the stop codon was removed from the reverse primer to prevent the transcript from terminating before the C terminal His-tag. By doing so, a fusion protein with two 6× His-tags in N and C-terminal would be generated, which potentially enhancing the affinity and efficiency of the purification process. Additionally, these primers were designed based on several criteria including no more than 5 °C in their melting temperature, absence of secondary structure (formation of hairpin, self-dimer, and heterodimer that can introduce primer dimer), non-self-complementary as well as GC content between 40-60%. In the present study, restriction enzyme cleavage sites were incorporated at 5' end of the forward and reverse primer. To facilitate efficient cutting, two or three nucleotides were added at the 5' end of both primers before the restriction site.

3.3.3 DNA Extraction

The extraction protocol was the same as the procedures described in Sub-section 3.1.1.

3.3.4 Polymerase Chain Reaction Amplification of Eight Candidate Vaccine Target Genes

The extracted DNA samples were assayed with PCR amplification using specific primers. Optimization was carried out using gradient PCR with the annealing temperature setting used calculated based on $(T_m - 5)$ to $(T_m + 5)$. Direct PCR was carried out in a 50 μL reaction volume per PCR tube with the components as shown in Table 3.1. The parameters of thermal cycling for 35 PCR cycles are shown in Table 3.2.

Table 3.1: Reaction components for PCR amplification of eight candidate vaccine target genes

Component	Final concentration	Volume (1X)	Volume (3X)
5X Green GoTaq®	1X	5.0 μL	15.0 μL
Reaction Buffer			
dNTP (10 mM)	0.2 mM	0.4 μL	1.2 μL
MgCl ₂ (25 mM)	1.5 mM	0.8 μL	2.4 μL
Forward primer (10 μM)	1.0 μM	1.0 μL	3.0 μL
Reverse primer (10 μM)	1.0 μM	1.0 μL	3.0 μL
DNA Template	<0.5 $\mu\text{g}/20 \mu\text{L}$	1.0 μL	3.0 μL
GoTaq® DNA Polymerase (5U/ μl)	1.25 U	0.25 μL	0.75 μL
Nuclease-Free water	-	10.55 μL	31.65 μL
Final volume	-	20.0 μL	60.0 μL

Table 3.2: Thermal cycling condition for 35 PCR cycles

Steps	Temperature ($^{\circ}\text{C}$)	Time	
Initial denaturation	95.0	3 minutes	
Denaturation	95.0	30 seconds	} 35 cycles
Annealing	64.0-70.0	30 seconds	
Elongation	72.0	30 seconds	
Final Elongation	75.0	5 minutes	

3.3.5 Detection of PCR Products and Purification

The amplified PCR products were evaluated by running 1.5% agarose gel electrophoresis. The gel extraction and purification were performed using FavorPrep™ GEL/PCR Purification kit (Favorgen, Ping-Tung, Taiwan) following manufacturer's protocol. Briefly, the DNA fragment of interest was excised from the agarose gel with a clean and sharp scalpel and transferred into a 1.5 mL microcentrifuge tube and incubated in 500 µL of FADF buffer at 55 °C for 10 min. The tube was vortexed every 2-3 min until the gel slice dissolved completely. A FADF column was placed into the Collection Tube for each dissolved gel slice. The 750 µL of the sample mixture was loaded onto the FADF column and centrifuged at 13,000 rpm for 30 s. The flow-through was discarded and the column was washed by adding 750 µL of Wash Buffer and centrifuged at 13,000 rpm for 30 s, and dried by centrifugation at 13,500 rpm for 3 min. The FADF column was transferred to a new 1.5 mL microcentrifuge tube and eluted with 20 µL of elution buffer. The microcentrifuge tube was incubated at room temperature for 1 min, and centrifuged at 13,500 rpm for 1 min. The eluted DNA was stored at -20 °C. A small portion of the eluted PCR product was run on 1.5% agarose gel electrophoreses to confirm the success of gel extraction.

3.3.6 Cloning of PCR Product into pGEM-T Easy Vector

3.3.6.1 Chemically Competent Cell Preparation

A 10 µL of cells were harvested from *Escherichia coli* XL1-Blue stock and then cultured into a 10 mL LB Broth and incubated overnight at 37 °C with continuous shaking at 160 rpm. On the following day, 1 mL of overnight cell culture was inoculated into a 20 mL fresh LB Broth and then it was incubated at 37 °C with continuous shaking at 160 rpm

until the reading at OD 600 (600 nm wavelength) reached the range between 0.4 to 0.5. Next, 1 mL of cells were aliquoted to a sterile ice-cold 1.5 mL microcentrifuge tube and centrifuged at 6,000 rpm for 10 min at 4 °C. The supernatant was decanted completely until sufficiently dry before placing it back on ice. The pellet was resuspended with 160 µL ice-cold 0.1 M CaCl₂ solution and put on ice for 10 min. Then, the tube was centrifuged at 6,000 rpm for 10 min at 4°C, and the supernatant was once again decanted completely from the cell pellet and placed on ice again. The pellet was resuspended with 80 µL of ice-cold 0.1 M CaCl₂ followed by 80 µL of ice-cold 40% glycerol to cryopreserve competent cells for long-term storage at -80 °C.

3.3.6.2 Transformation of Ligation Mixtures into Cloning Host, *Escherichia coli* XL1-Blue

Each of the purified PCR products was ligated into pGEM®-T Easy Vector (Promega, USA) (Appendices Figure S1). The ligation reaction mixtures were prepared using components listed in Table 3.3. The reaction mixtures were mixed thoroughly and incubated at 4°C overnight to maximize the yield of transformants.

Table 3.3: Components of ligation reaction

Component	Standard Reaction	Positive Control
2X Rapid Ligation Buffer, T4 DNA ligase	5.0 µL	5.0 µL
pGEM®-T Easy Vector	1.0 µL	1.0 µL
PCR product	3.0 µL	-
Control Insert DNA	-	2.0 µL
T4 DNA Ligase	1.0 µL	1.0 µL
Nuclease-free water	-	1.0 µL
Final volume	10.0 µL	10.0 µL

Before the transformation took place, the LAIX (LB agar + Ampicillin + IPTG + X-Gal) plates were first prepared in the laminar flow hood with a HEPA filter according to the

steps mentioned below. Sterile warm LB agar supplemented with 100 µg/ mL ampicillin was poured into petri dishes. After solidification, 100 µL of 100 mM IPTG solution was spread onto the surface of the agar and was left to dry with the aid of a sterile hockey stick. After that, 20 µL of 20 mg/mL X-Gal was also spread onto the agar surface following the previous spreading and drying strategy.

The transformation procedures began with the addition of 5 µL of each ligation mixture into an ice-cold 1.5 mL microcentrifuge tube containing 80 µL of freshly thawed XLI-Blue *E. coli* competent cells. The tube was left on ice for 10 min. The tube containing both the competent cells and ligation mixture was heat-shocked in the water bath at 42 °C for 50 sec followed by immediately placing back the tube on ice for 2 min. A 900 µL ice-cold LB broth medium was added to each tube of transformants, and the tubes were then incubated at 37 °C in an incubator orbital shaker with constant agitation at 160 rpm for 60 min. The 80 µL of transformant culture was spread onto the LAIX plates. The surface-dried plates were incubated at 37 °C overnight.

3.3.6.3 Blue-White Screening and Colony PCR

Blue/white colony screening observation was done on the overnight cultured bacterial agar plates to distinguish the desired transformants (white colonies) from the unsuccessful ones (blue colonies). The blue and white colonies were enumerated.

Colony PCR was conducted using T100™ Thermal Cycler (Biorad, USA) for confirmation of the desired inserts among the white colonies grown on the plates. Briefly, each of the white colonies was picked from the LAIX plates using a sterile pipette tip and transferred into a PCR tube containing 10 µL of ultrapure water and pipetting up and down to mix evenly. Reaction components for colony PCR were prepared according to Table 3.2

(refer to Sub-section 3.3.4). Then, the colony PCR was carried out with the parameters as shown in Table 3.3 (refer to Sub-section 3.3.4). Subsequently, PCR reactions were checked on 1.5% agarose gel at 90V for 60 min to examine the presence of the insert.

3.3.6.4 Extraction of Recombinant Plasmid and DNA Sequencing

Colony containing the desired insert was cultured in LB Broth supplemented with 100 µg/ mL of ampicillin at 37 °C for 16 hours with continuous shaking. The overnight bacterial culture was subjected to plasmid miniprep using PrimeWay Plasmid DNA Extraction Kit (1st BASE, Singapore) according to the manufacturer's protocol with some modifications on the elution step. The elution step was done by adding 30 µL Buffer EB to the middle of the spin column and incubating the tube at room temperature for 10 min. Then, the tube assembly was centrifuged at 13,500 rpm for 1 min. The elution step was repeated by applying another 20 µL elution buffer, incubating for 10 minutes, and centrifugation at 13,500 rpm for 1 min.

A 10 µL of the plasmid containing the desired inserts was sent to a commercial laboratory (Apical Scientific Sdn. Bhd., Malaysia) for DNA sequencing using T7 forward and SP6 reverse universal primers. The electropherogram files were examined using Chromas 2.6.6 (<https://technelysium.com.au/wp/chromas/>) and similarity analysis of the obtained DNA sequence was carried out using MEGA X (<https://www.megasoftware.net/>) to check whether the positive clone was in the correct reading frame and code for the correct protein. The sequence-verified clones were stored as glycerol stocks in *E. coli* XL1-Blue and stored at -80 °C.

3.3.7 Subcloning of Gene of Interest into pET32-a Vector

3.3.7.1 Restriction Digestion

The restriction digestion was conducted using the reagents listed in Table 3.4. A total of eight tubes were prepared for the digestion of each pGEM[®]-T Easy Vector containing each PVCs respectively. The mixture was incubated at 37 °C water bath for 5 minutes. After that, the mixture was subjected to enzyme inactivation by incubation at 80 °C. The incubation time for the reaction tube containing FastDigest HindIII was 10 min, whereas a 5-minute incubation period was needed for the tube containing the remaining three restriction enzymes. Finally, the reaction mixtures were analyzed on 1% agarose gel electrophoresis. The desired insert was cut from the gel and gel purified using the protocol as mentioned in Sub-section 3.3.5.

Table 3.4: List of restriction digestion reagents

Component	Volume (1X)
10X FastDigest Green Buffer	2.0 µL
Plasmid DNA (up to 2 µg)	3.0 µL
FastDigest BamHI/ FastDigest HindIII/ FastDigest KpnI/ FastDigest XhoI (Only two enzymes were used for each reaction)	2.0 µL (1 µL of each enzyme)
Nuclease-free water	13.0 µL
Final volume	20.0 µL

3.3.7.2 Transformation of Ligation Mixtures into Expression Host, *Escherichia coli* BL21 (DE3)

Each of the target genes was ligated into pET-32a(+) (Appendices Figure S2). The reagents listed in Table 3.5 were added into a 1.5 mL microcentrifuge tube and incubated at 4°C overnight for maximum transformant yield. Before transforming into the expression cells, *Escherichia coli* BL21 (DE3), the plasmid was first transformed into *E. coli* XL1-Blue competent cells for plasmid maintenance and stored as glycerol stocks at -80 °C.

Table 3.5: Components of ligation reaction

Component	Standard Reaction	Positive Control
2X Rapid Ligation Buffer, T4 DNA ligase	5.0 µL	5.0 µL
pET-32a (+)	1.0 µL	1.0 µL
DNA insert	3.0 µL	-
Control Insert DNA	-	2.0 µL
T4 DNA Ligase	1.0 µL	1.0 µL
Nuclease-free water	-	1.0 µL
Final volume	10.0 µL	10.0 µL

The transformation procedures began with the addition of 5 µL of each ligation mixture into an ice-cold 1.5 mL microcentrifuge tube containing 50 µL of freshly thawed *E. coli* BL21 (DE3) competent cells (Thermo Fisher, USA). The mixture was mixed well by gently flicking the tube several times and incubated on ice for 30 minutes. The cells were heat-shocked for exactly 30 seconds in a 42 °C water bath and incubated on ice for 2 min. A 250 µL of room-temperature S.O.C. medium was added to the tubes and placed on a shaking incubator at 225 rpm for 1 hour at 37 °C. A 70 µL of cells from each transformation reaction was spread onto separate LB plates containing the 100 µg/ mL. The plates were inverted and incubated overnight at 37 °C.

3.3.7.3 Colony PCR, Extraction of Recombinant Plasmid, and Plasmid DNA Sequencing

The colony PCR was completed with testing of white colonies only to identify colonies containing the desired recombinant plasmids. Then, plasmid miniprep was done according to protocols as described in Sub-section 3.3.6.4 to extract the plasmid from the positive white colonies for each potential vaccine candidates (PVCs). Positive recombinant plasmids were sent for DNA sequencing using T7 promoter primer. The electropherogram files were examined using Chromas 2.6.6 (<https://technelysium.com.au/wp/chromas/>) and similarity

analysis of the obtained DNA sequence was carried out using MEGA X (<https://www.megasoftware.net/>) to check whether the positive clone was in the correct reading frame and code for the correct protein. The sequence-verified clones were stored as glycerol stocks in *E. coli* XL1-Blue and stored at -80 °C.

3.3.8 Protein Expression

3.3.8.1 Isopropyl β - d-1-thiogalactopyranoside (IPTG) Induction

A 5 ml of LB medium supplemented with 100 μ g/ mL ampicillin was inoculated with a fresh bacterial colony harboring the expression plasmid and allowed to grow at 37 °C overnight. On the next day, the overnight culture was diluted 1:60 with fresh LB medium supplemented with 100 μ g/ mL ampicillin and grown at 37 °C in a shaking incubator with vigorous shaking until the OD600 reached 0.6. at which point a 1 ml bacterial culture was taken as an uninduced sample. IPTG was then added to the bacterial culture to a final concentration of 1 mM and cells were incubated for a further 4 hrs. The cells were harvested by centrifugation at 4,000 rpm for 10 min. The supernatant was discarded, and the pellet was kept at -20 °C.

3.3.8.2 Recombinant Protein Expression and Solubility Analysis by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Expression and solubility of each of the recombinant proteins were evaluated by SDS-PAGE. It is important to check for the solubility of the protein as it is necessary to solubilize the protein if it is found in the pellet (inclusion body) and perform purification under denaturing conditions.

The stored pellets (refer to Sub-section 3.3.8.1) were resuspended in 1× PBS buffer (pH7). A 25 µL of freshly prepared lysozyme solution (10 mg/mL in 10 mM Tris-HCl, pH 8.0) was added to the mixture and then mixed by vortexing for 3 sec. The lysis mixture was incubated for 30 min at 37 °C. After incubation, the tube containing the lysis mixture was placed in a boiling water bath for exactly 40 sec. A portion of the resuspended pellet was used directly to check for the level of expression of the recombinant protein. The remaining pellet was sonicated using an ultrasonic bath in ice water for 30 min. The fraction of the lysate was then centrifuged at 12,000 rpm, for 20 min at 4 °C to separate soluble and insoluble fractions. After separating the supernatant, the pellet fraction was resuspended in 1× PBS buffer.

The profile of protein expression was checked on SDS-PAGE with 12% resolving gel (3 mL ultrapure water, 2.1 mL 40% acrylamide/bis-acrylamide, 1.75 mL 1.5 M Tris HCl pH 8.8, 70 µL 10% SDS, 70 µL 10% APS, and 3.5 µL TEMED and 4% stacking gel (0.2 mL ultrapure water, 0.2 mL 30% acrylamide/bis-acrylamide, 0.5 mL 0.5 M Tris-HCl pH 6.8, 20 µL 10% SDS, 20 µL 10% APS, and 2 µL TEMED).

Samples of resuspended whole lysate, supernatant, and pellet fractions were prepared by mixing with 4× SDS sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, glycerol, β-mercaptoethanol, 0.5% bromophenol blue) in 4:1 ratio, respectively. Then, 10 µl of each sample was loaded into the well and electrophoresis was carried out in a Vertical Gel Electrophoresis apparatus (plate size: 160 mm×160 mm) (ATTO Corp., Japan) at a constant voltage of 120 V in the 1X running buffer (prepared from 10X running buffer which composed of 0.25 M Tris HCl, 1.92 M Glycine, and 1% (w/v) SDS) for 90 minutes.

