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**Antibiotic Use in Shrimp Culture in the Mekong Delta, Vietnam:
Pharmacokinetics, Pharmacodynamics and Impact on Shrimp and
Consumer Health**

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Abbreviations

AHPND	Acute hepatopancreatic necrosis disease
ADI	Acceptable daily intake
ASC	Aquaculture Stewardship Council
AUC	Area under the plasma concentration–time curve
CFT	Cefotaxime
Cl	Clearance
C _{max}	Maximum concentration
DOX	Doxycycline
EDI	Estimated daily intake
EMA	European Medicine Agency
FF	Florfenicol
Global GAP	Global Good Agricultural Practices
HQ	Hazard Quotient
IS	Internal standard
k _a	Absorption rate constant
k _{el}	Elimination rate constant
LC ₅₀	Median lethal concentration, i.e. the concentration expected to cause death in 50% of the exposed organisms
LD ₅₀	Median lethal dose, i.e. the dose expected to cause death in 50% of the exposed experimental animals
MARD	Vietnamese Ministry of Agriculture and Rural Development

MHLW	Japanese Ministry of Health, Labor and Welfare
MIC	Minimum inhibitory concentration
MRL	Maximum residue limit
PD	Pharmacodynamics
PK	Pharmacokinetics
PK/PD	Pharmacokinetics/Pharmacodynamics
RASFF	Rapid Alert System for Food and Feed
$T_{1/2ab}$	Absorption half-life
$T_{1/2el}$	Elimination half-life
T_{max}	Time to maximal plasma concentration
VASEP	Vietnam Association of Seafood Exporters and Producer
V_d	Volume of distribution
WFD	White feces disease
WSD	White spot disease
WSSV	White spot syndrome virus
WT	Withdrawal time

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Résumé - Abstract

Résumé

L'élevage de crevettes blanches du Pacifique (*Penaeus vannamei*), également appelées crevettes à pattes blanches, s'est fortement développé au Vietnam au cours des dernières décennies, en particulier dans le delta du Mékong, où la production de crevettes contribue de manière significative au secteur aquacole. Cependant, l'aquaculture intensive de crevettes est souvent associée à l'utilisation de divers produits chimiques et antibiotiques pour le contrôle des maladies et la gestion des élevages. L'utilisation inappropriée des antibiotiques, notamment l'administration de doses élevées et le non-respect des temps d'attente, peut entraîner la présence de résidus d'antibiotiques dans les produits de crevettes ainsi qu'une augmentation de l'antibiorésistance, soulevant des préoccupations en matière de sécurité des aliments et de santé publique. Dans ce contexte, la présente thèse visait à étudier l'utilisation des antibiotiques en aquaculture de crevettes et à déterminer la pharmacocinétique ainsi que la déplétion de certains antibiotiques chez la crevette à pattes blanches.

La première partie de ce travail a examiné l'utilisation des produits chimiques et des antibiotiques dans les systèmes d'élevage super-intensifs de crevettes blanches dans le delta du Mékong, au Vietnam. Une enquête auprès de 210 exploitations a été réalisée afin d'étudier les pratiques d'élevage, l'utilisation des produits chimiques et l'occurrence des maladies. Les résultats ont montré qu'un large éventail de produits chimiques était utilisé pendant les phases de préparation des bassins et de grossissement, principalement à des fins de désinfection. Les probiotiques étaient également largement appliqués par les éleveurs pour améliorer la qualité de l'eau et la digestibilité des aliments. La nécrose aiguë hépatopancréatique et la maladie des fèces blanches étaient les maladies les plus fréquemment rapportées. Les éleveurs utilisaient plusieurs antibiotiques, principalement le céfotaxime, la DOX et l'oxytétracycline, pour contrôler les infections bactériennes. Il est à noter qu'un petit nombre d'éleveurs a également signalé l'utilisation d'antibiotiques interdits au Vietnam tels que l'énrofloxacin et la ciprofloxacine. Ces résultats suggèrent que l'utilisation des antibiotiques en aquaculture de crevettes reste une problématique importante et que de meilleures pratiques de gestion sont nécessaires afin d'assurer un usage responsable et la sécurité des aliments.

Sur la base des résultats de l'enquête, plusieurs antibiotiques couramment utilisés en élevage de crevettes ont été étudiés plus en détail afin de déterminer leur pharmacocinétique et leur temps d'attente. La DOX a été administrée par voie orale à une dose de 20 mg/kg de poids corporel. Les concentrations ont été mesurées dans l'hémolymphe, l'hépatopancréas et le muscle. Les résultats ont montré une absorption et une distribution rapides, avec des concentrations les plus élevées observées dans l'hépatopancréas ($C_{\max}$ de 2,37 $\mu\text{g/g}$ à un $T_{\max}$ de 0,23 h). Sur la base de l'exposition observée dans les tissus, la DOX a montré un potentiel limité pour atteindre une efficacité antibactérienne contre *V. parahaemolyticus* à la dose étudiée. Le temps d'attente dans le muscle variait de 8 à 10 jours selon le

schéma d'administration. L'analyse histologique a indiqué que l'administration de DOX provoquait des modifications temporaires de l'hépatopancréas, mais que sa structure se rétablissait dans les sept jours suivant l'arrêt du traitement.

Le florfénicol a également été étudié dans des conditions expérimentales similaires à une dose de 10 mg/kg de poids corporel. Comme pour la DOX, il a montré une absorption rapide et des concentrations plus élevées dans l'hépatopancréas. Les résidus dans le muscle ont diminué rapidement et sont passés sous la limite de quantification dans les 24 heures suivant l'arrêt du traitement. Sur la base de la limite maximale de résidus de 100 µg/kg, le temps d'attente a été estimé à environ deux jours à une température de l'eau de 26,5°C. Cependant, les concentrations mesurées dans le plasma et l'hépatopancréas étaient inférieures à celles nécessaires pour inhiber efficacement *Vibrio parahaemolyticus*, suggérant que la dose étudiée pourrait être insuffisante pour le traitement. Dans le plasma, la $C_{\max}$ atteignait 60,56 µg/L à 1,77 h, tandis que dans l'hépatopancréas, une $C_{\max}$ plus élevée de 386,92 µg/kg était observée à 0,19 h. Les observations histologiques n'ont révélé aucun effet négatif après l'arrêt du traitement.

La pharmacocinétique et la déplétion des résidus de céfotaxime ont également été étudiées après administration orale à une dose de 25 mg/kg de poids corporel. Comme pour les autres antibiotiques, la distribution dans les tissus était rapide, avec des concentrations plus élevées dans l'hépatopancréas ($C_{\max}$ de $19,45 \pm 2,10$ mg/kg à un $T_{\max}$ de $1,00 \pm 0,20$ h). Sur la base de la concentration minimale inhibitrice de *Vibrio parahaemolyticus*, les résultats suggèrent qu'une administration quotidienne pourrait être efficace pour le contrôle des maladies bactériennes. Le temps d'attente a été estimé entre environ 2,0 et 77,7 heures, en fonction de la limite maximale de résidus (LMR) applicable et du schéma d'administration, lorsque les crevettes recevaient un aliment médicamenteux pendant trois jours consécutifs. L'analyse histologique n'a montré aucun dommage détectable au niveau de l'hépatopancréas.

Enfin, la présence de résidus d'antibiotiques dans des produits commerciaux de crevettes ainsi que le risque potentiel pour les consommateurs ont été évalués. Trente échantillons ont été collectés sur des marchés et supermarchés à Cần Thơ et analysés par LC-MS/MS. Cinq antibiotiques ont été détectés, notamment la DOX, l'oxytétracycline, l'énrofloxacin, la ciprofloxacine et le sulfaméthoxazole. Parmi eux, la DOX présentait les concentrations les plus élevées. Sur la base des données de consommation de crevettes collectées auprès des consommateurs locaux, l'apport quotidien estimé, dans des conditions de forte consommation (P95), variait de 0,002 à 0,241 µg/kg de poids corporel par jour. Ces valeurs étaient nettement inférieures aux doses journalières admissibles, indiquant un risque négligeable pour les consommateurs. Néanmoins, la présence de résidus dans certains échantillons souligne la nécessité de poursuivre la surveillance de l'utilisation des antibiotiques et des résidus dans les produits de crevettes.

Dans l'ensemble, cette thèse apporte des informations complémentaires sur les pratiques d'utilisation des antibiotiques en élevage de crevettes dans le delta du Mékong, ainsi que sur la pharmacocinétique, la déplétion des résidus et les effets potentiels de certains antibiotiques chez la crevette à pattes blanches. Elle évalue également la présence de résidus d'antibiotiques dans les produits de crevettes et l'exposition alimentaire associée. Ces résultats peuvent contribuer à améliorer la gestion des antibiotiques et la sécurité des aliments dans la production de crevette.

Summary

Whiteleg shrimp (*Penaeus vannamei*) farming has developed rapidly in Vietnam during the last decades, especially in the Mekong Delta, where shrimp production has significantly contributed to the aquaculture sector. However, intensive shrimp aquaculture is often associated with the use of different chemicals and antibiotics for disease control and farm management. The improper use of antibiotics, including high doses and non-compliance with antibiotic withdrawal times, may lead to antibiotic residues in shrimp products and an increase in antimicrobial resistance, raising concerns about food safety and public health. In this context, the present thesis aimed to investigate antibiotic use in shrimp aquaculture and to determine the pharmacokinetics (PK) and depletion of selected antibiotics in whiteleg shrimp.

The first part of this research examined the use of chemicals and antibiotics in super-intensive whiteleg shrimp farming systems in the Mekong Delta, Vietnam. Based on sample-size calculation, a survey involving 210 randomly selected shrimp farming households across seven Mekong Delta provinces was conducted to investigate farming practices, chemical use, and disease occurrence. The results showed that a wide range of chemicals were used during both pond preparation and grow-out stages, mainly for disinfection purposes. Probiotic products were also mostly applied by farmers to improve water quality and feed digestibility. The acute hepatopancreatic necrosis disease and white feces disease were the most frequent diseases reported by farmers. Farmers used several antibiotics, mainly cefotaxime (CFT), doxycycline (DOX), oxytetracycline and florfenicol (FF) to control bacterial infections. It is worth noting that a small number of farmers still reported the use of antibiotics such as enrofloxacin and ciprofloxacin, which are prohibited for use in aquaculture production in Vietnam. These findings suggest that antibiotic use in shrimp farming remains an important issue and that better guidance and management practices are needed to ensure responsible use and food safety.

Based on the survey findings, several commonly used antibiotics in shrimp farming were selected for subsequent PK and withdrawal time investigations. For the PK studies, shrimp were fed antibiotic-medicated feed as a single oral dose, and tissue samples were collected at different time points up to 24 h after administration. For residue depletion studies, shrimp were fed medicated feed once or twice daily for three consecutive days before withdrawal time determination. Doxycycline was investigated at a dose of 20 mg/kg body weight. The DOX concentrations were measured in hemolymph, hepatopancreas and muscle tissues. The results showed that DOX was rapidly absorbed and distributed in shrimp tissues, with the highest concentrations observed in the hepatopancreas, where the maximum concentration ($C_{\max}$) reached 2.37 $\mu\text{g/g}$ at the time to reach maximum concentration ($T_{\max}$) of 0.23 h. Based on the obtained exposure in shrimp tissues, DOX showed limited potential to achieve effective antibacterial activity against *Vibrio parahaemolyticus* (*V. parahaemolyticus*) at the investigated dose.

The withdrawal time in shrimp muscle ranged from 8 to 10 days at the maximum residue limit (MRL) of 50 µg/kg, depending on the dosing regimen. Histological examination indicated that DOX administration caused temporary changes in hepatopancreatic tissue during treatment, but the hepatopancreas structure recovered within seven days after stopping medication.

Florfenicol was also investigated under similar experimental conditions at a dose of 10 mg/kg body weight. As observed for DOX, FF showed rapid absorption and higher concentrations in the hepatopancreas. Residues in shrimp muscle declined quickly and fell below the quantification limit within 24 hours after stopping medication. Based on the MRL of 100 µg/kg, the withdrawal time was estimated to be approximately two days at a water temperature of 26.5°C. However, the concentrations measured in plasma and hepatopancreas of whiteleg shrimp were lower than those required to effectively inhibit *V. parahaemolyticus*, suggesting that the investigated dose may not be sufficient for treatment. In plasma, $C_{\max}$ reached 60.56 µg/L at 1.77 h ($T_{\max}$), while in the hepatopancreas, a higher $C_{\max}$ of 386.92 µg/kg was observed at 0.19 h. Histological observations indicated that no negative effects on the shrimp hepatopancreas occurred after cessation of medication.

The PK and residue depletion of CFT were also studied in whiteleg shrimp following oral administration at a dose of 25 mg/kg body weight. Similar to the other antibiotics (DOX and FF), CFT was rapidly distributed in shrimp tissues, with higher concentrations observed in the hepatopancreas ($C_{\max}$ of 19.45 ± 2.10 mg/kg at $T_{\max}$ of 1.00 ± 0.20 h). Based on the minimum inhibitory concentration of *V. parahaemolyticus*, the obtained exposure suggested that CFT administered once daily could potentially be effective for bacterial disease control in shrimp. The withdrawal time was estimated to range from approximately 2.0 hours to 77.7 hours depending on the applicable MRL and the dosing regimen when shrimp were fed medicated feed for three consecutive days. Histological analysis indicated that CFT administration did not cause detectable damage to shrimp hepatopancreas.

Finally, the occurrence of antibiotic residues in commercial whiteleg shrimp products and the potential risk for consumers were evaluated. Thirty shrimp samples were collected from markets and supermarkets in Can Tho City and analyzed for antibiotic residues. Five antibiotics were detected by liquid chromatography-tandem mass spectrometry, including DOX, oxytetracycline, enrofloxacin, ciprofloxacin and sulfamethoxazole. Among them, DOX showed the highest concentration in shrimp samples. Based on shrimp consumption data collected from local consumers, the estimated daily intake values under high-consumption conditions corresponding to the 95th percentile consumption ranged from 0.002 to 0.241 µg/kg body weight per day. These exposure values were much lower than the acceptable daily intake values established for these antibiotics, indicating negligible health risks for consumers. However, the detection of antibiotic residues in some shrimp samples emphasizes the importance of continued monitoring and responsible antibiotic use in shrimp production.

In general, this thesis provides additional information on antibiotic use practices in shrimp culture in the Mekong Delta, as well as on the PK, residue depletion and histological effects of selected antibiotics in whiteleg shrimp. In addition, the occurrence and dietary exposure assessment of antibiotic residues in shrimp products were evaluated. These findings may be useful for improving antibiotic management and food safety in shrimp production.

1. Introduction

1.1 Aquaculture overview

Aquaculture has become one of the most dynamic and rapidly expanding food production sectors worldwide, contributing substantially to global food security, employment, and livelihoods. Over recent decades, the sector has experienced continuous growth, driven by increasing demand for aquatic products, population growth, and the limited capacity of capture fisheries to further expand (Naylor et al., 2021). According to the recent FAO assessments, global fisheries and aquaculture production has remained at historically high levels, with aquaculture supplying an increasingly large share of aquatic food for direct human consumption (FAO, 2025). This structural shift reflects not only production expansion but also technological intensification, improvements in feed efficiency, genetic selection, and farm management practices (Boyd et al., 2020; Naylor et al., 2021). Beyond food provision, aquaculture also generates significant socioeconomic benefits, particularly in developing countries, where aquaculture supports rural livelihoods, creates employment opportunities along the value chain, and contributes to export revenues (Belton & Thilsted, 2014; FAO, 2025). However, the intensification of aquaculture production systems has also been accompanied by increasing disease occurrence and greater reliance on antibiotics, particularly in intensive shrimp farming. Consequently, concerns related to antibiotic residues, food safety, and consumer health have become increasingly important in modern aquaculture production.

The growth of aquaculture has been particularly pronounced in Asia, which accounts for the majority of global farmed aquatic animal production, especially in countries such as China, India, and Vietnam (FAO, 2025). Favorable climatic conditions, extensive inland and coastal water resources, and long-standing traditions of aquaculture have enabled many Asian countries to develop large-scale and export-oriented production systems. Within this regional context, shrimp aquaculture represents one of the most economically valuable subsectors. Farm-raised shrimp, including species such as whiteleg shrimp (*Penaeus vannamei*), contributes a substantial proportion of global crustacean output and plays a key role in international seafood trade due to its fast growth, high market demand, and adaptability to diverse farming systems (Anderson et al., 2019; Boyd et al., 2020). Although global shrimp production has remained substantial in recent years, industry performance is influenced by multiple risk factors, including disease outbreaks, feed costs, trade policies and farming practices across producing regions (Anderson et al., 2019).

The modern aquaculture sector in Vietnam began to develop in the 1960s and subsequently evolved through different production phases, including extensive, improved extensive, semi-intensive, intensive, and super-intensive farming systems (Tuan, 2013). Vietnam benefits from favorable natural conditions, including a long coastline and extensive river and estuarine systems. These factors have supported the development of aquaculture, which is now a key pillar of the country's agricultural and food production system.

Shrimp farming plays a central role within Vietnam's aquaculture industry, particularly in coastal and delta regions. Between 2018 and 2024, the shrimp farming area expanded at an average annual rate of approximately 5%, while shrimp production increased by about 8.4% per year. This growth has been largely attributed to improvements in farming practices and productivity in whiteleg shrimp culture. During the same period, whiteleg shrimp production increased by approximately 82%, corresponding to an average annual growth rate of 13%, confirming its dominance among cultured shrimp species in Vietnam (VASEP, 2025; Figure 1). The strong performance of the shrimp sector has reinforced its importance as a major contributor to national seafood export value and has stimulated continued investment in farm infrastructure, production technologies, and management practices.

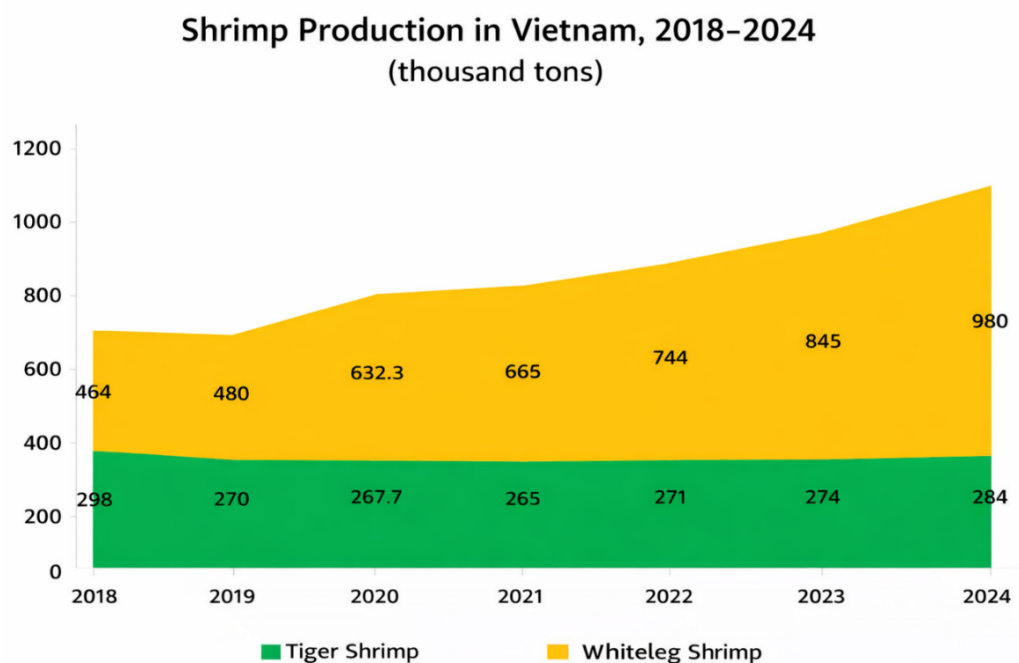


Figure 1. Trends in shrimp production in Vietnam (2018–2024). Values are expressed in thousand tons (VASEP, 2025)

Overall, the rapid expansion and intensification of aquaculture, particularly shrimp farming, underline the economic and social importance of this sector at both global and national levels. At the same time, the increasing adoption of intensive and super-intensive production systems has also been associated with greater pressure on farm management, particularly in relation to sustainability, production efficiency, and animal health management.

1.1.1 Global trends toward intensive and super-intensive aquaculture systems

Intensive and super-intensive aquaculture systems are characterized by high stocking densities, increased reliance on external inputs, and greater control of environmental conditions. Compared with extensive and semi-intensive systems, these production models require formulated feeds, aeration, and more active management to sustain productivity. Super-intensive systems further integrate advanced technologies such as recirculating water systems, automated feeding, and precise water quality control, enabling high yields within limited space (Oddsson, 2020) (Figure 2). As production intensity increases, these systems also become more dependent on continuous management and external inputs, which may create additional challenges in maintaining water quality, controlling disease outbreaks, and ensuring stable production performance (Boyd et al., 2020).



Figure 2. Super intensive whiteleg shrimp farming system in the Mekong Delta, Vietnam

In practice, intensive and super-intensive aquaculture production has expanded markedly in regions with strong market incentives and technological capacity, where economic returns from increased yields can justify higher input costs (Bush and Oosterveer, 2019). The adoption of these systems is often supported by improvements in production technologies, farm management practices, and access to export markets (Boyd et al., 2020).

While intensive and super-intensive systems can significantly enhance production efficiency and output, they also bring new challenges. High stocking densities and increased reliance on external inputs can increase vulnerability to disease outbreaks, elevate production costs, and require higher levels of technical expertise for effective management. These factors, coupled with environmental and sustainability concerns, have stimulated ongoing research and debates on how best to balance productivity with long-term resource stewardship and animal health (Verdegem et al., 2023).