For staining, the gel was removed from the gel cassette and immersed in a fixing solution (30% methanol, 10% acetic acid) for 10 min. A staining solution (30% methanol,

10% acetic acid, 0.1% Coomassie Blue Brilliant R-250) was added to the gel and stained for 1 hr with gentle shaking. After that, the staining solution was poured out and a destaining solution (30% methanol, 10% acetic acid) was added and left overnight or until the protein bands were seen without background staining of the gel. The result of the protein staining was observed and recorded.

3.3.9 Recombinant Protein Purification

In this study, six proteins were successfully expressed in *E. coli* BL21 (DE3). These recombinant proteins were either purified under native or denaturing conditions by a Ni-NTA Spin Kit (Qiagen, USA) according to the manufacturer's instructions (Ni-NTA Spin Kit Handbook).

Briefly, a 5 ml of LB medium supplemented with 100 µg/mL ampicillin was inoculated with a fresh bacterial colony harboring the desired expression plasmid and allowed to grow at 37 °C overnight. On the next day, the overnight culture was diluted 1:60 in 50 mL fresh LB medium supplemented with 100 µg/mL ampicillin and grown at 37 °C in a shaking incubator with vigorous shaking until the OD600 reached 0.6. IPTG was then added to the bacterial culture to a final concentration of 1 mM and cells were incubated for a further 4 hrs. The cells were harvested by centrifugation at 4,000 rpm for 10 min. The supernatant was discarded, and the pellet was kept at -20 °C if desired or processed immediately.

For denaturing condition, the cell pellet was thawed for 15 min and resuspended in 700 µL Buffer B (7 M urea, 0.1 M NaH₂PO₄; 0.01 M Tris·Cl (pH 8.0)) with 150 units of Benzonase Endonuclease (Merck, UK). The culture was incubated with agitation for 15 min

at room temperature. The lysate was centrifuged at 13,300 rpm for 30 min to pellet the cellular debris and the supernatant obtained was collected. A Ni-NTA Spin kit column was equilibrated with 600 μ L of Buffer B for 2 min at 3,600 rpm. The flow-through was discarded. A 600 μ L of supernatant containing 6xHis-Tagged proteins was added into a pre-equilibrated spin kit column and then centrifuged for 5 min at 2,000 rpm. The flow-through was discarded and the column was washed twice with 600 μ L Buffer C (8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-Cl (pH 6.3)) and centrifuged for 2 min at 3,600 rpm. The protein was eluted twice using 200 μ L of Buffer E (8 M urea; 0.1 M NaH_2PO_4 , 0.01 M Tris-Cl (pH 4.5)) and centrifuged for 2 min at 3,600 rpm. The eluted proteins were then analyzed using SDS PAGE (refer to Subsection 3.3.8.2).

While for native condition, the cell pellet was thawed for 15 min and resuspended in 630 μ L Lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole (pH 8.0)) with 150 units of Benzonase Endonuclease (Merck, UK) and 70 μ L lysozyme stock solution (10 mg/ml), and incubated on ice for 30 min. The lysate was centrifuged at 13,300 rpm for 30 min at 4 $^{\circ}\text{C}$ and the supernatant obtained was collected. A Ni-NTA Spin kit column was equilibrated with 600 μ L of Lysis buffer for 2 min at 3,600 rpm., and the flow-through was discarded. A 600 μ L of supernatant containing 6xHis-Tagged proteins was added into a pre-equilibrated spin kit column and then centrifuged for 5 min at 2,000 rpm. The flow-through was discarded and the column was washed twice with 600 μ L Wash Buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole (pH 8.0)) and centrifuged for 2 min at 3,600 rpm. The protein was eluted twice using 200 μ L of Elution Buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 500 mM imidazole (pH 8.0)) and centrifuged for 2 min at 3,600 rpm. The eluted proteins were then analyzed using SDS PAGE (refer to Subsection 3.3.8.2). Protein concentration was measured using the Nanodrop DS-11 Series (DeNovix, USA).

3.3.10 Preparation of Fish Vaccine Combination

Two experimental formulations containing three recombinant proteins were prepared. A total of 25 µg of an individual protein was prepared per fish in the vaccine mixture corresponding to a total of 75 µg of mixed proteins per fish. Each mixture was emulsified with one volume of Freund's Incomplete Adjuvant (FIA) to obtain a 1:1 oil in water preparation. The vaccine control containing only adjuvants was made accordingly without the protein constructs. The vaccines were stored at 4 °C until the further use.

3.4 Experimental Design and Vaccination Regime

3.4.1 Fish Maintenance

A total of 60 experimental fish *T. tambroides* (mean weight 25 g) was obtained from the local aquaculture farm and acclimatized at 25 ± 1 °C. All the fish were fed daily with commercial pellets for 2 weeks before the experiments. The fish were reared at 16 hours light: 8 hours darkness regime throughout the whole experiment. All procedures in this study were conducted according to the standard guidelines for the use of animal models, and the study was approved by the Animal Ethics Committee of Universiti Malaysia Sarawak (UNIMAS/AEC/R/F07/057).

3.4.2 Vaccination

The fish were randomly divided into four groups of 15 fish: (I) Control group (PBS); (II); Control group (adjuvant); (III) Vaccine A; and (IV) Vaccine B. This group size aligns with established standards and practices observed in a previous study (Yan et al., 2021). The fish were anesthetized (75 mg L⁻¹ MS222, Sigma-Aldrich, Denmark) and intraperitoneally

injected with 0.2 mL of different formulations warmed up to fish body temperature (14 °C) prior to the immunization. The intraperitoneal injection was selected as the preferred route of administration due to its ability to facilitate direct delivery of the vaccine into the peritoneal cavity of the fish and allow for precise and accurate dosing of the vaccine, crucial for eliciting optimal immune responses, as compared with the oral and immersion routes.

3.5 Bacterial Challenge Trial and Sampling

The experiment design of this study referred to previous studies with some modifications (Marana et al., 2017; Liu et al., 2022; Abotaleb et al., 2023). At 30 days post-immunization (dpi), the fish were challenged with 2.0×10^8 CFU/mL *P. koreensis* strain. Clinical signs and daily mortality were recorded for 7 days. The moribund fish that showed the symptoms of skin hemorrhages and complete loss of equilibrium was immediately removed for euthanasia (75 mg/L MS222, Sigma-Aldrich, Denmark). At 7 days post-challenge (7dpc), three individuals from each tank were randomly dissected after anesthetized with MS-222, and the spleens, kidneys, gills, and intestines were collected.

3.6 Efficacy of the Recombinant Protein Subunit Vaccines

3.6.1 Relative Percentage of Survival (RPS)

The protection elicited by the vaccine formulations was determined by comparing the cumulative mortality of treated and control groups. The relative percentage of survival (RPS) was calculated as $RPS = [1 - (\% \text{ mortality in vaccinated fish} / \% \text{ mortality in control fish})] \times 100$.

3.6.2 Histopathology

3.6.2.1 Agar Embedding for Cryostat Sectioning of Tissue Sample

Spleen, kidney, gills, and intestine tissue were washed with ice-cold phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde at 4°C for 48 to 72 hours. The tissues were washed again in 1X PBS after the fixing step at least 3 times for 20 mins to remove all possible carried-over PFA. The tissues were then placed in 15% sucrose in PBS solution for 6–12 h, or until the tissue had sunk. Next, the tissues were placed in 30% sucrose in PBS solution overnight or until the tissue had sunk. These tissues were then embedded in a melted agar solution (1.5% agar, 5% sucrose in water) and, after the blocks hardened, they were trimmed (leave at least 2-3 mm of agar around the tissue and 3-4 mm of agar below the tissue where it will be frozen to the cryostat chuck) and immersed in 30% sucrose in water solution overnight at 4 °C.

3.6.2.2 Cryostat Sectioning

The cryostat sectioning was carried out in Institute of Biodiversity and Environmental Conservation (IBEC), UNIMAS. When the blocks were ready to be sectioned, a slightly raised platform was created by freezing a layer of OCT medium to the cryostat chuck. A drop of OCT was added to the platform, and the agar block was positioned. Gradual even freezing is necessary to avoid cracking the block. The blocks were equilibrated to the cryostat temperature before sectioning. The prepared blocks were cut using a Leica CM 1850 cryostat microtome (Leica Biosystems, Germany). Sections were cut at 5 µm and mounted on microscope slides. The slides were then dried at room temperature till the sections were firmly adhered to the slides.

3.6.2.3 Hematoxylin and Eosin Staining

The slides were fixed with absolute methanol for 1 min and then rinsed with water. The slides were stained regressively with Gill's hematoxylin for 10 min and were then rinsed in tap water. The slides were decolorised in acid alcohol followed by rinsing in 4 changes of tap water. Dilute ammonia (0.1% ammonia) was added to the slides for 10 sec for bluing and rinsed with tap water to remove excessive ammonia. The slides were rinsed in 3 changes of 90% ethanol and stained with eosin for 2 min and then rinsed again with 3 changes of 90% ethanol. Slides were then dehydrated in 95% ethanol and absolute ethanol. Next, the slides were placed in two changes of xylene for 10 min each. After staining, 1-2 drops of mounting solution (50% glycerol in water) were added and mounted with coverslips. Random fields of each slide were captured (100×) using a light microscope (Leica Biosystems, Germany).

3.6.3 Analysis of Inflammation-Related Gene Expression using Quantitative real-time RT-PCR analyses

3.6.3.1 Total RNA Extraction

The spleen, kidneys, gills, and intestine tissues were homogenized with 1 mL TRI Reagent (Sigma Aldrich, USA). The tubes were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube and 200 µL of chloroform was added to the supernatant. The tubes were covered tightly and were shaken vigorously for 15 seconds. The tube was left for 5-10 minutes. Then, the tubes were centrifuged at 13,000 rpm for 15 minutes at 4 °C. The aqueous phase was transferred to a new tube and 500 µL of ice-cold isopropanol was added. The sample was inverted gently 5 times and incubated at room temperature for 10 min. Then, the tubes were centrifuged at 13,000 rpm for 10 minutes at 4

°C. The supernatant was discarded, and white pellet was washed with 1 mL of 75% ethanol followed by centrifugation at 12,000 rpm for 5-10 minutes at 4 °C. The ethanol was then discarded, and the white pellet was air-dried for 15-30 minutes. The pellet was dissolved in 20 µL of nuclease-free water and was stored at -80 °C. The concentration and purity of the total RNA were determined by Nanodrop DS-11 Series (DeNovix, USA).

3.6.3.2 cDNA Synthesis

The extracted RNA was reverse transcribed to synthesize the first-strand cDNA using a ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) using 500 ng total RNA according to the manufacturer's instructions. Briefly, a 1 in 50 volume of gDNA remover was first added to 4× DN Master Mix. Then, it was added to RNA in order to increase DNase I reaction and incubated at 37 °C for 5 min. After that, 2 µL of 5× RT Master Mix was added to the reaction mixture. Reverse transcription response was performed by following steps; incubation at 37 °C for 15 min, 50 °C for 5 min, 98 °C for 5 min.

3.6.3.3 Quantitative real-time RT-PCR

The immune-related genes included *TNF-α*, *IL-1β*, and *IL-8*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene for normalization. RT-qPCR was performed using ThunderBird SYBR Green qPCR master mix (Toyobo Life Science, Japan) on a Roche LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) with the following procedures: 95 °C for 1 min and 45 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 60 s. Specific primers (Table 3.6) for the amplification of these genes were designed by NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Reactions were performed in a final volume of 20 μ L containing 10 μ L of 1x THUNDERBIRD® SYBR® qPCR Mix, 0.6 μ L each of 0.3 μ M forward and reverse primers, 7.8 μ L of distilled H₂O, and 1 μ L of the cDNA template. Fluorescence measurements were taken at the end of the elongation step of each PCR cycle. To evaluate amplification specificity, melting curve analysis was performed at the end of each PCR run. Melting analysis was performed by first heating to 95 °C for 5 s, cooling to 40 °C for 30 s, heating to 65 °C for 1 min, and then melting with continuous acquisition (5 readings/°C) of fluorescence signal until 97 °C. The expression level of immune genes was calculated using the $2^{-\Delta\Delta C_t}$ method. Gene expression levels of the control group were set as 1, and that of the vaccinated groups were presented as fold change. All data were expressed as the mean $\pm$ SD. Statistical analyses were performed with one-way analysis of variance (ANOVA) and Dunnett's test to determine the significant differences in immune gene expression levels among control and vaccinated groups. Results with $p < 0.05$ or lower were designated statistically significant (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, or **** indicates $p < 0.0001$).

Table 3.6: Primer sequences of target genes and *GAPDH* for real-time PCR assay

Gene	Primer sequences (5'-3')	Amplicon size (bp)
<i>GAPDH</i>	F: GACAAGGCCTCAGCTCACAT R: TGCAGGATGCATTGCTGACT	145
<i>TNF-α</i>	F: CGCTGGAAGGGGTTTCAAGA R: CAACTGCTGCAAGGAGGACT	156
<i>IL-1β</i>	F: TCCTCGCAGCATGAGACAAG R: CAGGTGACGCTCTTCGATCA	142
<i>IL-8</i>	F: AGAAGTTCACCGCATCTGCC R: TAGGAATTGCTGGCCCTGAA	132

CHAPTER 4

RESULTS

4.1 Genomic Features of *P. koreensis* CM-01

Whole-genome sequencing was employed to investigate the genomic features of *P. koreensis* CM-01 (Figure 4.1). This whole genome sequence data has been deposited at GenBank under the Accession Number JAMSLY0000000000 (Table 4.1). The final assembly yielded 89 contigs with an average sequence coverage of 96.5×. The genome of *P. koreensis* CM-01 comprises 6,171,880 bp with a GC content of 60.5%. For assessment of our genome assembly, we searched a total of 782 BUSCO (Benchmarking Universal Single-Copy Orthologs) groups and determined that 778 (99.5%) were complete (with 777 single-copy BUSCOs and 1 duplicated BUSCOs), suggesting high completeness of genome assembly for *P. koreensis* CM-01. Based on NCBI Prokaryotic Genome Annotation Pipeline (PGAP) annotation, 5,538 coding sequences (CDSs with protein), 54 tRNAs, 3 rRNAs, and 61 pseudogenes were predicted.

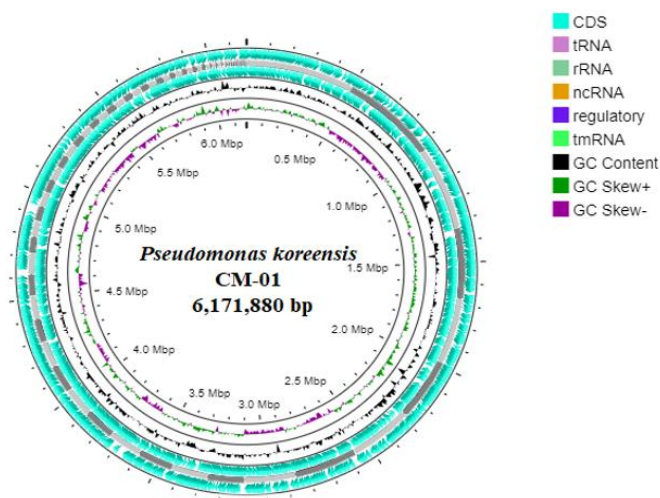


Figure 4.1: Circular representation of the draft genome of *P. koreensis* CM-01. From outer circle to inner circle, representation is as follows: 1. Forward strand CDSs (turquoise blue arrow); 2. Contigs (Gray arrow); 3. Reverse strand CDSs (turquoise blue arrow); 4. GC

plot (black); 5. GC skew (green and magenta colour correspond to CG skew +and−, respectively)

Table 4.1: Whole genome assembly and annotation statistics, and BUSCO v5.2.2 scores of *P. koreensis* CM-01

CM-01 genome	
Sequencing and assembly	
No. of Illumina reads	4,725,557
Total no. of Illumina bases (bp)	708,833,550
K-mer size (bp)	77
Average coverage (x)	96
Number of contigs	89
Contig maximum length (bp)	498,557
Contig N50 (bp)	233,601
L50	9
Genome size (bp)	6,171,880
GC content (%)	60.5
Genome annotation	
No. of genes (total)	5,661
Genes (coding)	5,538
CDSs (total)	5,599
CDSs (with protein)	5,538
Genes (RNA)	62
No. of rRNAs (5S, 16S, 23S)	3 (1, 1, 1)
No. of tRNAs	54
No. of ncRNAs	5
No. of pseudogenes	61
BUSCO Assessment Results	
Complete BUSCOs	99.5% (778)
Complete and single copy BUSCOs	99.4% (777)
Complete and duplicated BUSCOs	0.1% (1)
Fragmented BUSCOs	0.4% (3)
Missing BUSCOs	0.1% (1)

4.2 Selection of Potential Vaccine Candidates (PVCs)

4.2.1 Identification of Potential Virulence Proteins

VFAnalyzer systematically screened the query protein sequences against the Virulence Factor Database (VFDB) to identify potential virulence based primarily on homologies with known virulence factors in the *Pseudomonas* genus. Of the 5538 proteins, 177 (3.2%) proteins were found to be virulent (Figure 4.2a). These virulent proteins were categorized into 18 major virulence factor classes, including adherence, antiphagocytosis, iron uptake, protease, quorums sensing, regulation, secretion system, toxin, biofilm formation, cell surface components, efflux pump, immune evasion, invasion, lipid and fatty acid, magnesium uptake, serum resistance, stress adaptation, and others (Figure 4.2b). Notably, proteins associated with adherence were the most prevalent (n=88, 49.72%), followed by those involved in antiphagocytosis (n=24, 13.56%), and iron uptake (n=23, 12.99%).

4.2.2 Prediction of the Subcellular Localization of the Virulent Proteins

The subcellular localization analysis revealed the number of proteins localized in various bacterial cellular regions (cytoplasmic, inner membrane, periplasmic, outer membrane, and extracellular) (Figure 4.2c). The cytoplasmic region has the highest number of proteins, while extracellular proteins are the least abundant. Among the 177 virulent proteins selected, 66 (37.28%) proteins localize to the cytoplasm, 58 (32.77%) proteins localize to the membrane (inner and outer), 17 (9.60%) proteins localize to the periplasm, 11 (6.21%) proteins are extracellular, and 25 (14.12%) proteins have unknown localization. Proteins found in the extracellular, periplasm, outer and inner membrane were prioritised

due to their closer contact with host cells and hence their ability to initiate immune responses. Thus, 86 membrane, periplasmic, and extracellular proteins were selected for further analysis.

4.2.3 Transmembrane Helix, Antigenicity and Essentiality Prediction of the Selected Membrane (Inner and Outer), Periplasmic, and Extracellular Proteins

In the prokaryotic expression system, multiple transmembrane helix structures will affect the recombinant expression and purification of proteins; thus, 29 (33.72%) proteins with multiple transmembrane helix structures (>2) were excluded, and the remaining 57 (66.27%) proteins (of which 41 proteins had no transmembrane and 16 had one transmembrane) were forwarded for further analysis (Figure 4.2d).

The potential antigenicity of these 57 candidate proteins was assessed using VaxiJen with a cutoff value of ≥ 0.4 . Analysis revealed 51 (89.47%) proteins were found to score higher than the critical value, indicating that most of the identified proteins were potential antigenic proteins (Figure 4.2e) ensuring its capacity to provoke the immunity of the host.

The Database of Essential Genes (DEG) is a database of essential genes and proteins. From the 51 proteins obtained in the previous step, 9 (17.65%) proteins that aligned with DEG entries were retained, these proteins may play crucial roles in the microorganism's biology (survival, growth, and reproduction) (Figure 4.2f). All the parameters used are the literature cut off value.

4.2.4 Physicochemical Properties of the Proteins and Homology Check Against Fish Proteome

One protein was removed from the candidate protein list as its molecular weight and instability index exceeded the acceptable threshold of 110 kDa and 40, respectively. This suggests that the protein would be unstable upon expression. The remaining eight proteins were subjected to BLASTp to determine the non-homologous protein sequences against the fish proteome. The BLASTp analysis revealed no protein showed significant similarity to fish proteome.

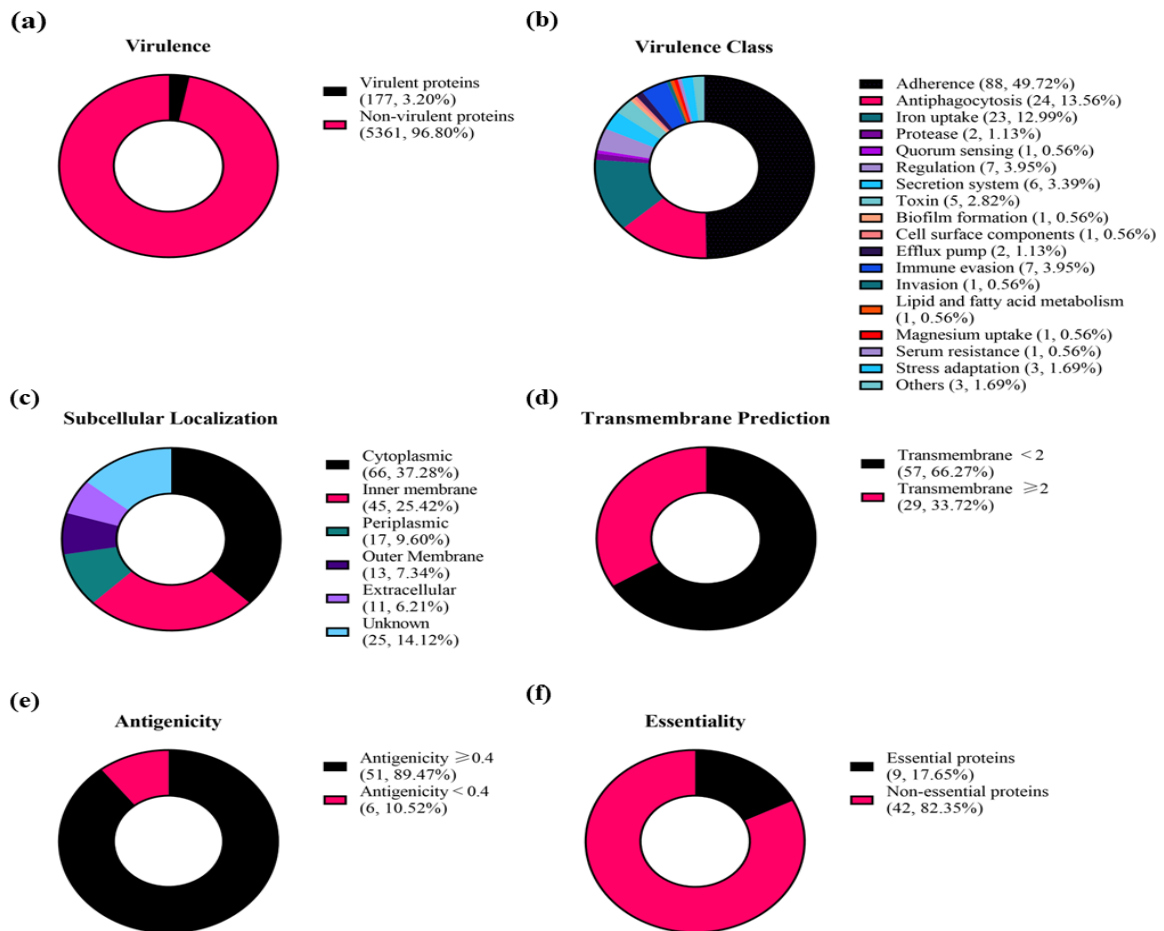


Figure 4.2: Prioritization of the PVCs against *P. koreensis* using reverse vaccinology. (a) Protein virulence prediction using VFDB (b) Virulence factor classes of the virulent proteins (c) Prediction of subcellular localization of virulent proteins (d) Transmembrane helix prediction of membrane and extracellular proteins (e) Antigenicity analysis of proteins with transmembrane helix <2 (f) Essentiality of proteins with antigenicity ≥0.4

4.2.5 Vaccine Targets

A total of eight proteins met all the filters and were selected as potential vaccine candidates (PVCs) for the development of subunit vaccines (Table 4.2). These proteins are FliL, EpsE, FleS, EmhA, MucD, FliN, AlgW, and SugC. Among these proteins, three are involved in bacterial adherence, two in antiphagocytosis, and one each contributed to immune evasion, efflux pump, and cell surface components. It is noteworthy that the majority of these proteins are situated within the inner membrane, with only two being located in the periplasmic space. The antigenicity of these proteins ranged from 0.4398 to 0.8306, showing they are antigenic in nature. Particularly, EmhA had the highest antigenic scores (0.8306).

ExPASy ProtParam server was applied to determine the vaccine candidates' physicochemical behaviour (Table 4.3). The molecular weight of individual proteins ranged from 16.35 kDa to 50.50 kDa, which is ideal because vaccine development has typically focused on smaller proteins (less than 110 kDa). Notably, these proteins displayed a high aliphatic index, ranging from 90 to 108.89, indicating these proteins could be stable across a broad thermal range. The instability index is computed to be in the range between 27.73 and 39.22 (< 40), so these peptides are classified as stable proteins. Finally, the estimated half-life for all of them is estimated to be more than 10 hours in *E. coli* (*in vivo*), suggesting that these proteins have expression capability in prokaryotic expression systems.

Table 4.2: Information about the PVCs

Protein	Protein name	Gene encoded	Virulence Class	Localization	Transmembrane helix	Antigenicity
MCM8743768.1	flagellar basal body-associated protein FliL	<i>fliL</i>	Adherence	Inner membrane	1	0.5644
MCM8744397.1	sugar transferase	<i>epsE</i>	Immune evasion	Inner membrane	1	0.4942
MCM8743780.1	ATP-binding protein	<i>fleS</i>	Adherence	Inner membrane	0	0.5379
MCM8744539.1	efflux RND transporter periplasmic adaptor subunit EmhA	<i>emhA</i>	Efflux pump	Inner membrane	0	0.8306
MCM8743049.1	DegQ family serine endoprotease	<i>mucD</i>	Antiphagocytosis	Periplasmic	0	0.4398
MCM8743766.1	flagellar motor switch protein FliN	<i>fliN</i>	Adherence	Inner membrane	0	0.6833
MCM8744045.1	Do family serine endopeptidase	<i>algW</i>	Antiphagocytosis	Periplasmic	1	0.5662
MCM8740928.1	ABC transporter ATP-binding protein	<i>sugC</i>	Cell surface components	Inner membrane	0	0.4409

Table: 4.3: Physicochemical characteristics of the eight potential vaccine proteins

Protein	Molecular weight (kDa)	Instability index	Aliphatic index	Half-life
FliL	18.09	39.07	100	>10 h in <i>E. coli</i>
EpsE	28.12	32.03	90	>10 h in <i>E. coli</i>
FleS	43.56	37.39	102.02	>10 h in <i>E. coli</i>
EmhA	41.02	37.05	90.73	>10 h in <i>E. coli</i>
MucD	50.50	35.24	100.17	>10 h in <i>E. coli</i>
FliN	16.35	27.73	108.37	>10 h in <i>E. coli</i>
AlgW	40.74	34.28	102.08	>10 h in <i>E. coli</i>
SugC	37.60	39.22	108.89	>10 h in <i>E. coli</i>

4.3 Vaccine Construction

4.3.1 Primer Design

A total of eight specific primers were designed to amplify the eight target genes of the vaccine candidates from the genomic DNA of *P. koreensis* (Table 4.4). To be able to clone the target genes into pET-32a (+) vector, the gene sequences had to be modified with primers to contain appropriate restriction sites. The restriction sites used in this study were selected on the rationale that restriction sites had to be present in the pET-32a (+) vector but absent in the pGEMT-Easy cloning vector. Furthermore, the chosen restriction enzymes would not cleave the target gene sequences. As a result, four restriction sites-BamHI, HindIII, KpnI, and XhoI-were strategically employed in three combinations: BamHI/ XhoI, KpnI/ HindIII, and BamHI/ HindIII.

The length of primers was in the range between 20 and 30 base pairs. The GC content of forward and reverse primers was designed in such a way within the range of 40 to 60%. There are no secondary structures or self-complementary conditions predicted to occur

within these primers by using OligoAnalyzer™ Tool. The difference between melting temperatures of forward and reverse primers was not more than 5°C.

Table 4.4: The list of restriction-site-introducing primers used for PCR amplification of the eight candidate vaccine target genes. The restriction enzyme sites are underlined and letters in bold indicate additional base pair for optimal restriction digestion

Gene		5'-3'	Restriction site	Locus Tag	Annealing temperature (°C)	Expected Amplicon size (bp)
<i>fliL</i>	F	AGT <u>GGATCC</u> ATGGCGAAGAGCGAAGCAGCA	BamHI	NFC82_22445	64.0	501
	R	GGT <u>CTCGAG</u> CTGCAGTACGAAATTAGTGAAAAGCAACTG	XhoI			
<i>epsE</i>	F	AAGGAATC ATGACTGGACATGAGCAAGGTGTACCG	KpnI	NFC82_25630	64.0	744
	R	TTT AAGCTT GCTTGCTCCGTGGCCGCTGAAT	HindIII			
<i>algW</i>	F	AAGGTACC ATGCTCAAGGCGCTGCGTTTTT	KpnI	NFC82_23855	66.0	1152
	R	TTT AAGCTT CTCTTCTTTGACCGGCGCC	HindIII			
<i>emhA</i>	F	AAGGTACC ATGCAATTCAAGCCAGCTGTTACCGCTC	KpnI	NFC82_26360	66.0	1155
	R	TTT AAGCTT CTCCCCTTTGCCGCCAGCGG	HindIII			
<i>mucD</i>	F	AGT <u>GGATCC</u> ATGTCGATACTTAGCTTGAAGTCTT	BamHI	NFC82_18770	64.0	1428
	R	GGT <u>CTCGAG</u> TTTCAGCCAGTTTGAAGGTGA	XhoI			
<i>fliN</i>	F	AGT <u>GGATCC</u> ATGATGAACGACGAAATGAACGCCCAAG	BamHI	NFC82_22435	66.0	459
	R	TTT AAGCTT GCGCAGCTTCTTGATGCGTTTCGC	HindIII			
<i>fleS</i>	F	AGT <u>GGATCC</u> ATGTCTTCTGCCTCCAATGCCGAGG	BamHI	NFC82_22505	70.0	1191
	R	CCC AAGCTT CTCAGCTCCCTGTGCAGCGGAAAA	HindIII			
<i>sugC</i>	F	AAGGTACC ATGACTGGTCTGATTCTGGAAAACGTC	KpnI	NFC82_07945	70.0	1053
	R	TTT AAGCTT AGCCCCCAACACCCGACATG	HindIII			

4.3.2 PCR Amplification

All eight gene-specific primers were utilized for gradient PCR optimization to determine the optimum annealing temperature for each gene (Table 4.4). The PCR products were resolved on 1.5% agarose gel for 90V for 60 minutes, followed by post-staining of the gel in ethidium bromide solution for 20 min. The gel was then visualized under a UV transilluminator to verify the presence of the target genes. A discrete single band was found in all amplifications and the size was consistent with the theoretical size of each of the candidate vaccine target genes (Figure 4.3).