1.2 Shrimp aquaculture situation

1.2.1 The aquaculture situation of shrimp in the world

According to Felix et al. (2020), black tiger shrimp (*Penaeus monodon*) was the dominant cultured species in most Southeast Asian countries like Thailand, Vietnam, and Indonesia for several decades. However, since 2001 most of them have shifted to culture of exotic whiteleg shrimp (*Penaeus vannamei*), a species that originated from the Pacific coast of Mexico and Central and South America. This transition has been primarily driven by the commercial availability of specific pathogen-free (SPF) and specific pathogen-resistant (SPR) shrimp broodstock, which has enabled more stable seed supply and improved production reliability under intensive farming conditions. Whiteleg shrimp exhibits several biological and production characteristics that favor its adoption in semi-intensive and intensive aquaculture systems. The availability of pathogen-free seed and the relatively large body size of whiteleg shrimp, with maximum wild weights of approximately 120 g for females and 60–80 g for males, are important factors enabling semi-intensive and intensive culture systems. The species also demonstrates broad tolerance to environmental conditions. *L. vannamei* can tolerate temperatures as low as 15°C, although growth performance is considerably enhanced at higher temperatures, with an optimal range reported between 25 and 35°C (Ponce-Palafox et al., 1997; Felix et al., 2020). Similarly, the species can survive across a wide salinity range from 0 to 50 g/L, while maintaining relatively stable growth rates within the range of 10–40 g/L. It can grow in freshwater, and the growth is slow if the salinity is below 10 g/L. In addition to temperature and salinity tolerance, *L. vannamei* can grow under a relatively wide pH range from 7.0 to 9.0 with optimal growth at pH 8.0 and water oxygen levels must be above 4.5 mg/L for optimal growth (Wyban & Sweeney, 1991; Felix et al., 2020). These physiological flexibilities have allowed whiteleg shrimp to be cultured in diverse farming environments, including coastal, brackish, and inland areas, thereby supporting the global expansion of shrimp aquaculture beyond traditional coastal zones. Generally, it is an easy species to breed and hence domestication of the species is very successful, commercial availability of specific pathogen free and high-health stock is also an advantage, selective breeding work is easier because of the short generation period and easier captive breeding. As shrimp aquaculture expanded rapidly worldwide, production systems also evolved from traditional extensive and semi-intensive practices toward more intensive and technologically advanced farming models. While these developments have significantly increased productivity, they have also introduced new challenges related to biosecurity and water quality management during culture operations. Intensive shrimp farming systems require a high level of technical expertise and careful management to sustain productivity in a reliable and environmentally responsible manner.

Due to the advantage characteristics of whiteleg shrimp, e.g., tolerance to a wide range of salinity and diseases, rapid growth, suitable survival, and ability to grow in high-density, it becomes the most common intensively cultured species in Vietnam and worldwide (Directorate of Fisheries, 2013; Tam

et al., 2021). In practical culture operations, shrimp health, survival, and growth are influenced by multiple interacting factors, including alkalinity, temperature, dissolved oxygen, pH, salinity, feed quality, and stocking density (Silva et al., 2015; Suriya et al., 2016; Samadan, 2018). The increasing complexity of managing these factors under intensive production conditions highlights the need for improved farm management strategies to ensure sustainable shrimp aquaculture development at the global scale.

1.2.2 The current shrimp aquaculture in Vietnam

Aquaculture production shares a large proportion of the economical resources of Vietnam. With a coastline of 3260 km long and large areas covered by saline water, Vietnam has developed into one of the world's leading shrimp producers and exporters. In 2025, the country's shrimp export value reached a record high of USD 4.6 billion, demonstrating the economic importance of this industry (VASEP, 2026). Many shrimp farms have been developed in southern Vietnam, particularly in the Mekong Delta (area of 40,000 km²), which accounts for approximately 80% of the country's exported shrimp value and is one of the most productive fishing and shrimp-farming regions in the world. The Mekong Delta of Vietnam has the greatest potential for shrimp aquaculture, an activity that plays a vital role in rural development and helps approximately one million shrimp farmers achieve a sustainable livelihood (Duc, 2010; Phuong & Oanh, 2010). Besides the large income, shrimp farming in Vietnam reduced poverty remarkably, provided employment opportunities, and increased exports (Ha et al., 2013). In addition, Vietnamese shrimp is mainly exported to international markets with strict quality and food safety standards, such as the European Union, the United States, and Japan.

The shrimp culture in Vietnam started with extensive farming systems in the early 1970s, and was then replaced by the improved extensive farming systems in the early 1980s, semi-intensive and intensive farming systems since 1985, and now super-intensive farming systems have become dominant (Tien et al., 2013). The shrimp industry began early, and one of the most recent shrimp culture systems is the intensive system, which makes up only 5.6% of shrimp farms but has high productivity of 8–10 tons per ha, whiteleg shrimp being the most predominantly farmed species in the Mekong Delta. According to Thien (2019), intensive whiteleg shrimp farming in Vietnam generally involves a production cycle including hatchery, nursery, grow-out, and harvest stages. In earthen pond systems, the farming infrastructure commonly consists of settling ponds, grow-out ponds, and wastewater discharge areas. Prior to stocking, ponds are prepared through sludge removal, liming, water filling, and disinfection. Water from settling ponds is subsequently transferred into grow-out ponds, where shrimp are stocked at densities of approximately 60–100 shrimp/m². Following nursery rearing, shrimp are generally cultured in grow-out ponds for approximately 3–4 months until market size is achieved, resulting in an overall production cycle of approximately 4–5 months from post-larvae to harvest. During the culture period, chemicals, disinfectants, and biological products are frequently applied to maintain water

quality and prevent disease outbreaks. In lined pond systems, additional water treatment ponds are generally included, and grow-out ponds are lined with plastic sheets and equipped with aeration systems. These systems typically operate at higher stocking densities (approximately 150–300 shrimp/m²) and involve more intensive environmental management practices. Partial harvesting or shrimp transfer between ponds may also be performed during the production cycle to optimize stocking density and productivity (Thien, 2019). Besides monoculture, integrated farming systems such as mangrove-shrimp systems and rice-shrimp systems were developed in the early 1980s and have played a significant role in the sustainable development of aquaculture in Vietnam (Quynh, 1992; Preston & Clayton, 2003; Minh et al., 2013; Tien et al., 2013).

In Vietnam, shrimp farming is largely dominated by small-scale producers. Results from the 2006 Rural, Agricultural and Fishery Census, conducted by the Vietnam General Office of Statistics, also confirm the importance of the Mekong Delta in terms of the quantity of shrimp farming households. Out of 337,614 households in Vietnam engaged in shrimp farming, 292,522 (86%) of households were from the Mekong Delta. Among these households, 53% operated farms with an average area of approximately 0.5 ha, while only 16% practiced intensive or semi-intensive shrimp culture (Lan, 2013). Unfortunately, the Vietnamese shrimp sector has faced major problems since the introduction and growth of the intensive systems. The most prominent issues are related to shrimp disease outbreaks, such as acute hepatopancreatic necrosis disease (AHPND), white feces disease (WFD) and white spot disease (WSD) (Thinh & Phu, 2021), together with rejection by import markets (Nguyen et al., 2020). The reason for rejections is related to food safety and quality, because there are many standards that differ between importers which are not met by the farmers. Moreover, disease outbreaks has resulted in growing debts among farmers, as income decreases due to more failing harvests, shrimp diseases caused losses of 28–50 million USD for the Mekong Delta in the 2000s, and many farmers gave up production and abandoned their farms (Chanratchakool & Phillips, 2002).

Although shrimp farming in Vietnam is still characterized mainly by small-scale household farming, many other management systems have been established including cooperatives, or large companies, which resulted in shrimp culture being operated with different systems. Consequently, international standards, which include organic farming, Global Good Agricultural Practices (Global GAP), Aquaculture Stewardship Council (ASC) and Best Aquaculture Practices (BAP), which are aquaculture certifications as well as international standards that ensure food safety, health and safety of workers, animal welfare, and environmental protection, have been introduced, applied, and promoted in the shrimp culture industry (Hai et al., 2015). Many large companies run all activities along the production chain from hatcheries, farms, and processing factories to export. These companies often hold international certifications from Global GAP, BAP, or ASC. Generally, cooperatives, co-management or cluster production could be an important solution and trend for small-scale household production in the near future.

1.2.3 The common diseases occurring in cultured shrimp

Shrimp aquaculture industry around the world has faced many serious diseases due to pathogens such as viruses, bacteria, parasites, water quality parameters, or some unidentified pathogens. Among these, viral and bacterial diseases are generally regarded as the most severe constraints to shrimp production (Stentiford et al., 2012; Thitamadee et al., 2016). Over the past few decades, several major disease outbreaks have led to the collapse, or near collapse, of shrimp farming industries in many countries, causing substantial economic losses and long-term impacts on production stability (Lightner, 2012). Whiteleg shrimp is highly susceptible to several viral pathogens such as white spot syndrome virus (WSSV), white feces disease (WFD), taura syndrome virus, yellow head virus, infectious hypodermal hematopoietic necrosis virus, lymphoid organ vacuolization virus, roe-like viruses (Felix et al., 2020). These viral pathogens differ in their epidemiology and virulence; however, they commonly result in reduced growth performance, mass mortalities, and severe economic consequences for shrimp farmers.

In addition to viral diseases, bacterial infections represent a major health challenge in shrimp aquaculture. According to Kumar et al. (2021), members of the genus *Vibrio* are among the most common bacterial pathogens associated with shrimp diseases, particularly under intensive culture conditions. One of the most devastating bacterial diseases is early mortality syndrome, also known as AHPND (Kumar et al., 2021; Thitamadee et al., 2016). In Vietnam, AHPND, WFD, and WSD are still common diseases in the Mekong Delta reported by farmers (Thinh & Phu, 2021). The AHPND disease was first reported in 2009 in China, and later spread to other countries such as Vietnam, Thailand and Malaysia, and then to South and Central America (Felix et al., 2020). The AHPND was first reported in Vietnam in 2010, caused by *V. parahaemolyticus*, resulting in massive mortality of farmed shrimp in the Mekong Delta (Tran et al., 2013; Nghia et al., 2015). Chi et al. (2017) reported that AHPND was also the main serious disease in shrimp aquaculture in the North of Vietnam. Flegel & Sritunyaluucksana (2018) confirmed that AHPND was caused by *V. parahaemolyticus* that carries a pVA plasmid containing genes for PirAvp and PirBvp toxins. The common clinical signs found in AHPND infected shrimp include lethargic motility, empty midgut, pale coloration and shrinking of the hepatopancreas (Zorriehzahra & Banaederakhshan 2015). In addition, AHPND normally occurred in the period of 20 and 40 days after stocking post-larvae (Phu et al., 2019a).

The WFD and WSD are two other common diseases reported in intensive shrimp farming systems, typically occurring between 30 and 60 days after stocking (Thinh & Phu, 2021). WFD is characterized by the presence of floating white fecal strings in culture ponds, which represent one of the most recognizable clinical signs of the disease (Tangprasittipap et al., 2013; Sriurairatana et al., 2014). It was identified as a multi-factor disease caused by various microorganisms, including bacteria, viruses, parasites, and particularly the microsporidian parasite *Enterocytozoon hepatopenaei* (Ha et al., 2011). Microsporidian spores have also been detected in shrimp affected by WFD in several shrimp-producing countries, including Thailand, further supporting the complex etiology of this disease (Flegel &

Sritunya-uucksana, 2018). Besides, dissected midguts are distended and full of white to yellow contents. Sriurairatana et al. (2014) reported that WFD in shrimp farms led to enormous economic losses worldwide yearly. According to Hou et al. (2018), the mortality rate of WFD in the summer may be as high as 50% in ponds, however, the specific reasons for the WFD outbreak remain unclear.