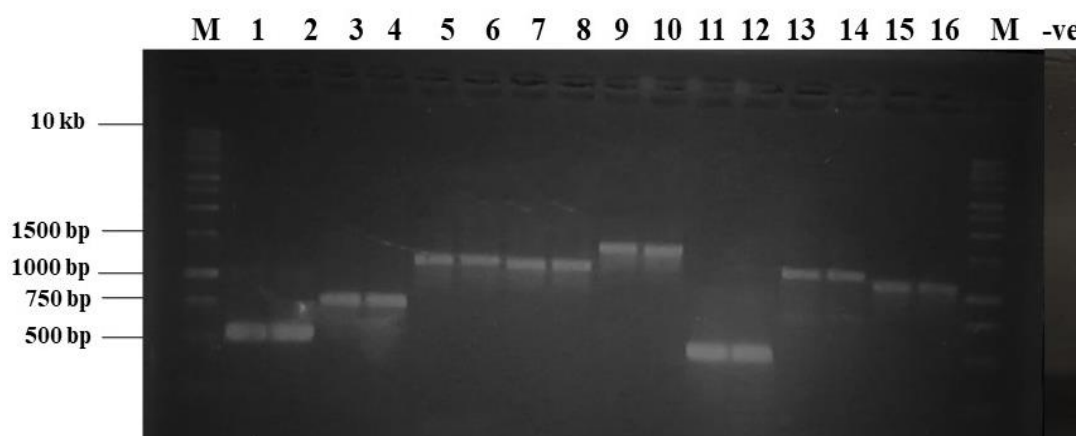


Figure 4.3: PCR amplification of eight candidate vaccine target genes. Lane M: Promega 1 kb DNA ladder; Lane 1-2: *fliL*; Lane 3-4: *epsE*; Lane 5-6: *fleS*; Lane 7-8: *emhA*; Lane 9-10: *mucD*; Lane 11-12: *fliN*; Lane 13-14: *algW*; Lane 15-16: *sugC*; -ve: Negative control

4.3.3 Cloning and Subcloning of the Eight Candidate Vaccine Target Genes

The amplified products of the selected candidate vaccine target genes were first cloned into pGEM-T Easy vector (Promega, USA) and sequenced accordingly. Sequencing was conducted from two directions using a T7 and SP6 universal primer. From the sequencing file, the existence of target DNA sequences was identified by locating their respective restriction sequences. After that, each sequence was then aligned to their

corresponding reference gene sequences, and results revealed that the positive clones showed that the length and sequence of each gene was consistent with the theoretical prediction, and the reading frame was correct (without insertion or deletion in the nucleotide sequenced that may interfere with the expression). Then, these recombinant pGEM®-T Easy vector constructs with the candidate vaccine target genes inserts were double digested using respective restriction enzymes. Figure 4.4 shows two distinct bands were observed in each lane: one band corresponds to the size of the vector which is about 3000 bp and the other band corresponds to the size of each of the candidate vaccine target genes (ranging from 459 bp to 1428 bp).

Subsequently, each of the candidate vaccine target gene inserts was subcloned into pET-32a (+) (Novagen, Germany). The recombinant plasmids were prepared for the positive clones and sequencing was done once again which confirmed that the gene cloned was correct and in frame with the vector. The recombinant plasmids were then introduced into *E. coli* BL21 (DE3) (Figure 4.5). The large number of transformants (200-300) seen in these plates indicates that these cells contain high levels of transformation efficiency. Two random colonies for each gene were then subjected to colony PCR and restriction digestion in order to identify the clones that harbor recombinant plasmid. Figure 4.6 shows the amplicons generated through colony PCR on 1.5 % agarose gel. All colonies generated bright bands with the expected size. As seen in Figure 4.7, there were two different sizes of cleaved fragments in each lane: one at approximately 6000 or just under, and the other at somewhere between 450 and 1500, which corresponds to the size of each of the candidate vaccine target genes (ranging from 459 bp to 1428 bp). Finally, clone 1 from each of the eight recombinant plasmids was chosen for expression study.

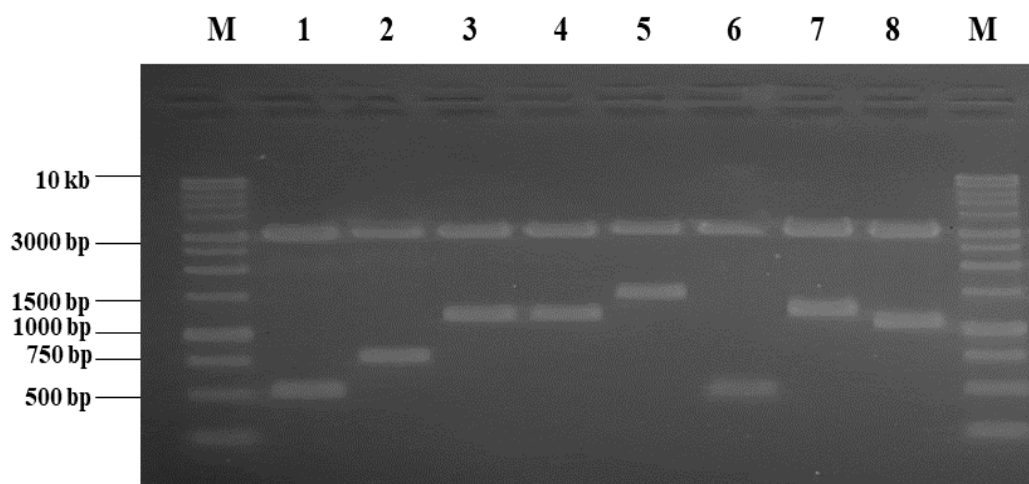


Figure 4.4: Agarose gel electrophoresis of the recombinant plasmid (pGEM-T Easy vector with target genes) digested by restriction enzymes. Lane M: Promega 1 kb DNA ladder; Lane 1: BamHI and XhoI digested pGEM-T Easy vector with *fliL*; Lane 3-4: KpnI and HindIII digested pGEM-T Easy vector with *epsE*; Lane 5-6: BamHI and HindIII digested pGEM-T Easy vector with *fleS*; Lane 7-8: BamHI and Xho I digested pGEM-T Easy vector with *mucD*; Lane 9-10: BamHI and HindIII digested pGEM-T Easy vector with *fliN*; Lane 11-12: KpnI and HindIII digested pGEM-T Easy vector with *algW*; Lane 13-14: KpnI and HindIII digested pGEM-T Easy vector with *sugC*

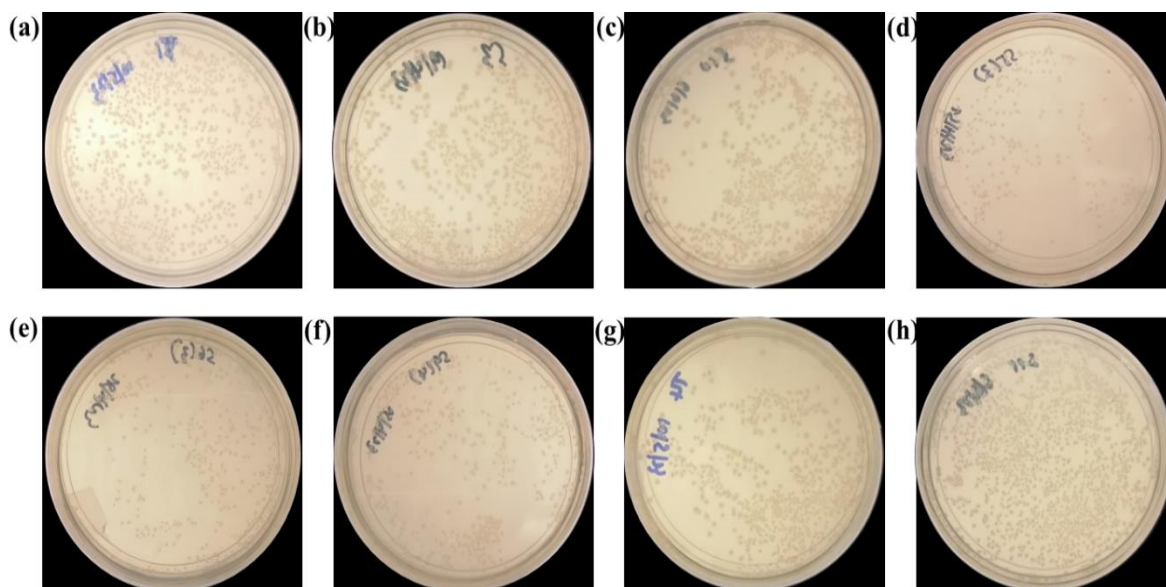


Figure 4.5: Agar plates showing the transformed *E. coli* BL21 (DE3) containing recombinant plasmid of (a) pET-32a-*fliL* (b) pET-32a-*epsE* (c) pET-32a-*fleS* (d) pET-32a-*emhA* (e) pET-32a-*mucD* (f) pET-32a-*fliN* (g) pET-32a-*algW* and (h) pET-32a-*sugC*

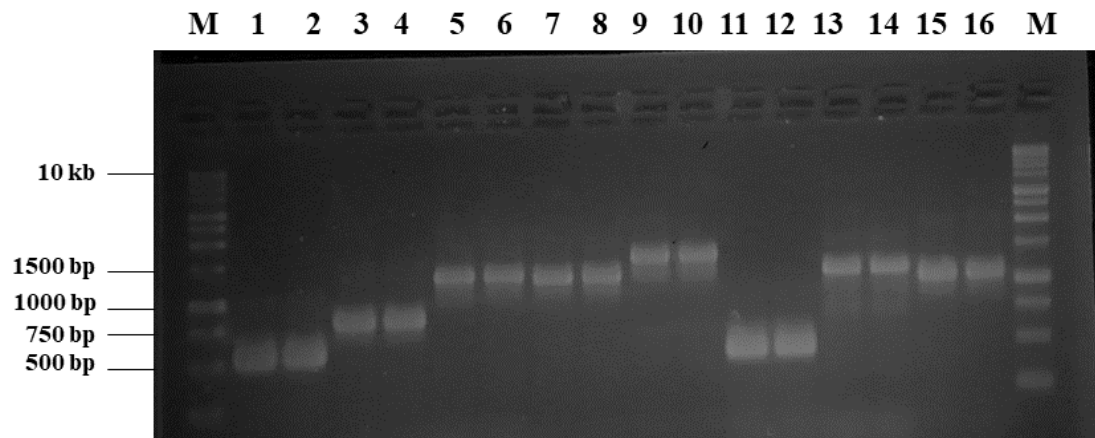


Figure 4.6: Colony PCR results obtained from transformed colonies of *E. coli* BL21 DE3 cells. Lane M: Promega 1 kb DNA ladder; Lane 1-2: *fliL*; Lane 3-4: *epsE*; Lane 5-6: *fleS*; Lane 7-8: *emhA*; Lane 9-10: *mucD*; Lane 11-12: *fliN*; Lane 13-14: *algW*; Lane 15-16: *sugC*

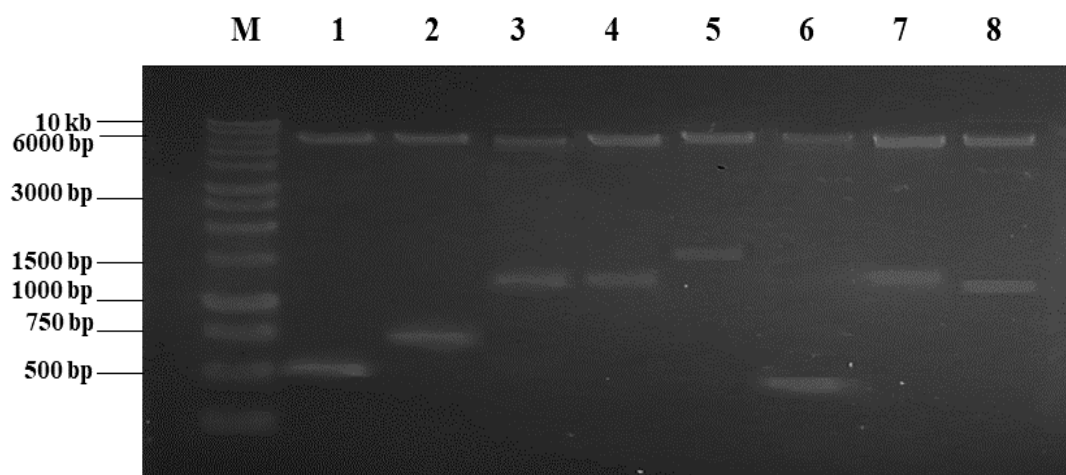


Figure 4.7: Agarose gel electrophoresis of the recombinant plasmid (pET-32a vector with target genes) digested by restriction enzymes. Lane M: Promega 1 kb DNA ladder; Lane 1: BamHI and XhoI digested pET32a vector with *fliL*; Lane 3-4: KpnI and HindIII digested pET32a vector with *epsE*; Lane 5-6: BamHI and HindIII digested pET32a vector with *fleS*; Lane 7-8: BamHI and Xho I digested pET32a vector with *mucD*; Lane 9-10: BamHI and HindIII digested pET32a vector with *fliN*; Lane 11-12: KpnI and HindIII digested pET32a vector with *algW*; Lane 13-14: KpnI and HindIII digested pET32a vector with *sugC*

4.3.4 Protein Expression

The expression of verified positive clones for each of the recombinant proteins in *E. coli* BL21(DE3) competent cells was performed with IPTG induction. As shown in Figure 4.8, protein expression by IPTG induction was clearly observable compared to that of uninduced cultures. The SDS-PAGE electrophoresis results showed that the molecular weight for each protein was consistent with their predicted weights (including the molecular weight of Trx-His-S Tag fusion protein of the pET-32a which is 20.4 kDa). However, the expected band was not observed in cases of BL21 (DE3) strain harbouring that plasmid of *pet32a-fliL* and *pet32a-mucD* even after optimization of culture condition such as induction concentration, time, and temperature (result not shown). As a result, only six proteins were purified.

The solubility of the six recombinant proteins was tested by the method described in Section 3.3.8.2. The information on the solubility of the protein is important before protein purification. It is necessary to solubilize the protein and perform protein purification under denaturing conditions if the protein was found in the pellet (inclusion body), conversely, soluble proteins are purified under native conditions (without denaturant). Samples of the whole cell, centrifuged supernatant, and corresponding pellet for each culture were treated with reducing sample buffer and heated prior to SDS-PAGE. As shown in Figure 4.8, five protein was observed to be overexpressed in the pellet fraction as insoluble inclusion bodies, including EpsE (Figure 4.8a), FleS (Figure 4.8b), EmhA (Figure 4.8c), AlgW (Figure 4.8e) and SugC (Figure 4.8f). For the purification of these proteins, the samples were solubilized in the presence of a denaturant, 6 M urea. For flagellar motor switch protein FliN, the expression was found in both supernatant and pellet fractions, but the protein band was more intense in the supernatant. Hence, this protein was purified under native conditions.