The WSD is caused by the WSSV, which is a crustacean-infecting, double-stranded DNA virus, and is the most serious virus in farmed shrimp worldwide (Kawato et al., 2019). The first reported WSD outbreak occurred in 1992 in China (Lo et al., 2005), and then WSD quickly spread throughout Asia including Japan, Korea, and Thailand (Chou et al., 1995, Wongteerasupaya et al., 2003, Walker & Mohan 2009). WSD outbreaks were also detected in other shrimp farming areas, i.e., South, North and Central America, Europe and the Middle East (Leu et al., 2009; Walker & Mohan, 2009; Sánchez-Paz, 2010; Lightner et al., 2012). The virus caused by WSD is highly virulent and may cause up to 100% mortality within 3-7 days after infection (Loy, 2014). In addition, WSSV is known to infect not only farmed shrimp but also a wide range of wild crustaceans and aquatic invertebrates, facilitating its persistence and spread in aquaculture environments (Walker & Mohan, 2009).

The various diseases and pathogens in shrimp culture have stimulated the research and development of pathogen-free seed and improved biosecurity strategies (Lightner, 2012). However, stocking pathogen-free post-larvae alone will not ensure a disease-free crop since pathogens may still enter the culture system horizontally and infect cultured shrimp (Felix et al., 2020). Viral pathogens still enter the culture by persisting in the soil, through water vectors, or other animals like contaminated crabs and birds, contaminated farm inputs, and contaminated farm implements.

1.2.4 Disease management strategies in shrimp aquaculture and reliance on antibiotics

Preventive disease management strategies are generally regarded as the first line of defense in shrimp aquaculture (Lightner, 2011; Moss et al., 2012). These strategies include the use of specific pathogen-free post-larvae, pond preparation and disinfection, water treatment, and the control of vectors and carriers, such as birds, crabs, wild crustaceans, and contaminated water sources, that may introduce pathogens into culture systems. Improvements in farm design, such as lined ponds, controlled water exchange, and the application of biosecurity protocols, have been promoted to limit pathogen entry and spread within farms. In addition, nutritional management and the use of functional feeds, probiotics, and immunostimulants have been increasingly explored as supportive approaches to enhance shrimp health and resilience against disease (Nayak, 2010). Despite the application of these management measures, disease outbreaks continue to occur frequently in shrimp farming, especially bacterial infections associated with intensive production conditions. In practice, most farmers use antibiotics to prevent and treat bacterial infections in order to control diseases, reduce mortality during disease outbreaks, and, in some farming systems, maintain shrimp survival and production performance under

intensive culture conditions (Holmström et al., 2003; Rico et al., 2013; Chi et al., 2017). Antibiotics are typically administered through medicated feed or, less commonly, through water treatment, with the aim of suppressing pathogenic bacteria and improving shrimp survival (Cabello, 2006).

The reliance on antibiotics in shrimp aquaculture is driven by several practical factors, including the rapid progression of bacterial diseases, high economic pressure to maintain production, and the lack of equally effective alternative treatments once disease signs become apparent. Surveys conducted have documented the widespread use of different antibiotic classes, such as amoxicillin, cotrim (a combination product of sulfamethoxazole and trimethoprim), FF and oxytetracycline, which have been commonly used by shrimp farmers in Vietnam (Phu et al., 2019a; Thinh et al., 2020). However, improper use of antibiotics, including excessive dosages and non-compliance with withdrawal periods, has resulted in the detection of antibiotic residues in exported shrimp products, raising important concerns regarding food safety, consumer exposure, international trade compliance, and aquaculture sustainability (Rico et al., 2014). Moreover, repeated antibiotic exposure in aquaculture environments may contribute to the development of antimicrobial resistance (Chopra & Roberts, 2001). Therefore, understanding antibiotic use practices and their effects on shrimp health, residue depletion, and consumer safety is essential, providing an important foundation for the experimental studies presented in the subsequent chapters of this thesis.

1.3 Drug and chemical use in shrimp aquaculture in Vietnam

1.3.1 Antibiotic overview and antibiotic use in shrimp culture

Antimicrobials are substances that can inhibit the growth of or destroy microorganisms, including bacteria, fungi, viruses, and parasites (Russell, 2004). Antimicrobials include several categories such as antibiotics, disinfectants, antiseptics, and antiparasitic compounds. Among these, antibiotics are specifically active against bacteria and are commonly used in both human and veterinary medicine (Denyer et al., 2004; Russell, 2004).

Antibiotics are organic compounds, typically produced by or derived from microorganisms, that can inhibit the growth of or kill other microorganisms at low concentrations (Denyer et al., 2004; Russell, 2004). Currently, antibiotics are produced partly or completely synthetically. According to Walsh (2003), several antibiotics can completely kill bacteria (called bactericidal), while some antibiotics can only inhibit the growth of other bacteria (called bacteriostatic). The ability of an antibiotic to inhibit or kill bacteria represents the effectiveness of that antibiotic, while the overall effectiveness of the antibiotic is related to the interaction among the antibiotic, bacteria, and host (Doern & Brecher, 2011). Beyond their direct antimicrobial activity, antibiotics differ considerably in their physicochemical properties, antimicrobial spectrum, and pharmacological behavior, which together determine their clinical performance and suitability for use in different animal species and production systems

(Kümmerer, 2009). In aquaculture production systems, antibiotics are widely used for disease prevention and treatment (Chi et al., 2017; Phu et al., 2016). However, their improper use may result in residue accumulation in edible shrimp tissues, raising concerns regarding food safety and consumer exposure.

Antibiotic classification and modes of action

There are several ways of classifying antibiotics, but the most common classification is based on their molecular structures, spectrum of activity and mode of action. Some common classes of antibiotics based on chemical or molecular structures include beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulfonamides, glycopeptides and oxazolidinones. The effectiveness, toxicity and potential allergic side effects of antibiotics within the same structural class will overall be similar (Calderon & Sabundayo, 2007). This structural classification is particularly useful in veterinary and aquaculture contexts, as it provides an initial indication of the expected antimicrobial spectrum and potential residue behavior of antibiotics used in food-producing animals (Kümmerer, 2009). Nevertheless, antibiotics from the same class may still exhibit differences in absorption, tissue distribution, and elimination depending on their specific chemical substitutions and physicochemical properties.

Regarding their modes of action, most classes of antibiotics target some unique feature of bacterial structure or metabolism (Figure 3). The major mechanisms of antibiotic action include inhibition of cell wall synthesis, breakdown of cell membrane structure or function, inhibition of the structure and function of nucleic acids, inhibition of protein synthesis, or blockage of key metabolic pathways (Talaro & Chess, 2008; Madigan & Martinko, 2006; Wright, 2010). Particularly, β -lactam antibiotics, such as penicillins and cephalosporins, inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins, leading to structural instability and cell lysis (Walsh, 2003). In contrast, antibiotics targeting protein synthesis act at different ribosomal sites. Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit and preventing the attachment of aminoacyl-tRNA, whereas phenicols and macrolides interact with the 50S subunit, blocking peptide bond formation or ribosomal translocation (Madigan & Martinko, 2006; Wright, 2010). Antibiotics that interfere with nucleic acid synthesis, such as quinolones, inhibit bacterial DNA replication by targeting DNA gyrase and topoisomerase IV, while sulfonamides disrupt essential metabolic pathways by inhibiting folate synthesis (Talaro & Chess, 2008). These distinct modes of action not only determine the antimicrobial spectrum of each antibiotic class but also influence their bactericidal or bacteriostatic properties.

Understanding antibiotic classification and mechanisms of action is fundamental for interpreting differences in therapeutic efficacy, PK, residue depletion, and potential tissue effects in aquatic animals.

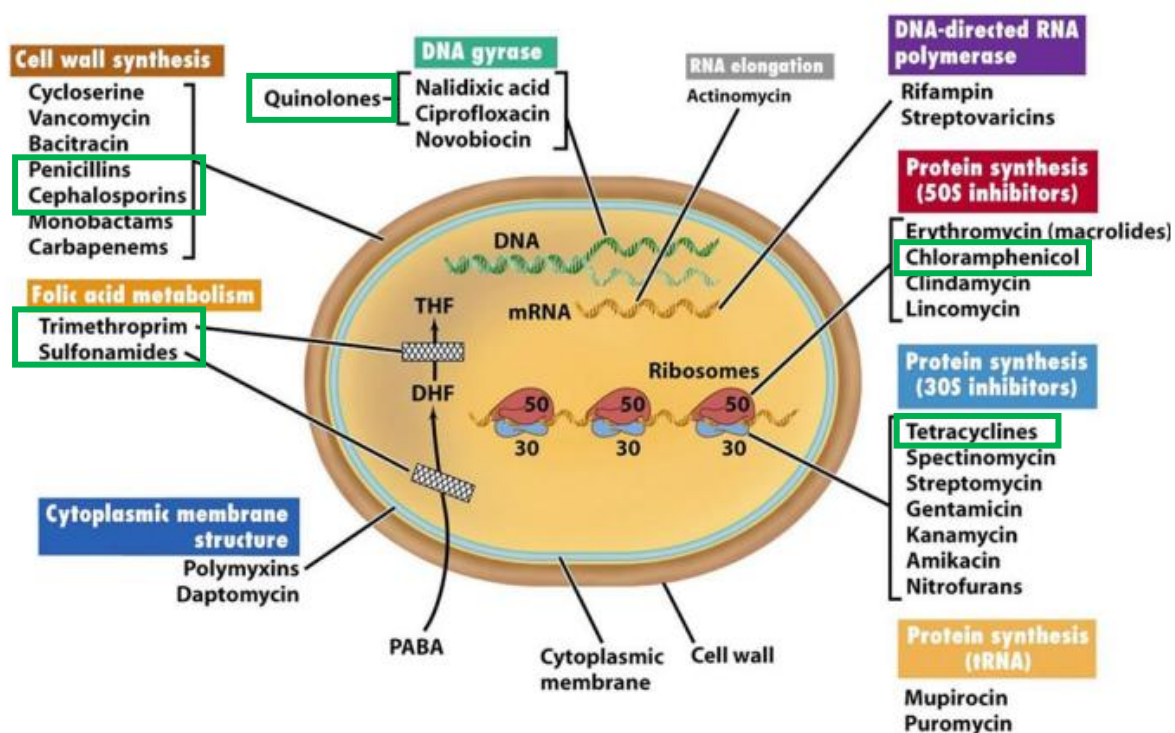


Figure 3. Major target sites and mechanisms of action of different antibiotic classes in bacterial cells, including inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, folic acid metabolism, and cytoplasmic membrane function. The antibiotic classes highlighted with green boxes correspond to antibiotics reported to be used by shrimp farmers in the Mekong Delta, Vietnam (Madigan & Martinko, 2006)

Global use of antibiotics in aquaculture

Antibiotics have been used in animal medicine since the 1940s (Lozano et al., 2018) and in aquaculture for more than 50 years (Shamsuzzaman & Kumar, 2012). Their application has expanded in parallel with the intensification of aquaculture systems, particularly in regions with high production density and disease pressure. Antibiotics are widely used in aquaculture in Asia, the USA, Canada, Europe and other countries (Tuševljak et al., 2013). In aquaculture production, antibiotics are primarily applied to control bacterial infections, improve short-term survival, and reduce economic losses during disease outbreaks, especially in intensive and super-intensive farming systems (Cabello, 2006; Rico et al., 2014).

In recent years, increasing attention has been paid to the consequences of antibiotic use in aquaculture, including the emergence of antimicrobial resistance, the presence of antibiotic residues in edible aquatic products, and potential environmental impacts associated with antibiotic release into surrounding ecosystems (Cabello, 2006; Kümmerer, 2009; Cabello et al., 2016). Within this global context, understanding country-specific patterns of antibiotic use is essential, particularly in major shrimp-producing countries such as Vietnam.

The use of antibiotics in shrimp culture in Vietnam

For many years, the shrimp industry in Vietnam has developed rapidly, driven by increasing global demand, technological advances, and the widespread adoption of intensive and super-intensive farming systems. This rapid development has been accompanied by higher stocking densities and greater production pressure, which have increased the risk of disease outbreaks in shrimp farms. As a result, antibiotics have been widely used as part of disease management practices in shrimp aquaculture. Antibiotics used in shrimp farming are very diverse and include multiple antibiotic classes. In a survey conducted in five provinces of the Mekong Delta performed by Thinh & Phu (2021), farmers used 10 types of antibiotics during cultivation shrimp in order to prevent and control disease, i.e., FF, enrofloxacin, ciprofloxacin, tetracycline, oxytetracycline, DOX, CFT, cotrim (containing sulfamethoxazole and trimethoprim), amoxicillin, and chloramphenicol.

This wide range of antibiotics reflects both the variety of bacterial disease challenges encountered in shrimp farming and the differences in farmers' knowledge, access to veterinary advice, and availability of pharmaceutical products. Recent field studies and monitoring work provide concrete evidence that antibiotic use in Vietnamese aquaculture is widespread, heterogeneous, and sometimes problematic in terms of product quality and compliance. Surveys and field assessments have documented frequent use of multiple antibiotic classes across small-scale and medium-scale farms, and they show that farmers often apply products empirically with variable dosing and treatment durations (Cuong et al., 2021). Additionally, quality assessments conducted in Northern Vietnam found that many antimicrobial products available to farmers are of inconsistent or poor quality and that labelled concentrations or instructions are sometimes inaccurate, which complicates proper dosing and withdrawal management (Tran et al., 2018). Environmental monitoring and literature reviews also report the occurrence of various antibiotic compounds in Vietnamese aquatic environments and farm effluents, indicating that antibiotic use at farm scale can lead to environmental dissemination and create selection pressure for resistance in surrounding ecosystems (Binh et al., 2018).

Beyond residue-related food safety concerns, the emergence and dissemination of antimicrobial resistance have become an increasingly important issue associated with antibiotic use in aquaculture. Antibiotic residues and resistant bacteria released into aquatic environments may contribute to the selection and spread of antimicrobial resistance across environmental, animal, and human interfaces. From a One Health perspective, inappropriate antibiotic use in shrimp farming may therefore have implications not only for shrimp health and aquaculture sustainability, but also for public health and the long-term effectiveness of antimicrobial therapies (Milijasevic et al., 2024).

According to regulations issued by the Ministry of Agriculture and Rural Development (MARD), several antibiotics are either restricted or prohibited for use in aquaculture in Vietnam (MARD, 2016). However, field surveys and residue monitoring data indicate that banned or restricted antibiotics have

occasionally been used in practice, often without strict compliance with recommended withdrawal periods. Such practices increase the risk of antibiotic residues remaining in shrimp products at the time of harvest. Vietnamese shrimp products occasionally face warnings and import rejections in major international markets. The Rapid Alert System for Food and Feed (RASFF) from the EU reported that shrimp products from several regions in Vietnam occasionally contain antibiotic residues exceeding permitted levels, e.g., from 2020 to 2024, residues of oxytetracycline, DOX, tetracycline, and sulfamethoxazole were reported in frozen shrimp products (RASFF, 2025). These notifications highlight persistent challenges in controlling antibiotic residues in shrimp production for export. Similarly, the Japanese market has maintained strict monitoring of antibiotic residues in imported shrimp. In 2024, the Ministry of Health, Labor and Welfare (MHLW) of Japan rejected multiple shrimp shipments originating from Vietnam due to residues of enrofloxacin, furazolidone and DOX (MHLW, 2025).

To address these issues, responsible antibiotic use in shrimp aquaculture requires adherence to several fundamental principles. Antibiotics should only be used after identification of the causative bacterial pathogens, ideally supported by bacterial isolation and antibiotic susceptibility testing. Accurate dosage, treatment duration, and strict observance of withdrawal periods are essential to ensure both therapeutic effectiveness and food safety. The inappropriate combination of multiple antibiotics without understanding their interactions should be avoided, as such practices may promote the development of antimicrobial resistance and increase the risk of toxic effects. Furthermore, antibiotics should not be used for disease prevention or treatment of viral infections, as these practices offer no therapeutic benefit and may exacerbate long-term sustainability issues (Te, 2003).

Antibiotics investigated in this thesis

Cefotaxime. Cefotaxime (Figure 4) is a commonly used antibiotic in shrimp culture and plays an important role in disease treatment (Plosker et al., 1998). Cefotaxime is a third-generation cephalosporin, which belongs to the class of beta-lactams; it has a broad spectrum of activity against aerobic Gram-negative as well as Gram-positive bacteria, while it shows variable activity against anaerobic bacteria (Dudley & Barriere, 1982; Carmine et al., 1983). Cefotaxime penetrates well through the cell walls of bacteria, shows high beta-lactamase stability, and strong affinity to the important target enzymes (Schrinner et al., 1980). This antibiotic is used in aquaculture, and its residue can be found in countries where aquaculture is developed, i.e., in China, the detection frequency of CFT was 40% in fish ponds around Tai Lake, with a level exceeding 10,000 ng/L (Song et al., 2016), and it was found in surface water of Xiangjiang River with a concentration of 830 ng/L (Lin et al., 2018).

In Egypt, CFT is used in bacterial pathogen management in tilapia (Ali et al., 2021). Cefotaxime is used to control susceptible bacterial infections in aquatic and terrestrial animals, such as tilapia, horses, birds,

dogs, cats and reptiles at the dose of 10–80 mg/kg body weight via intraperitoneal, intramuscular, intravenous or subcutaneous injection (Ali et al., 2021).

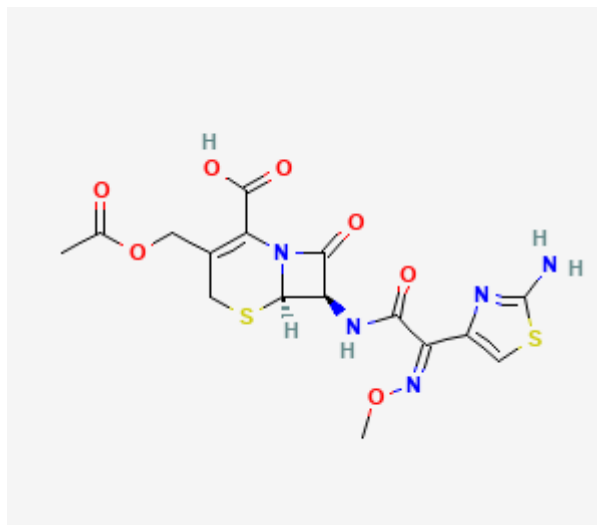


Figure 4. Chemical structure of cefotaxime (PubChem CID 5742673; NCBI, 2026a)

Regarding the toxicity, Berezhinskaia et al. (1990) demonstrated that CFT has low acute toxicity with median lethal dose (LD_{50}) value of 7000 (6295-7805) mg/kg on mice through intravenous administration. The chronic toxicity of CFT studied on rats at the human equivalent dose show that there was no significant effect on liver and kidneys, the count of the blood cells and the histologic pattern of the viscera, after intravenous and intramuscular administration. Similarly, CFT had no action on hematopoiesis, respiration and the central nervous system, after intravenous and intramuscular administration, but CFT had a slight irritating action on the tissues. In addition, Carmine et al. (1983) showed that CFT appeared to have no adverse effects on renal function in a renal tolerance study in rabbits at the dose of 750 or 1500 mg/kg/day for 7 days.

The exposure of humans and aquaculture environments to suboptimal antibiotic concentrations or prolonged low-level exposure may contribute to the development of antimicrobial resistance (Cattoir, 2016). In aquaculture systems, antibiotic application may select for resistant bacterial populations, including potentially zoonotic strains (Silbergeld et al., 2008; Martinez, 2009). Resistant bacteria originating from animal production systems may also be transmitted to humans (Marshall & Levy, 2011; Rico et al., 2012). Lulijwa et al. (2019) reported that among the 15 leading aquaculture-producing countries, China had the highest number of antimicrobial resistance reports between 2008 and 2018 (74 reports), followed by Vietnam (21 studies), India (20 studies), Chile (11 studies), Malaysia (10 studies), Brazil (9 studies), and Thailand (7 studies), highlighting the regional relevance of resistance-related concerns in aquaculture.

Moreover, CFT is a third-generation antibiotic which is not the first line antibiotic. The third generation of cephalosporin is considered a critically important antibiotic, and according to WHO, an antibiotic is classified as critically important if it meets the C1 and C2 criteria (C1, the antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people; and C2, the antimicrobial class is used to treat infections in people caused by either: bacteria that may be transmitted to humans from nonhuman sources, or bacteria that may acquire resistance genes from nonhuman sources) (WHO, 2019). From an international antimicrobial stewardship and One Health perspective, the use of critically important antimicrobials in aquaculture should be carefully evaluated to minimize the risk of antimicrobial resistance development and dissemination (Cabello et al., 2016; WHO, 2019). Thus, CFT should be used only when necessary and must be combined with PK information. Generally, to promulgate strict regulations on the use and application of new antibiotics like CFT in aquaculture, research on the dosage and elimination time of antibiotics is very important to ensure food safety for consumers as well as limiting the problem of antimicrobial drug resistance.

Florfenicol. Florfenicol (Figure 5) is one of the antibiotics commonly used for the treatment of bacterial diseases in whiteleg shrimp and other aquaculture species (Phu et al., 2016 & 2019a; Ren et al., 2016; Lillehaug et al., 2018). Florfenicol is a broad-spectrum synthetic bacteriostatic antibiotic belonging to the phenicol group. The mode of action of FF is similar to that of chloramphenicol, acting through the inhibition of protein synthesis via binding to the S50 ribosomal subunit of the target microorganism, resulting in the prevention of protein chain elongation in bacterial cells, and the inhibition of bacterial growth (Wang et al., 2009; Lambert, 2012; Nejad et al., 2017). Because chloramphenicol is prohibited for use in food-producing animals due to its association with severe adverse effects in humans, FF has been developed and widely adopted as a safer alternative phenicol antibiotic for veterinary applications, including aquaculture (Dowling, 2006). Florfenicol is one of the antibiotics approved by the US Food and Drug Administration for aquaculture that has been shown to be effective in treating diseases caused by Gram-positive and Gram-negative bacteria in aquaculture and livestock (Samuelsen & Bergh, 2004; Caipang et al., 2009; Huang et al., 2015). In Vietnam, FF is reported among those approved for aquaculture use within the regulatory framework of the Ministry of Agriculture and Rural Development (MARD), based on FAO assessments of antimicrobial use in the sector (Gaunt, 2020).