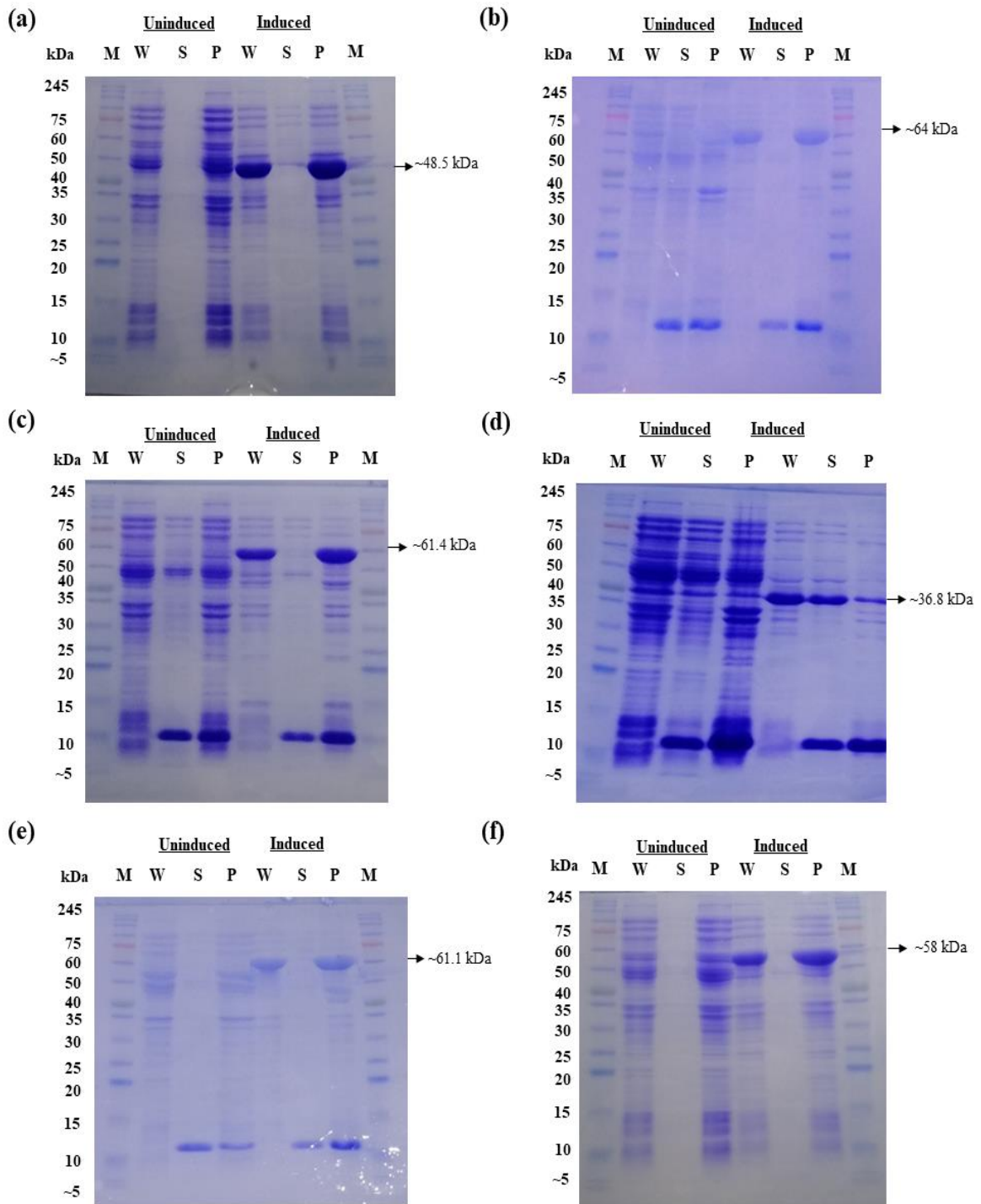


Figure 4.8: SDS-PAGE analysis of recombinant protein expression and solubility in *E. coli* BL21 (DE3). (a) EpsE (b) FleS (c) EmhA (d) FliN (e) AlgW, and (f) SugC. M: ExcelBand™ 3-color Pre-Stained Protein Ladder (3.5-245 kDa); W: Whole cell lysate; S: Supernatant; P: Pellet

4.3.5 Protein Purification

Purification of the six recombinant proteins was done by using a Ni-NTA Spin Kit (Qiagen, USA) under denaturing and native conditions as described in Section 3.3.9. Upon induction with IPTG, proteins were expressed in varying yields (ranging from 2.4 to 3.6 mg per liter of bacterial culture) (Table 4.5). The purified recombinant protein was analyzed by Coomassie brilliant blue staining. As shown in Figure 4.9, The expressed proteins with His-tagged were purified successfully via metal affinity chromatography using Ni-NTA resin.

Table 4.5: Protein yields per liter bacterial culture

Protein	Protein yield per litre
EpsE	4.2 mg
FleS	2.6 mg
EmhA	3.0 mg
FliN	3.6 mg
AlgW	3.4 mg
SugC	2.4 mg

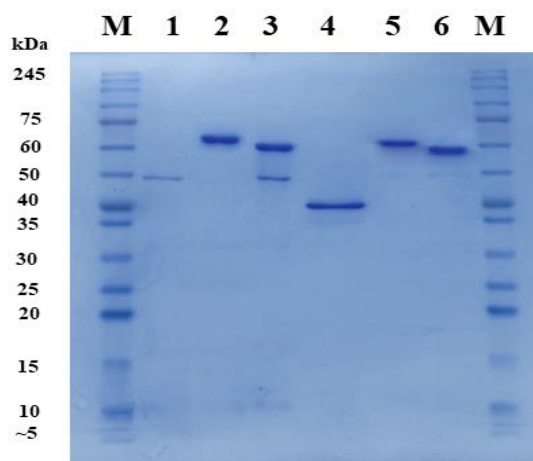


Figure 4.9: SDS-PAGE analysis of purified recombinant proteins. Lane M: ExcelBand™ 3-color Pre-Stained Protein Ladder (3.5-245 kDa); Lane 1: EpsE; Lane 2: FleS; Lane 3: EmhA; Lane 4: FliN; Lane 5: AlgW; Lane 6: SugC

4.3.6 Fish Vaccine Formulation

The six recombinant protein constructs were randomly allocated into two vaccine groups as follows: VacA and VacB (each containing three proteins). Vac A is composed of EpsE, FleS, and EmhA, while Vac B is composed of FliN, AlgW, and SugC. Each vaccine was mixed with FIA in a 1:1 (v/v) ratio.

4.4 Efficacy of the Recombinant Protein Subunit Vaccines

4.4.1 Relative Percentage of Survival (RPS)

The protective efficacy of the subunit vaccine was evaluated by observing the cumulative mortality and calculating the RPS of the fish at 7 dpc. The results of the immunization and challenge experiment are shown in Table 4.6 and Figure 4.10. The fish treated with the subunit vaccines (Vac A or Vac B) did not exhibit any observable adverse clinical signs compared to the control fish. Moreover, no mortalities were observed in either the control or vaccinated cohorts within the 30 dpi monitoring period, except for five fish that received the Vac B injection.

Table 4.6: Experimental setup presenting the different groups, sample size, mortality, and RPS

	Control (PBS)	Adjuvant	VacA	VacB
No. of immunized fish	15	15	15	15
No. of challenged fish	15	15	15	10
Mortality (%)	53.3	40.0	0	10.0
RPS (%)	-	25.0	100.0	81.2

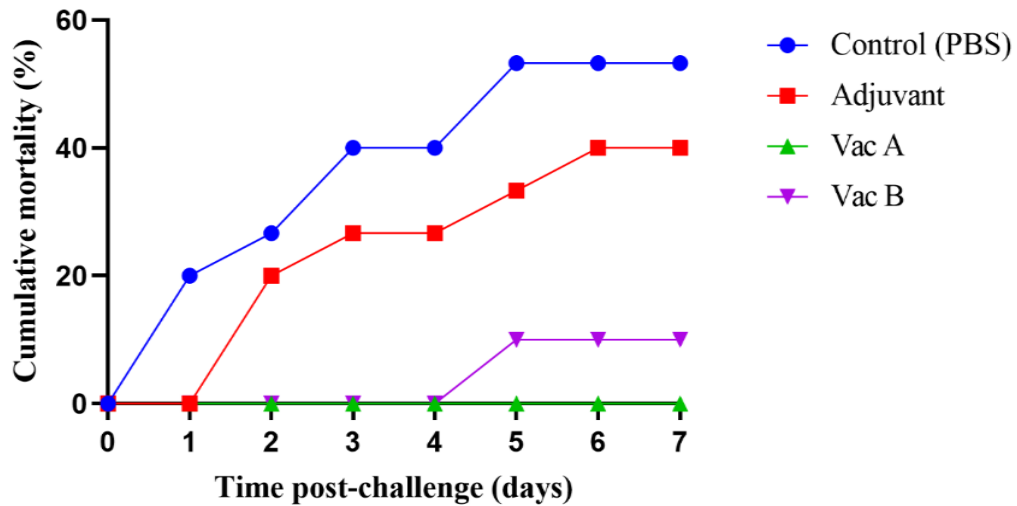


Figure 4.10: Cumulative mortality of control and immunized fish after being challenged with 2.4×10^8 CFU/mL *P. koreensis*

The immunized fish were challenged at 30 dpi with 2.4×10^8 CFU/mL of *P. koreensis*. Mortality of intraperitoneally injected *T. tambroides* in all groups began on the first day of experimental infection and persisted until 6 dpc. Most of the infected fish presented visible hemorrhaging at the body surface and tail rot, while a minority showed abdominal distention, loss of scale, displayed erratic swimming and thigmotaxis behaviors, and reduction in responsiveness from day 2 to day 6 post-challenge (Figure 4.11). Moreover, some of the fish remained without feeding for a few days after the challenge but started feeding normally at 5 dpc. It is worth noting that most of the non-vaccinated fish died within one to three days after the appearance of clinical signs while the vaccinated fish died at least three or more days after showing clinical signs. Furthermore, the fish in the control groups (both PBS and adjuvant injected) manifested most of pathological symptoms than fish in the vaccinated groups, and the severity was much more depicted in the control groups than in the vaccinated group.

The mortalities of PBS-injected control fish and adjuvant-injected control fish started on day 1 and day 2 after being challenged, respectively. The cumulative mortality of

PBS-injected control fish was the highest (53.3%) at 7 dpi followed by adjuvant-only control fish (40%, RPS of 25.0%). While for the vaccinated groups, no mortality was observed in the Vac A immunized fish throughout the challenge period, suggesting this vaccine elicited excellent protective efficacy (RPS of 100%) against *P. koreensis* infection in *T. tambroides*. Vac B also conferred promising protection to the fish, demonstrated by a low mortality rate of 10%, corresponding to a RPS of 81.2%. These results indicated that both vaccines are capable of protecting fish against infection (with Vac A showing better efficacy than Vac B), as evidenced by the relatively lower mortality rates compared to the non-vaccinated groups. No mortality was observed after day 6 for all control and vaccinated groups.

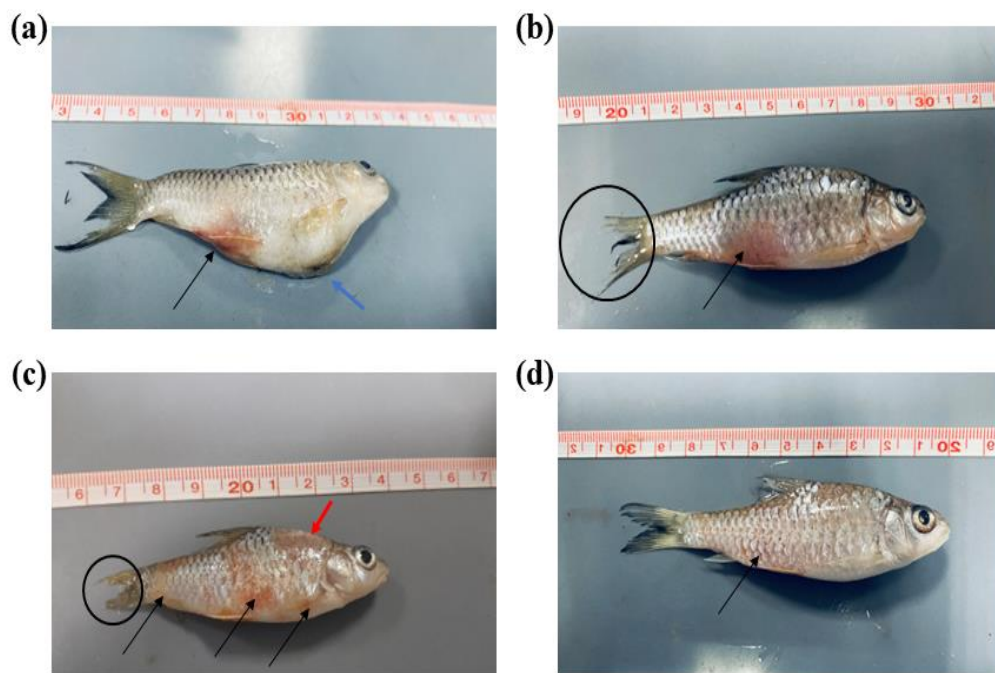


Figure 4.11: Clinical signs of non-vaccinated and vaccinated *T. tambroides* experimentally infected with *P. koreensis*. (a) Non-vaccinated fish showed hemorrhage in the abdomen (black arrow) and abdominal enlargement (blue arrow) (b) Non-vaccinated fish showed hemorrhage in the abdomen (black arrow) and tail rot (black circle) (c) Non-vaccinated fish showed widespread hemorrhages throughout body surface (black arrow), loss of scales (red arrow), and tail rot (black circle) (d) Vaccinated fish (Vac B) showed mild haemorrhage on the skin (black arrow)

4.4.2 Histopathology

Tissue samples including kidney, spleen, gills, and intestine at 7 dpc from both non-vaccinated (PBS) and vaccinated fish (Vac A and Vac B) were collected to determine changes and compare them between the groups to ascertain whether there is a difference in the damage caused by the bacterium (Figure 4.12).

Microscopic examination of the kidney section of non-vaccinated fish after infection with *P. koreensis* showed necrosis in renal tubular cells and interstitial congestion (Figure 4.12a, black arrow 1) while fish immunized with Vac A and Vac B showed almost normal organization of the kidney with many renal tubules and area of hematopoietic tissue in between without significant pathological alterations. In spleen, both non-vaccinated and vaccinated fish after experimental infection showed varying sizes and numbers of melanomacrophage centres (MMCs) (Figure 4.12b, black arrow 1), with the non-vaccinated fish displaying a higher count compared to the vaccinated groups. Notably, the gills demonstrate a significantly higher number of histological alterations in comparison to other organs across both non-vaccinated and vaccinated groups of fish. The prominent changes in the gills of non-vaccinated infected fish were telangiectasia at the tips of secondary lamellae (Figure 4.12c, black arrow 1), complete fusion of secondary lamellae (Figure 4.12c, black arrow 2), and dilatation of the venous sinus at the middle of the primary lamellae (Figure 4.12c, black arrow 3). Vac A immunized fish showed normal histologic limits of the central venous sinus, primary, and secondary lamellae while Vac B immunized fish showed shortening of secondary lamellae and fusion of the adjacent secondary lamellae resulting from hyperplasia of the epithelial cells. Lastly, the intestine of infected non-vaccinated fish showed epithelial necrosis with sloughed necrotic debris in its lumen (Figure 4.12d, black arrow 1) and structural disorder of the lamina propria of the villi with injured central

chylomicron (Figure 4.12d, black arrow 2). Vac A immunized fish presented normal structure of mucosal epithelium, submucosa, and serosa without major pathological alterations while Vac B vaccinated fish showed minor structural disorder of the lamina propria.

Overall, histopathological analysis revealed distinct differences between non-vaccinated and vaccinated fish following infection with *P. koreensis*. Immunized fish (particularly vaccinated with Vac A) displayed milder or fewer pathological changes in the examined organs, indicating the potential efficacy of vaccination in mitigating the observed tissue damage.

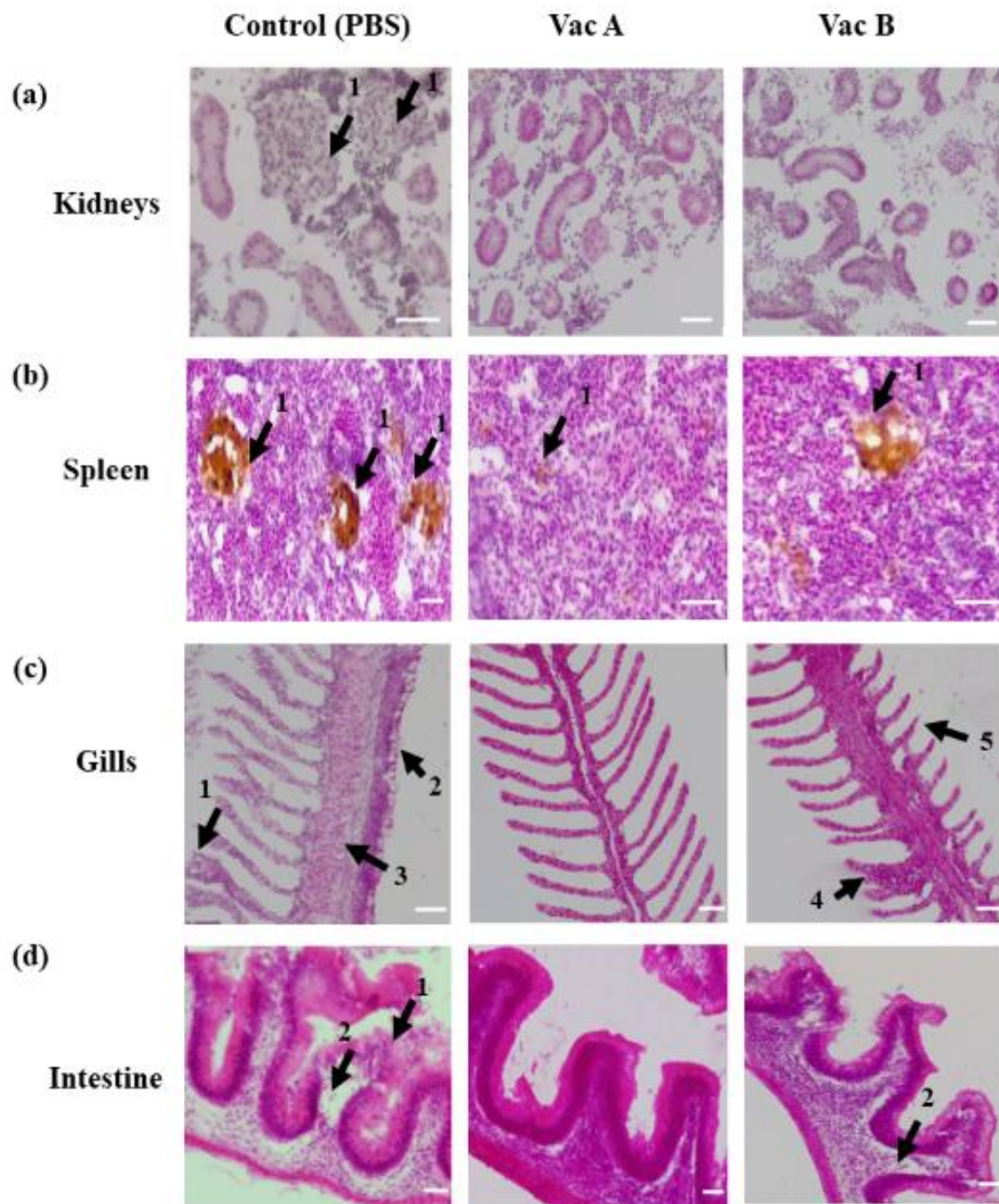


Figure 4.12: Histopathological changes in different tissues of non-vaccinated and vaccinated groups of fish after experimental infection with *P. koreensis*. (a) Kidneys: (1) renal tubular necrosis and interstitial congestion (b) Spleen: (1) Melanomacrophages centres (MMCs) (c) Gills: (1) Intestine (1) telangiectasia at the tips of secondary lamellae (2) total fusion of lamellae and disorganization of secondary lamellae (3) dilatation of the venous sinus of the primary lamellae. (d) Intestine: (1) sloughing of necrotic cells or cellular debris into the lumen (2) injured central chylomicron. Photomicrographs were taken at 100X magnification, and the white bar represents 100 μ m in all micrographs

4.4.3 Analysis of inflammation-related gene expression using RT-qPCR

The relative expression of *TNF- α* , *IL-1 β* , and *IL-8* in the spleen, kidney, gills, and intestine of non-vaccinated (PBS) and vaccinated groups (Vac A and Vac B) was checked using RT-qPCR (Appendices Figure S3 and Figure S4) at 7 dpc (Figure 4.13). The expression level differed from gene to gene in all tissues examined and most genes showed altered expression after infection.

Firstly, *TNF- α* exhibited significant upregulation in all studied tissues of the Vac A- and Vac B immunized fish following experimental infection with *P. koreensis* in comparison to expression levels in the PBS control group (Figure 4.13a). Specifically, the *TNF- α* gene transcript level was upregulated ≈ 3.4 -fold and ≈ 6.8 fold in kidneys, ≈ 5.7 -fold and ≈ 5.4 fold in the spleen, ≈ 2.5 -fold and ≈ 2.1 -fold in gills, and ≈ 2.8 -fold and ≈ 1.9 -fold in the intestine of the Vac A- and Vac B immunized fish, respectively.

Conversely, the mRNA expression of *IL-1 β* exhibited a consistent downregulation in the kidney, spleen, gills, and intestine of both Vac A- and Vac B immunized fish (Figure 4.13b). Specifically, the *IL-1 β* gene transcript level was downregulated ≈ 0.2 -fold in kidneys, ≈ 0.01 -fold and ≈ 0.04 fold in spleen, ≈ 0.03 -fold and ≈ 0.01 -fold in gills, and ≈ 0.2 -fold and ≈ 0.01 -fold in the intestine of the Vac A- and Vac B immunized fish, respectively.

Differential mRNA expression of *IL-8* was observed across tissues in vaccinated fish post-infection (Figure 4.13c). Notably, in the kidney, both Vac A- and Vac B-immunized fish exhibited an upregulation of *IL-8* by 5.4-fold and 2.9-fold. In contrast, the spleen showed a consistent downregulation by 0.27-fold and 0.13-fold in both Vac A- and Vac B immunized fish respectively. Furthermore, while both vaccinated groups displayed upregulation in the gills (5.6-fold in Vac A and 1.3-fold in Vac B) and intestine (87.4-fold

in Vac A and 18-fold in Vac B), statistical significance was exclusively observed in Vac A vaccinated fish.

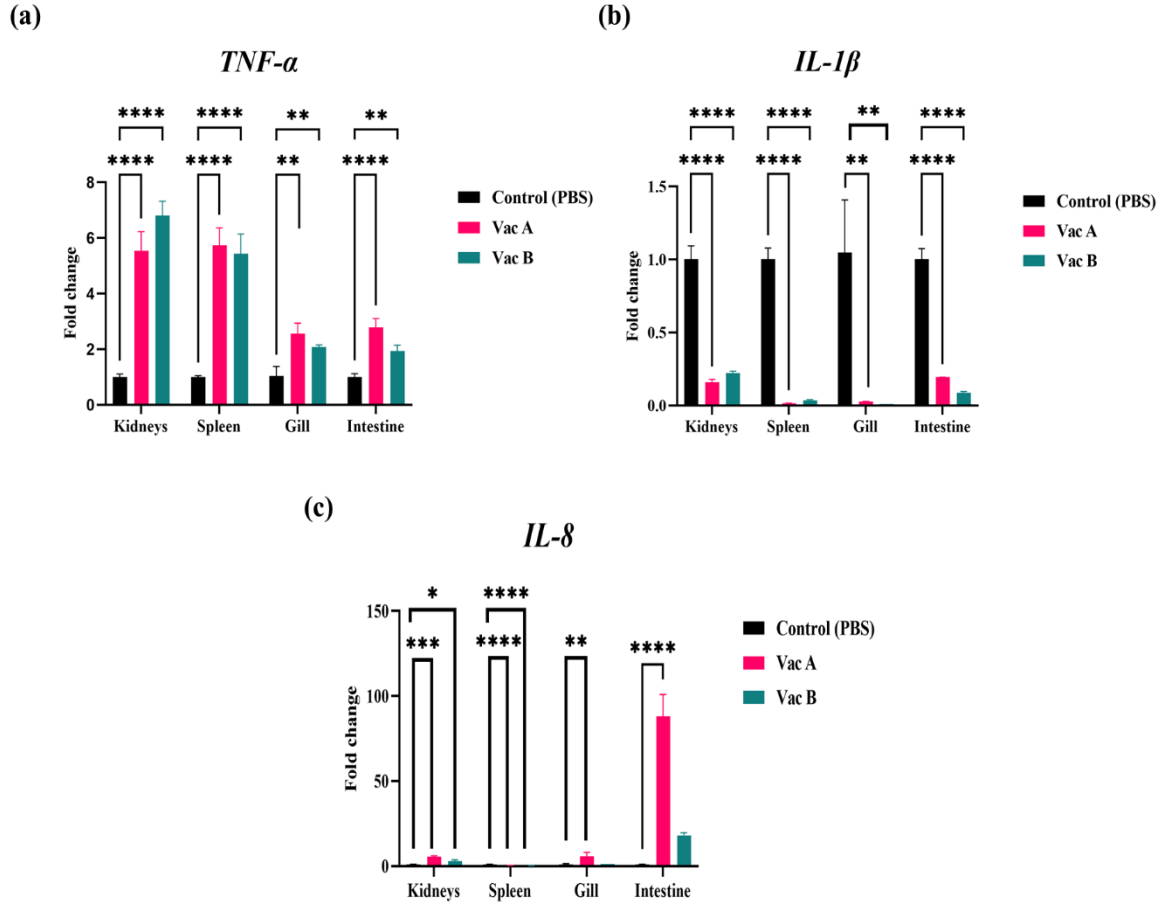


Figure 4.13: The relative gene expression of immune-associated genes (a) *TNF-α* (b) *IL-1β* (c) *IL-8* in kidneys, spleen, gills, and intestine of *T. tambroides* after experimental infection. Results with p<0.05 or lower were designated statistically significant (* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, or **** indicates p<0.0001)

CHAPTER 5

DISCUSSION

5.1 Genomic Features of *P. koreensis* CM-01

Differing from the conventional approach of 'isolate, inactivate, and inject' used in vaccine development (Braggazzi et al., 2018), this study adopted reverse vaccinology, which harnesses genomic data and computational analysis to develop targeted and specific subunit vaccines against *P. koreensis* infection in *T. tambroides*. To the best of our knowledge—this is the first time it has been applied for *P. koreensis*. The reverse vaccinology is a top-down approach that started from the determination of the genome sequence of a pathogen. In this study, the genome of the *P. koreensis* CM-01 was sequenced on the Illumina Novaseq platform. As mentioned earlier, 32 strains of the species *P. koreensis* have been sequenced, and all were found in association with plants, water, and in soil environments, none of which were from fish sources. Notably, the strain *P. koreensis* CM-01 was isolated from the internal organs of diseased *T. tambroides*, representing a distinct deviation from the species' typical environmental habitats. This finding underscores a significant degree of adaptability and versatility within the *P. koreensis* species. Moreover, the isolation of *P. koreensis* CM-01 from a fish host particularly in the diseased state, opens avenues for research into the strain's adaptation, potential pathogenicity, ecological flexibility, and its broader implications for the evolutionary dynamics of the *P. koreensis* species.

The Illumina sequencing has been used successfully in this draft genome project, which yielded 96-fold coverage. This signifies a robust depth of coverage that aids in minimizing errors and inaccuracies in the assembly, contributing to the reliability of the results (Desai et al., 2013). Furthermore, the high level of completeness reflects the accuracy

and comprehensiveness of the assembly (Simão et al., 2015). This high proportion of complete BUSCOs indicates that the majority of expected genes or conserved orthologs are present in the assembly, suggesting that essential genetic elements are intact in *P. koreensis* CM-01. This is crucial for downstream analyses, as a comprehensive representation of the genome allows for more accurate functional predictions and evolutionary studies (Waterhouse et al., 2018). In the present study, based on the genome assembly, the genome size of CM-01 was not different from the average genome size of *P. koreensis* in the NCBI genome database which ranges from 5.9 to 6.99 Mbp, except for *P. koreensis* P19E3, which has a genome size up to 7.5 Mbp and was flagged as contaminated sequences on the database. Furthermore, the GC content of *P. koreensis* CM-01 falls within the typical range observed for other *P. koreensis* strains. According to Ely (2021), bacterial genomic GC content varies from less than 15% to more than 75%. Hence, a GC content of 60% is considered high in this case. As indicated by Aliperti et al. (2023), high GC-content prokaryotes are often associated with adaptability to diverse and challenging environments. This association may be linked to the specific genetic makeup and functional traits encoded by a higher GC content, allowing these organisms to thrive in varying ecological niches, exhibit versatility in resource utilization, and potentially possess more robust defense mechanisms.

5.2 Selection of Potential Vaccine Candidates Criteria

The next step in the reverse vaccinology pipeline after whole genome sequencing involves the bioinformatic analysis of the genomic data obtained to identify high-priority vaccine candidates.

In this study, virulence is one of the most important screening criteria of the vaccine candidate selection as virulent proteins elicit stronger immune responses and initiate critical

disease pathways, making them good targets for vaccine development (Alsowayeh et al., 2022). The systematic screening conducted by VFDB identified substantial potential virulence factors in the *P. koreensis* CM-01. The identification of 177 virulent proteins aligns with previous studies highlighting the diverse nature of virulence factors in *Pseudomonas* species (Minandri et al., 2016; Reboud et al., 2017; Bonneau et al., 2020; Ozer et al., 2021; Yang et al., 2022). The sheer volume of virulence proteins indicates the complexity of pathogenicity in *P. koreensis* CM-01 and suggests the multifaceted nature of its potential pathogenic mechanisms. The distribution, especially with the predominance of adherence-associated proteins (49.72%), highlights the significance of these factors in pathogenesis, as observed in other *Pseudomonas* species like *P. aeruginosa* (Altaee et al., 2022), *P. tructae* (Oh et al., 2019); *P. plecoglossicida* (Jiao et al., 2021; He et al., 2023; Shi et al., 2023).

Assessing the subcellular localization of proteins within bacterial cells is fundamental to deciphering their roles in host interactions. Notably, when focusing on virulence-related proteins, a significant proportion was found to localize in the cytoplasm, aligning with the premise that intracellular processes play crucial roles in the pathogenic mechanisms of bacteria (Sharma et al., 2017). However, these proteins are more suitable as drug targets given their location. The strategic selection of membrane (inner and outer), periplasmic, and extracellular proteins for subsequent analysis underscores the significance of proteins in mediating interactions with the host and initiating immune responses. This aligns with the well-established roles of outer membrane proteins and secretory systems in host-pathogen interactions in gram-negative bacteria (Henderson & Nataro, 2001; Naz et al., 2019). Moreover, the presence of proteins with unknown localization warrants a deeper exploration using advanced methodologies or techniques that can precisely determine the

subcellular localization of these proteins, providing a more holistic understanding of their functions in bacterial physiology and pathogenicity.

The exclusion of proteins with multiple transmembrane domains, MW >110 kDa and instability index >40, aligns with the recognized difficulties in expressing and purifying such proteins within prokaryotic systems (Marius et al., 2010; Rodrigues et al., 2019). Hence, by prioritizing proteins without these structures, the study increases the feasibility of successful recombinant expression and subsequent functional analyses. Also, the selection of antigenic proteins aligns with the significance of antigenic proteins in eliciting host immune responses, crucial for the development of effective vaccines against infectious agents (Monterrubio-López et al., 2015; Alsowayeh et al., 2022). The retention of these proteins that align with DEG entries signifies their potential as fundamental components within the microbial system. Hence, they are less likely to mutate or change over time (Luo et al., 2015) due to their critical role in the pathogen's life cycle. Consequently, vaccines developed against these highly conserved targets are expected to provide longer-lasting protection against the pathogen. Finally, eight proteins have been screened from 5,538 proteins which demonstrated compliance with these essential parameters, ensuring their potential as vaccine candidates.

5.3 Molecular cloning, expression, and purification of the potential vaccine candidates

In this study, a prokaryotic *E. coli* system was used for the expression of recombinant proteins due to its inexpensiveness, and simple and fast high-density cultivation (Lipinszki et al., 2018). It is also a well-characterized and extensively studied organism, with a wealth of genetic tools and molecular techniques available for genetic manipulation and protein

expression (Bhatwa et al., 2021). Furthermore, *E. coli*-derived recombinant proteins are often highly stable and retain their immunogenicity, making them suitable candidates for vaccine development in fish diseases.

The six recombinant proteins (EpsE, FleS, EmhA, FliN, AlgW, and SugC) were successfully expressed upon IPTG induction indicating that T7 promoter controlling the expression of these genes responds appropriately to IPTG, leading to the production of the target proteins. In contrast, two proteins (FliL and MucD) appeared to be toxic for the host cells during expression despite employing different optimization parameters. In the future, further optimization can be undertaken, such as making changes in the expression vector, selecting a different host, and optimizing cell growth conditions and media.

The pET-32a (+) vector that was applied to overexpress the other six recombinant proteins is derived from the pET-32 series which is designed for cloning and high-level expression of peptide sequences fused with the 109 aa Trx•Tag™ thioredoxin protein. The vector also contains a His-Tag which is used for affinity purification. The 6× His-tag facilitates binding to Ni-NTA agarose and allows the immobilization of the protein on metal-chelating substances. NTA is a tetradentate chelating that occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two vacant sites available to interact with the 6× His-tag (Moore et al., 2021). In this study, most of the proteins expressed in the form of inclusion bodies; therefore, they required solubilization to expose their 6× His-tag before purification by affinity chromatography. The flagellar motor switch protein FliN was an exception: expressed in soluble forms and thus purified under native conditions. The 6× His-tag is relatively small in size (~2.5 kDa), possesses low immunogenicity and toxicity, and has minimal effects on the folding of the protein (Zhao et al., 2013), hence its removal is unnecessary.