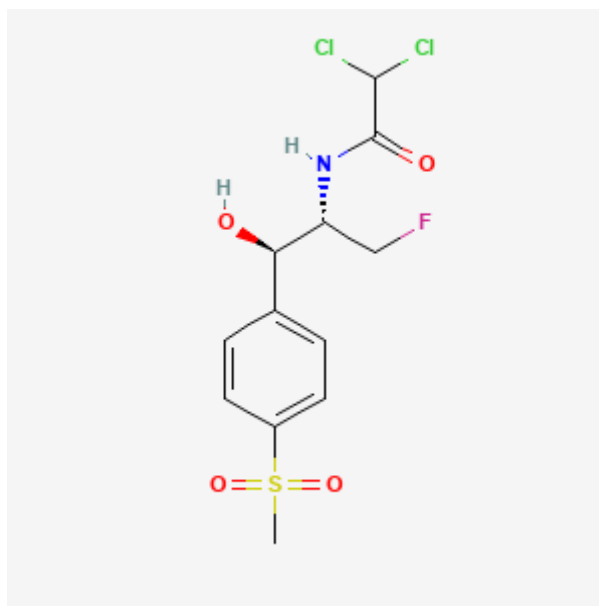


Figure 5. Chemical structure of florfenicol (PubChem CID 114811; NCBI, 2026c)

Toxicological evaluation of FF has primarily been conducted within veterinary regulatory frameworks, where safety assessments in laboratory mammals provide the basis for establishing ADI values. Acute toxicity studies indicate a low level of oral toxicity, particularly in mice and rats, no acute lethal dose (LD₅₀) could be established after oral exposure at doses up to 2000 mg/kg body weight, indicating a low level of acute oral toxicity. Following intraperitoneal administration, the LD₅₀ in rats was reported to be close to 2000 mg/kg body weight (EMA, 2014). Subchronic and chronic toxicity studies further indicate that FF does not exhibit genotoxic or carcinogenic potential, and no evidence of chloramphenicol-associated idiosyncratic aplastic anemia has been observed in animals exposed to FF. This improved safety profile is attributed to the absence of the p-nitro group present in chloramphenicol, which is considered responsible for severe hematological toxicity in humans (Schwarz et al., 2001; EMA, 2014).

Beyond mammalian regulatory data, experimental ecotoxicological studies have evaluated the acute toxicity of FF in aquatic organisms. In whiteleg shrimp, a 24 h median lethal concentration (LC₅₀) of 32.42 mg/L has been reported by Soto-Rodríguez et al. (2006), while in fish species higher LC₅₀ values have been observed, including a 96 h LC₅₀ of approximately 349.94 mg/L in Nile tilapia larvae (Mattioli et al., 2020), and a 48 h LC₅₀ of 337 mg/L for *Daphnia magna* (Martins et al., 2013). In carp, LC₅₀ values exceeding 1000 mg/L have been observed (Carraschi et al., 2011). These findings indicate that FF exhibits relatively low acute lethality in aquatic invertebrates and fish under short-term exposure conditions. Notably, FF concentrations detected in surface water typically range from ng/L to µg/L, although higher concentrations may occur in local aquaculture hotspots (Guo et al., 2024). Therefore, concentrations in the environment are generally low compared to experimentally determined LC₅₀

values, suggesting that the potential for acute mortality in aquatic organisms is not high at typical environmental concentrations.

Nevertheless, low acute toxicity does not eliminate ecological or public health concerns, as concentrations below the inhibitory threshold can exert selective pressure on microbial populations and promote the emergence and spread of antibiotic resistance. Additionally, FF belongs to the amphenicol class, which is classified by the World Health Organization as Highly Important Antimicrobials (HIA) for human medicine (WHO, 2019). Although FF itself is not approved for use in human medicine, concerns arise from potential cross-resistance with chloramphenicol, a structurally related compound that remains in limited clinical use. Therefore, this classification emphasizes the importance of prudent and responsible FF use in aquaculture to limit the development and spread of antimicrobial resistance (Cabello et al., 2016).

Doxycycline. Doxycycline (Figure 6) is a second-generation, broad-spectrum tetracycline antibiotic widely used in veterinary and aquaculture medicine. It exhibits bacteriostatic activity by targeting bacterial ribosomes, where it binds to the 30S subunit and inhibits protein synthesis through blocking the attachment of aminoacyl-tRNA to the ribosomal acceptor site, thereby preventing elongation of the polypeptide chain (Chopra & Roberts, 2001; Etebu et al., 2016). This mechanism suppresses bacterial growth rather than directly killing bacterial cells, thereby allowing host defense mechanisms to contribute to pathogen elimination. Compared with first-generation tetracyclines, DOX is characterized by improved antimicrobial potency and chemical stability, which has contributed to its extensive application in animal production systems, including aquaculture species (Etebu et al., 2016). Doxycycline is highly effective against Gram-positive and Gram-negative bacteria (Chopra & Roberts 2001). In aquaculture, DOX has been reported to be effective against several bacterial pathogens commonly associated with disease outbreaks in shrimp farming, particularly species of the genus *Vibrio* (Rico et al., 2013).

From a regulatory perspective, DOX is permitted for veterinary use in aquaculture in several countries under national regulatory frameworks, subject to dose limitations, withdrawal periods, and residue monitoring requirements. In Vietnam, DOX is included in the official list of veterinary drugs authorized for circulation and use in animal production, including aquaculture, under the regulations issued by the Ministry of Agriculture and Rural Development (MARD, 2024), and has also been reported among the antibiotics commonly applied in the aquaculture sector based on FAO workshop assessments (Gaunt, 2020).

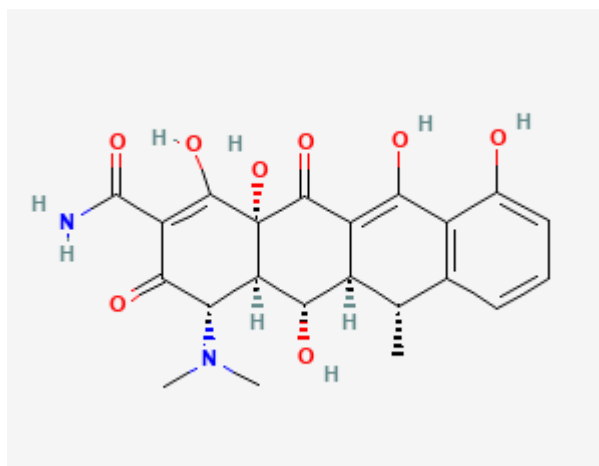


Figure 6. Chemical structure of doxycycline (PubChem CID 54685920; NCBI, 2026b)

Regarding toxicity and safety, DOX is generally considered to have low acute toxicity and is well tolerated when administered at appropriate doses. In veterinary medicine, DOX has been widely used due to its relatively low risk of severe adverse effects compared with some other antibiotic groups (Nelson & Levy, 2011). Regulatory toxicity assessments show that DOX has no genotoxic or carcinogenic properties, and ADI values for human consumers have been established based on long-term toxicity and reproductive studies conducted in laboratory animals (EMA, 2018). These assessments form the scientific basis for residue regulation and consumer risk assessment related to the use of DOX in aquaculture.

However, adverse effects associated with DOX exposure have been documented, particularly under conditions of overdose or prolonged use. In animals, reported effects primarily involve the gastrointestinal tract and liver, including digestive disturbances and hepatocellular alterations, reflecting the known class-related toxicological characteristics of tetracycline antibiotics (EMA, 2018). Additionally, the presence of DOX residues in aquatic environments, arising from medicated feed losses and farm effluents, has been shown to affect non-target organisms and alter microbial community structures, potentially facilitating the survival and spread of antibiotic resistance determinants (Djelal et al., 2024).

According to the World Health Organization, tetracyclines, including DOX, are classified as Highly Important Antimicrobials (HIA) for human medicine (WHO, 2019). Although DOX is not ranked among the Critically Important Antimicrobials, it remains an essential therapeutic option for the treatment of a wide range of bacterial infections in humans. Therefore, the use of DOX in aquaculture should be subject to prudent use principles and appropriate regulatory oversight to limit the emergence of antimicrobial resistance and ensure compliance with international food safety standards.

1.3.2 Chemicals for water quality treatment

During shrimp culture operations, farmers use several chemicals to maintain water quality, control pathogens, and stabilize pond conditions. A survey conducted in 2021 across five key shrimp farming provinces in the Mekong Delta, including Ca Mau, Bac Lieu, Soc Trang, Tra Vinh and Ben Tre found that calcium carbonate (CaCO_3) and calcium oxide (CaO) were widely used by farmers in pond preparation, initial water treatment and grow-out stages (Thinh & Phu, 2021). These compounds are primarily used to adjust pH, increase alkalinity, and improve buffering capacity, thereby creating more stable environmental conditions for shrimp growth.

After water is introduced into preparation ponds, or directly into grow-out ponds in some farming systems, chemicals are used to reduce microbial load and potential disease risks. According to Thinh & Phu (2021), chlorine and iodine disinfectants (typically at concentrations of 0.05–0.1 mg/L) and potassium permanganate (KMnO_4 ; 1–41 mg/L) were the most frequently reported agents for water disinfection. These compounds are favored for their broad-spectrum antimicrobial activity and rapid action against bacteria, viruses, and protozoa. However, their application requires careful dose management, as excessive concentrations may negatively affect shrimp health and beneficial microbial communities. In addition to commonly used disinfectants, quaternary ammonium compounds such as benzalkonium chloride (BKC; 0.32–1.6 mg/L) were frequently applied by farmers to treat pond water, owing to their broad antimicrobial activity and relatively low cost.

Regarding chemicals used for water treatment during the grow-out stage, according to Thinh & Phu (2021), most farmers used CaCO_3 , iodine, or BKC every 10 or 15 days to maintain water quality and suppress disease outbreaks. In contrast, chlorine was less frequently used during the grow-out period, likely due to concerns regarding its toxicity and potential adverse effects on shrimp health (Phu et al., 2019a). Virkon (containing hydrogen peroxide) was also used to disinfect the pond water. However, according to farmers' reports, it was used less frequently because of its higher cost despite its perceived efficacy (Phu et al., 2019a). The types of chemicals reported in the survey by Phu et al. (2019a) in Southern Vietnam were similar to those reported by Chi et al. (2017) in Northern Vietnam, indicating that the types of disinfectants applied and their modes of use are relatively consistent across different geographic regions of the country.

Generally, these surveys indicate that chemical water treatment remains an indispensable component of shrimp farming in Vietnam. While these practices play a crucial role in disease prevention and water quality management, their frequent and sometimes preventive use suggests the need for better guidance on appropriate application strategies to minimize potential negative impacts on shrimp health, pond ecosystems, and environmental quality.

1.3.3 Probiotics used in shrimp aquaculture

Probiotics are live microorganisms that can improve the health of the host organism or the culture environment when applied in adequate amounts (Verschuere et al., 2000). During the shrimp farming process, probiotics are widely used both as feed additives and water quality improvers.

Probiotics used in aquaculture can be classified into three groups based on their primary function and application in shrimp farming systems, rather than microbial taxonomy. Group 1 consists of live microorganisms that are incorporated directly into shrimp feed, including species from *Bacillus*, *Lactobacillus*, and *Saccharomyces*. These probiotics are primarily applied to improve feed utilization, enhance digestive enzyme activity, and promote gut health in cultured shrimp (Verschuere et al., 2000; Kesarcodi-Watson et al., 2008).

Group 2 includes microorganisms that antagonize or compete with pathogenic microorganisms in the culture environment, i.e., *B. licheniformis* and *Bacillus* sp., which are commonly applied to pond bottoms to suppress pathogenic bacteria through competitive exclusion, enzyme production, and the secretion of antimicrobial compounds (Verschuere et al., 2000; Nayak, 2010). Group 3 comprises microorganisms that primarily function as environmental bioremediators, contributing to improved water and sediment quality in shrimp ponds. This group includes *Nitrosomonas*, *Nitrobacter*, *Actinomyces*, *Bacillus*, *Rhodobacter* sp., *Rhodospirillum*, *Rhodopseudomonas viridis*. These microorganisms are applied to pond water and bottom substrates to enhance organic matter degradation, nutrient cycling, and the removal of toxic nitrogenous compounds (Defoirdt et al., 2011).