For purification, the protein yields ranged from 2.4 to 3.6 mg per liter of bacterial culture. The measured expression levels align with the lower range (2–5 mg/liter) specified in the Ni-NTA Spin Kit Handbook (Qiagen). The observed range of protein expression might indicate the need for further optimization in the expression system to achieve higher yields, aiming for improved protein production in future experiments.

5.4 Relative Percentage of Survival (RPS)

In this study, the vaccines were designed to contain three proteins instead of single protein as the combination of multiple recombinant proteins has been showed to induce a more robust immune response, as evidenced by studies conducted by Wilhelm et al. (2006) and Feng et al. (2009). As demonstrated in the study of Wilhelm et al. (2006), the highest protective response with a RPS of 95% was obtained with a vaccine formulation containing FlgG, Hsp60, and Hsp70, and their individual contributions to the survival of the fish was tested by single protein immunization, resulting in RPS of 94%, 88%, and 91%. This suggests that the inclusion of multiple recombinant proteins is needed to obtain a good protective effect against targeted disease.

The results of the study on the protective efficacy of the two formulated subunit vaccines against *P. koreensis* infection in *T. tambroides* exhibit promising outcomes in fish health and survival. FIA was used along with the recombinant proteins in this study as FIA has proven highly effective in fish vaccination and provides a significant reduction in toxicity compared with FCA (Raman & Kumar, 2022). Biswas et al. (2021) showed that recombinant proteins mixed with FIA and intraperitoneal injected provided very strong protection to the fish (survival rate = 80%) against *Aeromonas* disease.

Notably, the lack of mortality within the 30-day monitoring period for both adjuvant-only and vaccinated (Vac A and Vac B) groups, except for a few incidents in the Vac B vaccinated group, indicates the overall robustness of the vaccine candidates. A similar incident was observed in the study conducted by Siti-Zahrah et al. (2003), where aberrant mortality was noted within 24 and 48 hours after vaccination. Besides that, the fish that received the Vac B injection may have had an adverse reaction to the vaccine components which could trigger an exaggerated immune response leading to severe inflammation or an autoimmune reaction in fish.

Upon challenge with *P. koreensis* at 30 dpi, a clear distinction in response to infection between vaccinated and non-vaccinated groups emerged. The non-vaccinated fish experienced rapid mortalities, with notable clinical signs such as hemorrhaging, tail rot, and varied behavioral abnormalities occurring as early as the first-day post-challenge. In contrast, the vaccinated groups displayed delayed onset and reduced severity of symptoms, demonstrating a substantial increase in survival time post-infection. This delayed mortality, as well as the milder manifestation of symptoms in vaccinated fish compared to controls, highlights the effectiveness of the subunit vaccines in conferring protection against *P. koreensis* infection.

The detailed comparison of the mortality rates underscores the superior efficacy of Vac A, which prevented any mortality during the challenge period, resulting in a notable RPS of 100%. Vac A consists of EpsE, FleS, and EmhA. The EpsE serves dual functions by acting both as an enzyme in the synthesis of the biofilm EPS and as a flagellar clutch, inhibiting motility through its interaction with the flagellar rotor. These two functions of EpsE are genetically separable, and through separate and distinct mechanisms, EpsE promotes matrix synthesis and inhibits motility to synergistically stabilize the biofilm (Peng

et al., 2020). EPS and biofilm are the important virulence factors in *Pseudomonas* spp., which enable bacteria to form resilient communities, resist environmental stressors, evade the immune system, and contribute to pathogenicity in the context of infections (Mirghani et al., 2022).

The FleS is part of a two-component signal (TCS) transduction system in *Pseudomonas* spp., utilized to modulate gene expression in response to changing environmental conditions. Generally, the histidine kinase-like ATPase (HATPase) domain in the sensor histidine kinase FleS transduces the signal from the specific stimulus by autophosphorylation at a conserved histidine residue using adenosine triphosphate (ATP) as the substrate. The phosphoryl group is then transferred to a conserved aspartate residue on the response regulator protein, FleR (Zhou et al., 2021). Once activated, FleR then regulates the expression of genes involved in flagellar synthesis, adhesion, swarming, motility, and antibiotic resistance (Sultan et al., 2021; Zhou et al., 2021). As indicated in the study of Sultan et al. (2021), more than 26 genes in *P. aeruginosa* are regulated directly or indirectly by FleS/R.

EmhA is a periplasmic adaptor protein that facilitates the function of the EmhABC efflux pump by acting as an intermediary between EmhB (RND transporter) and the EmhC (outer membrane protein), contributing to the export of toxic substances out of the bacterial cell (Adebusuyi & Foght, 2011). According to Auda et al. (2020) and Colclough et al. (2020), efflux pumps of the Resistance-Nodulation-Division (RND) superfamily contribute to the pathogenesis, virulence, and stress response of bacteria as well as the well-known function, antimicrobial resistance in many Gram-negative bacteria, including *Pseudomonas*.

Vac B, which is composed of FliN, AlgW, and SugC, also exhibited considerable efficacy with a low mortality rate and an RPS of 81.2%. The FliN is one of three flagellar

motor switch proteins (FliG, FliN, FliM) that form the rotor-mounted switch complex (C ring), located at the base of the flagellar basal body of many bacteria. The C-ring is essential for flagellar assembly, rotation, and clockwise (CW)-counterclockwise (CCW) switching (Chang et al., 2021). As indicated in the study of Bouteiller et al. (2021), FliN is present in the most copies (>100) per flagellum, approximately three times greater than the number of its counterparts. In other bacteria, FliN has a critical role in switching, interacting with the chemotaxis signaling molecule phospho-CheY (CheY^P) to trigger or enable the switch to CW rotation, leading to bundle dispersal and reorientation of the cell (Sarkar et al., 2010). However, this is not the case for *Pseudomonas* spp. Bouteiller and his colleagues (2021) mentioned that CheY^P interacts with FliM rather than FliN based on the sequence homology with *E. coli* CheY. The exact function of FliN is yet to be determined, but it has been shown to play a role in providing a binding site for chaperone/substrate complexes before their secretion (Bouteiller et al., 2021). FliN, along with some other proteins, has been found to be less effectively processed or presented to the immune system during an *A. hydrophila* infection in channel catfish, as indicated by the lack of reactivity of the infected catfish serum to this specific protein. The absence of a strong reaction to fliN by the infected catfish serum is probably due to limited access or exposure to the immune system of the infected fish for proper antigen processing and subsequent immune response generation (Yeh et al., 2012).

AlgW is a member of a subgroup of the Do/DegP/HtrA2 family of serine proteases. Generally, *Pseudomonas* spp. synthesizes an exopolysaccharide called alginate in response to environmental conditions. Alginate functions to protect the bacteria from various environmental stresses and aids in the adhesion of bacteria to solid surfaces, which is important for biofilm formation (Gheorghita et al., 2022). The major biological function of AlgW is to detect C-terminal sequence signals that become accessible during stress and to

initiate a proteolytic cascade that inactivates MucA (a negative regulator of AlgU that prevents AlgU from binding to the RNA polymerase for transcription), thereby activating the AlgU transcription factor to synthesis alginate (Cezairliyan et al., 2009).

SugC is one of the components of the trehalose-recycling ABC transporter, working in coordination with other integral parts: the periplasmic sugar-binding lipoprotein LpqY, the transmembrane proteins SugA and SugB. This transporter exhibits high specificity for trehalose uptake. In bacterial cell walls, trehalose has a structural role in adaptive responses to stress such as osmotic differences and extreme temperature (Iturriaga et al., 2009). Previously conducted phylogenetic analyses demonstrated that all the mycobacterial SugC proteins cluster together and these proteins branched out together with homologous proteins from *P. syringae* and *Klebsiella pneumoniae*. This pattern underscores the protein's significance as a key sugar transporter among major bacterial species (Sabharwal et al., 2020). The SugC has been proposed to function as a power source for the SugABC transporter (De la Torre et al., 2021). To date, there have been no comprehensive studies conducted on its functional characterization in SugABC transporter.

To our knowledge, none of these proteins has been tested in a fish vaccine study except FliN. The absence of these proteins as current antigens in fish vaccines suggests unexplored potential for innovation and novel approaches to vaccine formulation. Overall, these results affirm the potential of both vaccines in protecting against *P. koreensis*, with Vac A showing a stronger defense mechanism. To gain a more comprehensive understanding of the safety profile of Vac B, a detailed analysis of vaccine formulations to identify potential triggers for exaggerated immune responses, inflammation, or autoimmune reactions would be needed. Furthermore, the cytokine profiles, antibody titers, and cellular immune

responses in fish that received the Vac B should be investigated to understand how the proteins interact with the fish's immune system.

5.5 Histopathology

The histopathological studies on different target tissues including the kidneys, spleen, gills, and intestine of both non-vaccinated (PBS control) and vaccinated fish (Vac A and Vac B) following infection with *P. koreensis* provide crucial insights into the protective efficacy of vaccination against tissue damage. Fish lack lymph nodes and bone marrow, and leukocyte–antigen interactions take place in immunological sites such as the kidneys, spleen, gills, and intestine (Smith et al., 2019; Mitchell & Criscitiello, 2020). Hence, this work focused on the histological alterations in these few organs.

In teleost fish, the kidney functions similarly to the bone marrow of higher vertebrates and is the largest site of hematopoiesis (Sahoo et al., 2021). In the present study, the non-vaccinated fish displayed significant renal damage characterized by necrosis in renal tubular cells and interstitial congestion. The compromised integrity of renal tubular cells can disrupt vital processes such as filtration, reabsorption, and secretion, all of which are essential for maintaining the internal environment and homeostasis within the fish's body (Flerova et al., 2023). Furthermore, interstitial congestion can impede the blood flow within the kidney, further exacerbating tissue damage and impairing renal function. Conversely, the presence of a nearly normal kidney profile indicated that both subunit vaccines played a pivotal role in protecting the kidney tissue from the damaging effects of the *P. koreensis* infection.

Spleen is the major secondary lymphoid organ in fish. It plays a pivotal role in the induction of adaptive immune responses and contributes significantly to the elimination of immune complexes (Sayed et al., 2022). In this study, both non-vaccinated and vaccinated

fish displayed MMCs in their spleens following experimental infection. However, the key observation was the variability in both the size and the quantity of MMCs between the two groups. The non-vaccinated fish showed a higher count of MMCs compared to the vaccinated groups. MMCs are discrete aggregates of melanomacrophages mainly found in the spleen, head kidney, and liver of teleost fish (Agius & Roberts, 2003; Barst et al., 2015; Steinel et al., 2017). According to Ledic-Neto (2014), these MMCs are typically present in small quantities in healthy fish, but their size and/or number can increase significantly in the presence of a pathological condition or disease. These MMCs serve as sites for antigen trapping and presentation, suggesting their involvement in the adaptive immune response. It is believed that they share functional similarities with mammalian germinal centers (GCs), albeit with distinct characteristics (Kembou-Ringert et al., 2023). Our observation aligns with the established research, affirming their association with the immune response variation between vaccinated and non-vaccinated fish.

The gills, being a primary portal for pathogen entry and a crucial site for immune defense in fish (Yu et al., 2020; Tongsri et al., 2023), showcased a significantly higher degree of changes, highlighting the vulnerability of this organ to infection. Non-vaccinated infected fish displayed severe histological changes in their gills: telangiectasia, fusion of secondary lamellae, and dilatation of the venous sinus indicated substantial disruption to the gill structure, likely impeding the gills' respiratory function and compromising the fish's health (Ramudu et al., 2020). In contrast, the vaccinated groups displayed varying responses. Vac A vaccinated fish exhibited normal gill structures, suggesting a protective effect of this specific vaccine on maintaining the structural integrity of the gills. However, the Vac B vaccinated fish exhibited shortening of secondary lamellae and fusion of adjacent secondary lamellae. This response, although different from the damage seen in non-vaccinated fish,

could potentially affect respiratory efficiency and overall gill function, albeit to a lesser extent. According to Parra et al. (2015), an accumulation of immune cells has been described in trout gills in the interbranchial lymphoid tissue (ILT). Such aggregations are exclusive to gills; other mucosa-associated lymphoid tissues (MALTs) have not demonstrated similar aggregations thus far. IgT⁺ B cells are the main population of B cells in gills and IgT constitutes the main humoral component after infection. This finding provides valuable insights into how vaccines might leverage this specialized immune environment to bolster gill integrity and enhance protective effects.

Similar to gills, the intestine is also the first line of defense against inflammation and pathogen infection (Zhang et al., 2022). It is immunologically active and armored with various immune cell types, including T cells, B cells, granulocytes, and macrophages (Wu et al., 2016). In the present study, the infected non-vaccinated fish showed structural disorder of the lamina propria of the villi with injured central chylomicron. Chylomicrons are essential for the absorption and transport of dietary lipids within the body (Yan et al., 2015). Injury or impairment to these structures could disrupt the lipid metabolism and overall energy balance in the infected fish. Conversely, vaccinated fish showed no or very little abnormality, akin to other organs mentioned above. Upon intraperitoneal immunization, mucosal IgM antibodies are generated, alongside responses from polymeric immunoglobulin receptors (pIgR). The pIgR responses facilitate the transport of IgM-antigen complexes across the intestinal epithelium and into the intestinal mucus, where they can neutralize pathogens and prevent their attachment to the intestinal lining. This process aids in maintaining the structural integrity of the intestine and prevents the development of structural disorders (Du et al., 2022).

Several studies conducted a similar investigation into the effects of vaccination on tissue damage in fish infected with other bacterial pathogens (Silvaraj et al., 2021; Sun et al., 2021; Liu et al., 2022; Monir et al., 2022 Zhang et al., 2023). Notably, these studies, similar to ours, emphasized the potential of vaccination in moderating the immune response and reducing the severity of tissue damage caused by bacterial infections.

5.6 Analysis of inflammation-related gene expression using RT-qPCR

Proinflammatory cytokines and chemokines like *TNF- α* , *IL-1 β* , and *IL-8* are crucial components of host defense against microbial pathogens. They play pivotal roles in recruiting and activating phagocytes at the site of infection, as well as in controlling the balance between the humoral and cellular immune response (Zou & Secombes, 2016; Sakai et al., 2021, Monir et al., 2022).

In the present study, *TNF- α* was significantly upregulated in all the studied tissues of vaccinated fish (Vac and Vac B) following infection with *P. koreensis*. This simultaneous upregulation across diverse organs may imply the systemic nature of the immune response generated by both subunit vaccines, highlighting their efficacy in activating macrophage and phagocyte activity in multiple organs to mount a strong defense against bacterial infection (Zou & Secombes, 2016). Similar phenomena have also been documented in vaccinated blunt snout bream (*M. amblycephala*) challenged with *A. hydrophila* (Zhang et al., 2023), red tilapia (*Oreochromis* spp.) challenged with *Streptococcus iniae* and *A. hydrophila* (Monir et al., 2022), and silver catfish (*Rhamdia quelen*) infected with *A. hydrophila* (de Figueiredo Soveral et al., 2022).

Contrastingly, *IL-1 β* exhibited a marked downregulation in all studied tissues in vaccinated fish after infection. A similar trend has been observed in the study of Khaj et al. (2021). The observed downregulation of *IL-1 β* might be a part of the regulatory effect of *IL-10*. Although the gene expression of *IL-10* was not tested in this study, it has been reported that *IL-10* modulates inflammatory responses and inhibits the production of various cytokines including *IL-1 β* to prevent excessive inflammation or maintain immune homeostasis in the fish upon infection (Popoola et al., 2021; Yang et al., 2021). Future research could focus on directly investigating the involvement of *IL-10* in modulating *IL-1 β* expression during the course of infection.

The differential expression of *IL-8* was observed across tissues in vaccinated fish after infection, with Vac A and Vac B eliciting distinct patterns of *IL-8* expression. The substantial upregulation in the kidney, gills, and intestine for Vac A, particularly with the remarkable 87.4-fold increase in the intestine, implies a robust localized immune response, potentially offering better protection against *P. koreensis*. However, *IL-8* was significantly downregulated in the spleen for both Vac A- and Vac B immunized fish. Previous studies have highlighted the varying expression of chemokine genes in different organs in response to infection (Montero et al., 2011; Bird et al., 2015). The differential magnitude of expression between the two vaccines suggests that Vac A induces a stronger *IL-8*-mediated response compared to Vac B.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The objectives of this study were achieved with the identification and development of the potential vaccine candidates through the reverse vaccinology approach, and subsequent evaluation of the immune responses and protective efficacy of the subunit vaccines against *P. koreensis* infection in *T. tambroides*. In this approach, the sequences of the whole genome of *P. koreensis* CM-01 were examined. WGS analysis revealed that the genomic size of *P. koreensis* CM-01 is 6,171,880 bp and has a G+C content of 60.5%.

The proteome of *P. koreensis* CM-01 comprises 5,538 proteins and was subjected a series of bioinformatics analyses including assessment of virulence, subcellular localization, transmembrane helical structure, antigenicity, essentiality, non-host homology, molecular weight, and stability, resulting in the identification of eight proteins as potential vaccine candidates. These eight candidate vaccine target genes were successfully cloned into pGEM-T and subsequently subcloned into pET 32a (+) vector. However, only six of them were successfully expressed and purified.

The protective properties of these six recombinant protein antigens were tested by *in vivo* challenge experiment. These six recombinant protein antigens were formulated in two mixtures (Vac A: EpsE, FleS, and EmhA; Vac B: FliN, AlgW, and SugC). Both Vac A and Vac B elicited strong protective responses in an experimental challenge against *P. koreensis* (100% and 81.2%, respectively). Histological examination highlighted that immunization with both Vac A and Vac B demonstrated protective effects in the kidneys, spleen, gills, and intestine of fish following *P. koreensis* infection, as indicated by reduced tissue damage

compared to non-vaccinated fish. However, upon comparative evaluation, Vac A demonstrated no adverse effects on the tissues, while Vac B showed mild degenerative changes. The immune-related genes expression analysis revealed significant upregulation of *TNF- α* and downregulation of *IL-1 β* in the kidneys, spleen, gills, and intestine in both Vac A and Vac B immunized fish following *P. koreensis* infection. Additionally, *IL-8* exhibited differential expression across tissues with distinct patterns observed between Vac A and Vac B. While both vaccines modulate immune gene expression, the magnitude of response by Vac A is generally greater, indicating its potentially superior efficacy in activating the immune system against *P. koreensis* infection.

To the best of our knowledge—this is the first time reverse vaccinology has been applied to *P. koreensis*. The overall results observed in this experiment are promising since the two formulated subunit vaccines administered through the intraperitoneal route succeeded in protecting against infection in *T. tambroides*, with Vac A subunit vaccine emerging as a more ideal vaccine option against *P. koreensis*.

6.2 Recommendations

In the study, the two formulated subunit vaccines have been shown to induce protection against *P. koreensis* infection in *T. tambroides*. While the initial results are promising, continued research, including field trials and further optimization, will be essential to fully realize the potential benefits of these vaccines.

Before advancing to field trials, further studies may be needed to assess the long-term durability of protection, and analysis of cellular immune response of the two formulated recombinant subunit vaccines in *T. tambroides*. Specifically, the IgM and IgT (which are

indicative of the humoral immune response) antibody levels in fish sera needs to be measured at multiple time points post-vaccination using enzyme-linked immunosorbent assay (ELISA) to track changes in antibody titers. It helps in detecting any decline in antibody levels, indicating a potential decrease in immunity. This is particularly important for determining the need for booster vaccinations to maintain protection. It is not enough to only look at the antibody-mediated response. The cellular immune response, particularly T-cell mediated immunity, is also vital. Measuring the expression levels of T-cell markers like CD4-1 and CD8 α using real-time PCR will provide insights into the cellular immune responses post-vaccination.

Given the varying degrees of protection that can be achieved with the different preparations, the proper selection of antigens appears crucial for successful protection. Particularly, the protective input of each protein can be evaluated by single protein immunization to confirm their individual contribution to protection. This could help refine the vaccine formulation for enhanced protective efficacy.

On the whole, this study has pioneered the use of reverse vaccinology for the identification and development of subunit vaccines against *P. koreensis*, yielding two formulations with demonstrable protective efficacy in *T. tambroides*. This marks a significant step forward in aquaculture pathogen management. It is hoped that the findings of this study will not only contribute to the development of effective prophylactic strategies for *T. tambroides* but also establish a framework for combating other pathogens through genomic and proteomic-guided vaccine design.

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APPENDICES

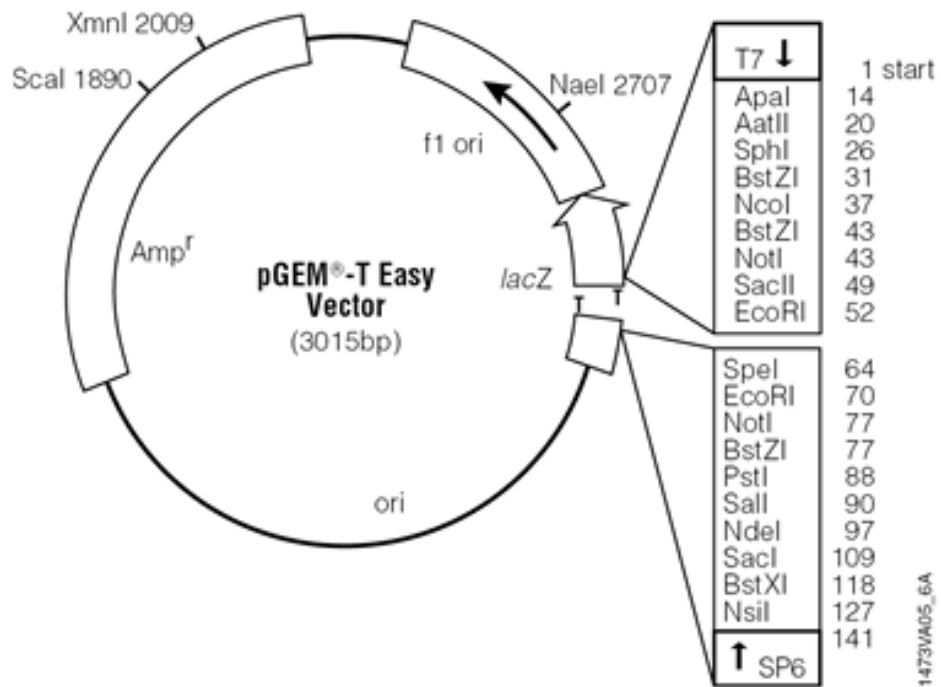


Figure S1: The plasmid map for pGEM-T Easy Vector.

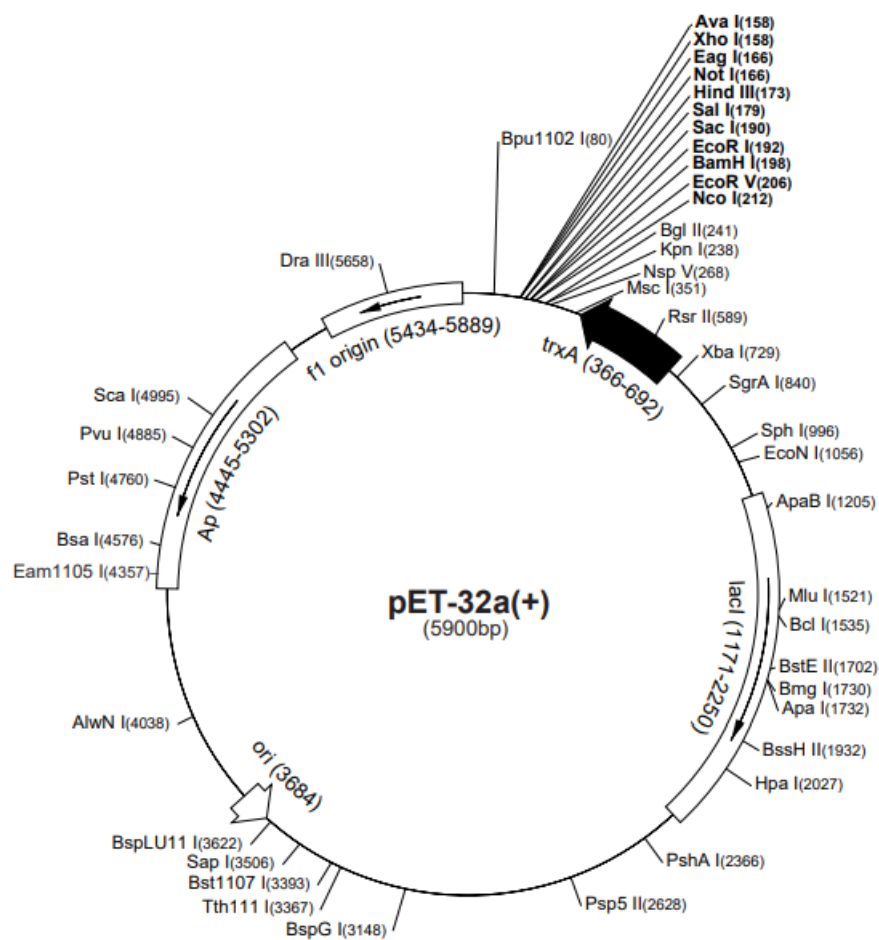


Figure S2: The plasmid map for pET32a (+).

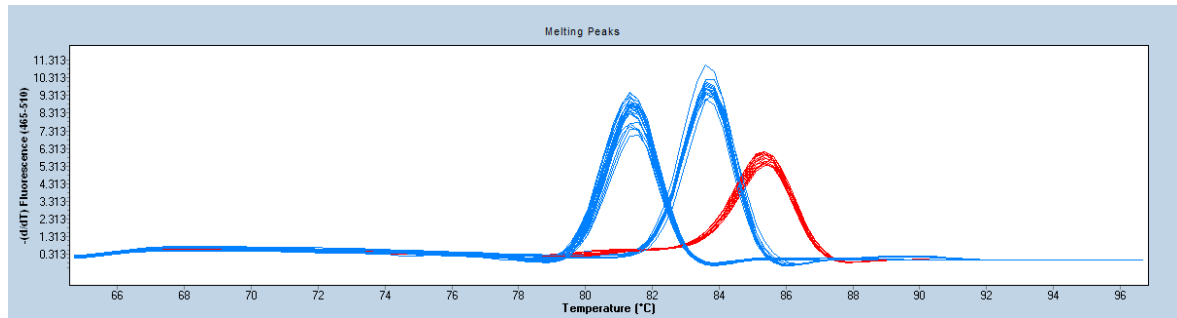
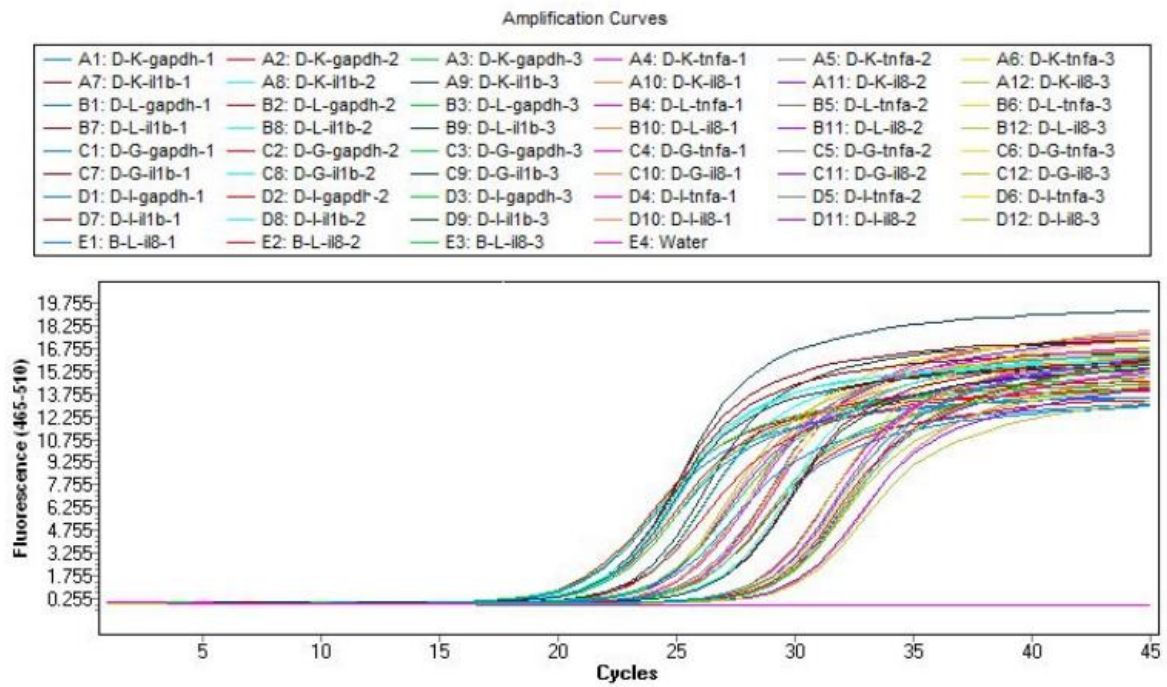


Figure S3: qPCR melt curve plot for four different primer pairs. First peak represents *TNF- α* and *IL-1 β* ; second peak represents *IL-8*, and third peak represents *GAPDH*.

(a)



(b)

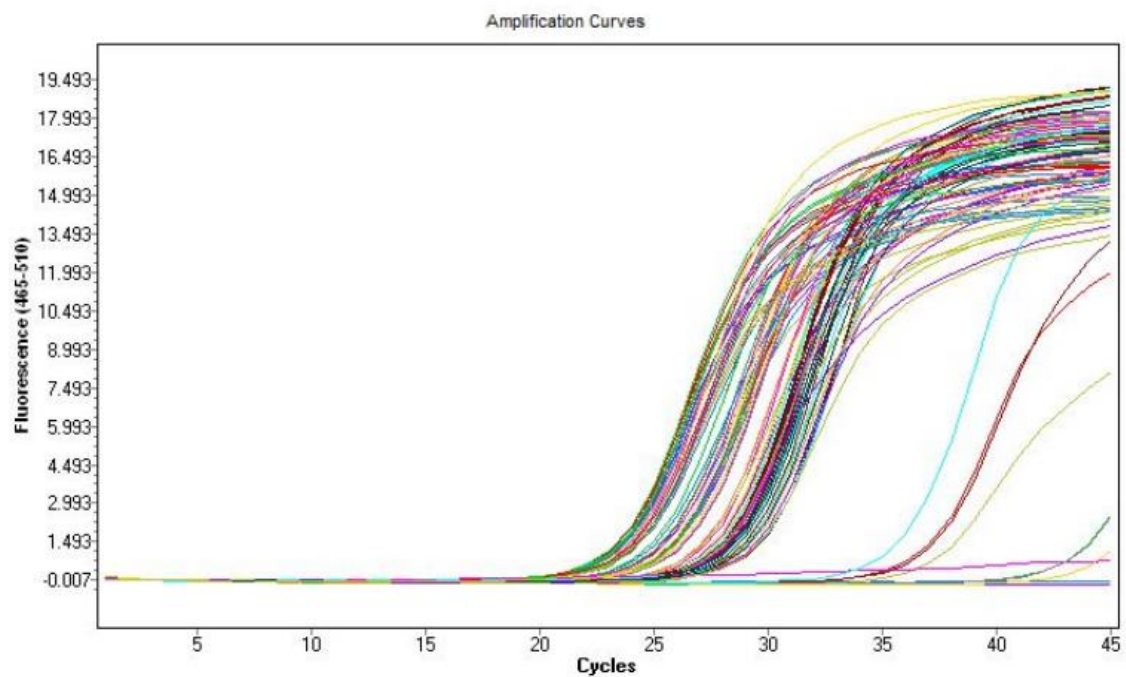


Figure S4: qPCR amplification plot of (a) Control (PBS) and (b) Vac A-and Vac B immunized fish samples.