Surveys conducted in Vietnam have shown that farmers routinely apply probiotic products to enhance shrimp digestibility and growth performance, as well as to improve water quality and reduce the accumulation of organic waste in ponds (Rico et al., 2013, Chi et al., 2017, Thinh & Phu, 2021). Common bacteria listed in those products include *B. subtilis*, *B. licheniformis*, *B. megaterium*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*. Other species such as *B. amyloliquefaciens*, *B. pumilus*, and *B. firmus* are less frequently used, but have also been reported in some products.

Other studies reported that using *Bacillus* probiotics in shrimp culture can reduce suspended solids and nutrient loads, as well as improve water quality through the removal of toxic metabolites such as ammonia (NH₃), hydrogen sulfide (H₂S), and nitrite (NO₂⁻) (Wang et al., 2005; 2007; 2008). In addition, *Bacillus* probiotic strains when added to feed can increase feed digestibility and stabilize intestinal flora (Balcázar et al., 2006; Newaj-Fyzul et al., 2014).

Despite their widespread use, farmers reported that the quality of probiotic products was not always guaranteed. Uddin et al. (2015), who analyzed probiotic products, reported that these products contained bacteria with lower concentrations than claimed and the bacterial composition was also different when compared to the claim on the product. In some cases, bacterial species listed on the label were not detected, while unlisted microorganisms were present. These findings underscore the need for stricter

quality control, standardization, and regulatory oversight of probiotic products used in shrimp farming to ensure their efficacy and safety.

Overall, probiotics play a multifaceted role in shrimp culture by simultaneously improving host nutrition, enhancing disease resistance, and stabilizing the culture environment. However, the effectiveness of probiotic applications largely depends on product quality, the selection of appropriate microbial strains, and proper management practices.

1.4 Anatomical and physiological characteristics of whiteleg shrimp

The whiteleg shrimp (*Penaeus vannamei*) exhibits a relatively simple but functionally specialized internal anatomy adapted to aquatic life. The digestive system consists of a foregut (including the mouth, esophagus, and stomach), a midgut associated with the hepatopancreas (digestive gland), and a hindgut terminating at the anus (Figure 7A). The hepatopancreas of shrimp (Figure 7B) plays a central role in digestion, absorption, and storage of nutrients, and is also considered the primary organ involved in xenobiotic metabolism and detoxification, including antimicrobial compounds (Duan et al., 2024).

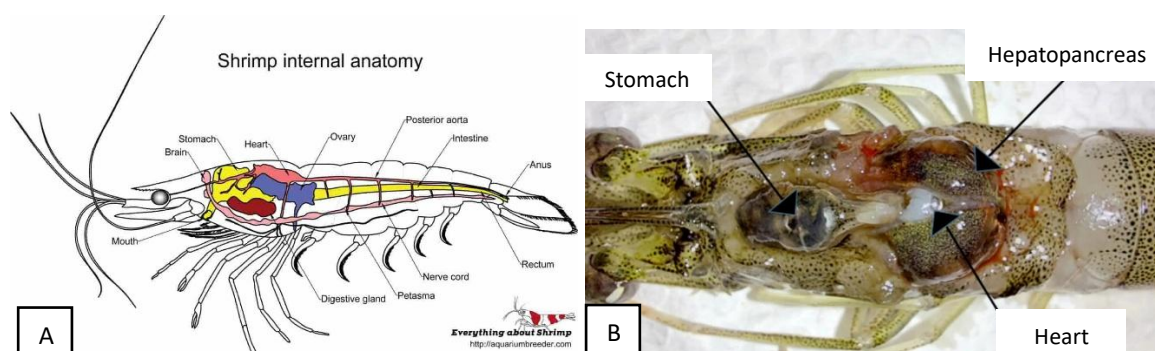


Figure 7. Internal anatomy of *Penaeus vannamei*. (A) Schematic representation of shrimp internal anatomy. (B) Dissection image highlighting key organs.

(*Aquariumbreeder, n.d.*)

The circulatory system of whiteleg shrimp is of the open type, meaning that hemolymph circulates freely within body cavities rather than remaining confined within closed blood vessels. The dorsal heart contracts to pump oxygenated hemolymph through several arteries (typically eight major arteries) to different tissues, facilitating the distribution of nutrients, oxygen, and xenobiotics (Dall et al., 1990). This system directly influences PK behavior, as demonstrated by the preferential accumulation of compounds in the hepatopancreas compared to muscle (Ma et al., 2019; Xu et al., 2022).

The nervous system is composed of a supraesophageal ganglion, which functions as the main neural ganglion (brain) coordinating sensory and physiological responses, and a ventral nerve cord. From a physiological perspective, whiteleg shrimp exhibits adaptive mechanisms such as osmoregulation and

metabolic adjustment to cope with environmental fluctuations. These processes are regulated at both molecular and systemic levels and are essential for maintaining homeostasis under varying salinity and stress conditions (Chen et al., 2015). In the context of this thesis, the hepatopancreas and hemolymph circulation are particularly important because they strongly influence the absorption, distribution, metabolism, and elimination of antibiotics in shrimp.

1.5 Pharmacokinetics of antibiotic in shrimp

1.5.1 Basic concepts of pharmacokinetics and pharmacodynamics in aquatic species

Pharmacokinetics

For a drug to be effective in a living organism, it must go through many processes. Once inside the body, the drug must be absorbed and the body must react to its effects. Pharmacokinetics is the science of the kinetics of absorption, distribution, metabolism, and excretion (ADME) of drugs in the body (American Pharmaceutical Association, 1972; Rodgers & Rowland, 2006; Jambhekar & Bree, 2009).

Absorption is the process by which a drug proceeds from the site of administration to the site of measurement (usually blood, plasma or serum) (Jambhekar & Bree, 2009). In aquatic species such as shrimp, drugs are commonly administered orally via medicated feed. Oral drug absorption is highly dependent on the structure and function of the gastrointestinal tract. Shrimp lack a true vertebrate stomach and possess a relatively short and structurally distinct digestive tract, which may influence drug dissolution, transit time, and extent of systemic uptake compared to other species (Vinarov et al., 2021). These anatomical features, along with environmental factors such as water temperature and feeding behaviour, can influence the rate and extent of drug absorption, and thus affect systemic bioavailability (Björklund & Bylund, 1991).

Distribution is the process of reversibly transferring the drug to and from the site of measurement. Any drug portion that leaves the site of measurement and does not return has undergone elimination. The rate and extent of drug distribution are a function of how well the tissues and/or organs are perfused with blood, the binding between drug and plasma proteins or tissue components, and the permeability of tissue membranes to the drug (Jambhekar & Bree, 2009). In shrimp, drug distribution occurs via the hemocirculatory system rather than a closed circulatory system. In species with an open circulatory system like shrimp, all of the organs and internal structures are constantly bathed in hemolymph, potentially leading to different equilibrium dynamics compared to vertebrates (Fang et al., 2018). The rate and extent of distribution are influenced by the physicochemical properties of the drug, particularly lipophilicity and degree of ionization. Lipophilic compounds may preferentially accumulate in the hepatopancreas, which plays a central role in storage and metabolism of xenobiotics. Tissue distribution is particularly important in aquaculture because edible muscle tissue determines food safety compliance and establishes drug withdrawal times.

Metabolism is defined as the conversion of one chemical species to another chemical species. Normally, metabolites show little or none of the activity of the parent compound; however, there are some exceptions, e.g., the metabolite of enrofloxacin is ciprofloxacin, which possesses higher antimicrobial activity than the parent compound (Jambhekar & Bree, 2009). In shrimp, drug metabolism primarily occurs in the hepatopancreas, a central organ involved in digestion, nutrient storage, and xenobiotic biotransformation, and is functionally comparable to the vertebrate liver in terms of detoxification processes (Livingstone, 1998; Vogt, 2019). Biotransformation typically involves Phase I reactions (oxidation, reduction, and hydrolysis), often associated with cytochrome P450 enzymes (James & Boyle, 1998). These reactions may be followed by Phase II conjugation processes that enhance water solubility and facilitate subsequent excretion (Taxak & Bharatam, 2014; Currie, 2018). Compared with terrestrial vertebrates, metabolic pathways in crustaceans are less extensively studied, and available evidence suggests interspecies variability in biotransformation capacity, leading to differences in elimination half-life and residue persistence, highlighting the need for species-specific PK studies.

Elimination is the irreversible loss of drug from the site of measurement and includes both metabolism and excretion. In vertebrates, the principal organs involved in drug elimination are the kidney and liver. The kidney is the primary site for excretion of a drug (in a chemically unchanged form) or its metabolites, while the liver is the primary site of drug metabolism, contributing to the overall elimination process. In shrimp, elimination occurs primarily through hepatopancreatic metabolism and excretion across the gills and digestive tract, reflecting fundamental physiological differences from finfish and terrestrial animals (Tang et al., 2022). In aquatic species, including shrimp, PK principles are generally consistent with those established for terrestrial animals; however, species-specific characteristics may lead to distinct drug disposition patterns that must be considered when interpreting PK data.

Several routes of drug administration are available in veterinary medicine, including oral, topical, intramuscular, subcutaneous, intravascular, nasal, pulmonary, and rectal routes. However, in shrimp aquaculture, oral administration via medicated feed represents the most practical and widely used method, while injection and immersion are less commonly applied. For intramuscular and subcutaneous routes of administration, bioavailability is generally high and often exceeds 75%, although it may vary depending on the drug formulation and physiological factors affecting absorption. Drugs that are administered by the intravascular route do not involve an absorption step. Oral administration is the most commonly used extravascular route. Oral drug absorption generally follows first-order kinetics. Figure 8 represents the plasma concentration–time curve for a drug administered by the oral route, characterized by parameters such as maximum concentration ($C_{\max}$) and time to reach maximum concentration ($T_{\max}$).

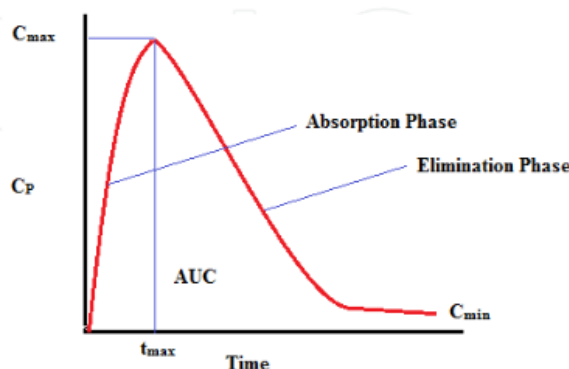


Figure 8. Schematic plasma concentration–time curve of a drug following oral administration, illustrating the absorption and elimination phases, maximum plasma concentration ($C_{\max}$), minimum plasma concentration ($C_{\min}$), time to reach maximum concentration ($T_{\max}$), plasma concentration (C_p), and area under the concentration–time curve (AUC) (Ahmed, 2015)

Once a drug is applied into the body, it will distribute into the different organs and body compartments. Analysis of the drug kinetics may be performed using compartmental or non-compartmental models (Ahmed, 2015). The compartment model describes drug disposition (distribution and elimination). In this model, when drug concentration in plasma and highly perfused organs change, it is assumed that the changes are equivalent in plasma and in tissue, and the elimination process occurs from the central compartment. Non-compartmental analysis is used to estimate PK parameters without deciding on a particular compartmental model.

In PK analysis, several parameters are investigated such as maximal concentration ($C_{\max}$), time to reach maximum concentration ($T_{\max}$), area under the concentration–time curve (AUC), the absorption half-life ($T_{1/2ab}$), the absorption rate constant (K_{ab}), the elimination half-life ($T_{1/2el}$), the elimination rate constant (K_{el}), volume of distribution (V_d) and total body clearance (CL) (Jambhekar & Breen, 2009; Zhang et al., 2017). These parameters are fundamental for understanding drug exposure and optimizing dosing regimens. Nevertheless, PK parameters can differ significantly between or within species, influenced by factors such as species specificity, health status, geographical location, age, size, salinity, water temperature, drug administration routes, and other experimental conditions (Björklund & Bylund, 1991; Zhang & Li, 2007).

Pharmacodynamics

While PK describes what the body does to a drug, pharmacodynamics (PD) refers to the relationship between drug concentration at the site of action and the resulting biological or antimicrobial effect. For antibacterial agents, PD activity is commonly characterized using *in vitro* susceptibility parameters such

as the MIC. Integration of PK and PD concepts (pharmacokinetics/pharmacodynamics, PK/PD) provides a quantitative framework for linking drug exposure to antimicrobial efficacy and has become a cornerstone for the rational design of dosing regimens. Key PK/PD indices, including the ratio of peak concentration to MIC ($C_{\max}/\text{MIC}$), the ratio of area under the concentration–time curve to MIC (AUC/MIC), and the duration for which drug concentrations exceed the MIC ($T>\text{MIC}$), are closely associated with the antibacterial activity of different classes of antibiotics (Toutain et al., 2002; Nielsen & Friberg, 2013).

In aquaculture, establishing appropriate PK/PD indices is essential for optimizing antibiotic dosing strategies against bacterial infections, ensuring therapeutic effectiveness while minimizing the risk of antimicrobial resistance development. Integrating PK parameters such as $C_{\max}$ and $\text{AUC}_{0-24\text{h}}$ with appropriate PD parameters, particularly MIC values, to determine the appropriate therapeutic dose and treatment interval for antibiotics ensures their effective application in controlling bacterial infections in aquatic species (Mothadaka et al., 2023). In shrimp aquaculture, optimization of antimicrobial therapy based on PK/PD principles is essential for improving treatment outcomes and supporting the responsible use of antibiotics. Because antibiotics used in shrimp farming are often administered through medicated feed, dosing regimens need to be designed based on the specific PK characteristics of each shrimp species and the culture environmental conditions. However, the practical application of PK/PD principles in shrimp farming is still limited at present.

1.5.2 Factors affecting antibiotic pharmacokinetics in shrimp

The PK of antibiotics in shrimp is influenced by a complex interplay of biological, environmental, and management-related factors. As ectothermic aquatic invertebrates, shrimp are strongly affected by external conditions, which can influence drug absorption, distribution, metabolism, and elimination. Furthermore, PK parameters can differ significantly between or within species, influenced by factors such as species specificity, health status, geographical location, age, size, salinity, water temperature, drug administration routes, and other experimental conditions (Björklund & Bylund, 1991; Zhang & Li, 2007).

Species-specific physiology and anatomical characteristics. In shrimp, the open circulatory system allows hemolymph to directly bathe internal organs and tissues, facilitating rapid distribution of drugs (Wu et al., 2006; Fang et al., 2018). In addition, the relatively simple digestive tract may contribute to faster but potentially less consistent oral absorption compared to vertebrates (Vinarov et al., 2021). These physiological characteristics are consistent with the relatively short $T_{\max}$ values observed in the present study. Furthermore, there was evidence that drug absorption also depends on physiological differences between species, specifically, the structure of the intestine and the species-specific response to environmental factors (Kverme et al., 2019). For example, at the same applied dose (10 mg FF/kg

bw), the $T_{\max}$ value in plasma of striped catfish was 3.70 h (Pham et al., 2023), while in the present study, the $T_{\max}$ value in shrimp plasma was 1.77 h (Huynh et al., 2025).

Route of administration and feeding behavior. In shrimp aquaculture, oral medication administration is widely used through the addition of drugs to feed. This is a non-invasive method; therefore, it has the advantage of not causing stress to the animals and allows for large-scale treatment (Bianchini & Baldisserotto, 2021). However, drug absorption is closely linked to feeding behavior, feed intake rate, and feeding frequency. Uneven feed consumption, feed loss to the water column, and competition among shrimp may result in variable individual drug exposure within the same population (Rico et al., 2013). These factors may contribute to variability in PK parameters such as $C_{\max}$ and AUC.

Environmental conditions. Environmental factors, particularly water temperature, play a critical role in shaping antibiotic PK in shrimp. As ectothermic organisms, shrimp exhibit temperature-dependent metabolic rates; increases in water temperature generally accelerate drug absorption and elimination, whereas lower temperatures may prolong drug persistence in tissues (Feng et al., 2019). Several studies have demonstrated that changes in temperature and salinity can lead to marked differences in antibiotic depletion rates in shrimp, underscoring the need to consider environmental conditions when interpreting PK data and establishing withdrawal times (Rairat et al., 2023).

Health status and pond management practices. Shrimp health status is another important determinant of antibiotic PK. Disease conditions affecting the hepatopancreas or digestive tract can impair drug absorption and metabolism, leading to altered systemic exposure (Bianchini & Baldisserotto, 2021). In addition, pond management practices, including water exchange rate, stocking density, and water quality management, can indirectly affect PK by impacting shrimp stress levels, feeding behavior, and metabolic activity of shrimp (Campa-Córdova et al., 2022; Lin et al., 2024).

1.5.3 Variations in reported pharmacokinetic parameters of different antibiotics in whiteleg shrimp

Although PK studies have provided essential information on the absorption, distribution, and elimination of antibiotics in shrimp, the available PK data remain limited and heterogeneous. Most published studies focus on individual antibiotics under specific experimental conditions, including particular doses, routes of administration, and sampling designs, which restricts direct comparison across studies. As illustrated in Table 1, PK parameters such as $T_{\max}$, $C_{\max}$, AUC, and elimination half-life show substantial variability among different antibiotics in whiteleg shrimp, depending on the route of administration, target tissue, and dosage regimen. This difference reflects both drug-specific properties and tissue-specific disposition patterns in shrimp. In addition, variability in experimental design, including single versus multiple dosing, sampling intervals, analytical methodologies, and PK modeling approaches, further contributes to differences in reported PK parameters.

Table 1. Pharmacokinetic parameters of antibiotics in whiteleg shrimp following different routes of administration in muscle, hepatopancreas, and hemolymph

Antibiotic	Muscle				Hepatopancreas				Hemolymph				References
	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	
	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/L)	(mg h/L)	(h)	
Single dose													
Oxytetracycline* 100 mg/kg bw	6	0.73	32.63	23.53	1	149.5	1015	14.89	6	27.77	691.3	11.01	(Ma et al., 2019)
Oxytetracycline** 10 mg/kg bw	6	7.94	166.5	20.90	-	-	-	-	0	32.22	266.1	20.74	(Chiayvare esajja et al., 2006)
Thiamphenicol* 10 mg/kg bw	2	2.98	29.33	6.84	1	204.2	1381	31.29	2	7.96	43.96	10.66	(Fang et al., 2013)
Florfenicol* 150 mg/kg bw	0.5	5.94	7.96	1.44	-	-	-	-	0.4	6.84	8.25	0.75	(Chuchird et al., 2025)

Antibiotic	Muscle				Hepatopancreas				Hemolymph				References
	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	
	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/L)	(mg h/L)	(h)	
Florfenicol* 10 mg/kg bw	2	1.91	16.04	18.32	0.5	164.2	902.2	41.38	2	5.53	41.27	17.36	(Fang et al., 2013)
Sulfamethoxazole* 83.3 mg/kg bw	1	21.5	203.9	6.53	0.5	89.54	381.1	8.22	1	36.52	395.3	13.76	(Ma et al., 2017)
Trimethoprim* 16.7 mg/kg bw	1	0.78	1.90	2.12	0.5	73.17	321.3	4.53	1	0.89	4.82	7.38	(Ma et al., 2017)
Enrofloxacin*** 10 mg/kg bw	0.25	5.81	90.1	52.3	0.5	11.23	274.2	75.8	0.08	4.87	75.8	19.8	(Fang et al., 2018)
Multiple dose													
Enrofloxacin* 30 mg/kg bw	1	1.96	34.75	10.92	3	16.29	314.9	15.86	1	19.64	299.6	16.07	(Ma et al., 2018)

Antibiotic	Muscle				Hepatopancreas				Hemolymph				References
	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	
	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/kg)	(mg h/kg)	(h)	(h)	(mg/L)	(mg h/L)	(h)	
Oxytetracycline* 100 mg/kg bw	6	1.67	66.05	35.39	6	124.3	1858	18.89	6	68.03	2049	20.52	(Ma et al., 2019)
Sulfamethoxazole* 83.3 mg/kg bw	1	47.8	403.5	10.92	1	73.73	756.5	11.33	1	85.89	1493	11.03	(Ma et al., 2017)
Trimethoprim* 16.7 mg/kg bw	1	0.80	3.03	2.71	1	46.50	190.2	9.65	1	1.14	4.84	5.25	(Ma et al., 2017)

T_{max} : mean time to maximal concentration (h), C_{max} : mean maximal concentration (mg/kg or mg/L), $AUC_{0-\infty}$: mean area under the concentration-time curve from 0 h to ∞ (mg h/kg or mg h/L), $T_{1/2el}$: mean elimination half-life of drug (h), * oral administration, ** intra-sinus administration, *** intramuscular administration

Overall, the currently available PK studies demonstrate that antibiotic disposition in whiteleg shrimp is highly variable and influenced by multiple biological, environmental, and experimental factors. These findings highlight the complexity of predicting antibiotic behavior in shrimp and emphasize the need for carefully designed PK studies under relevant culture conditions.

1.6 Impact of antibiotics on shrimp health and hepatopancreas histology

In addition to influencing antibiotic pharmacokinetics, antimicrobial exposure may also affect shrimp health and tissue integrity, particularly in the hepatopancreas, which is a major organ involved in metabolism and detoxification. Therefore, understanding the structure and histological responses of the hepatopancreas is important for evaluating the biological effects of antibiotics in shrimp.

1.6.1 Shrimp hepatopancreas structure and function

The hepatopancreas (also called the midgut gland) of shrimp and other crustaceans is the most sensitive organ to drug and chemical exposure. In shrimp, the hepatopancreas performs multiple physiological functions that are comparable to those of the liver, pancreas, and intestinal glands in vertebrates, including digestion, nutrient absorption, metabolism, and detoxification. Owing to its central physiological role and high metabolic activity, the hepatopancreas has been a major focus of histological and biological investigations for more than a century (Caceci et al., 1988). Under the microscope, the organ is composed of several blind-ended, finger-like tubules, which together form a highly branched tubular system (Figure 9). Each tubule represents a functional unit of the organ and is lined by a simple epithelial layer surrounding a central lumen (Caceci et al., 1988). This tubular organization provides an extensive surface area that facilitates the secretion of digestive enzymes into the lumen and the subsequent absorption of digested nutrients.

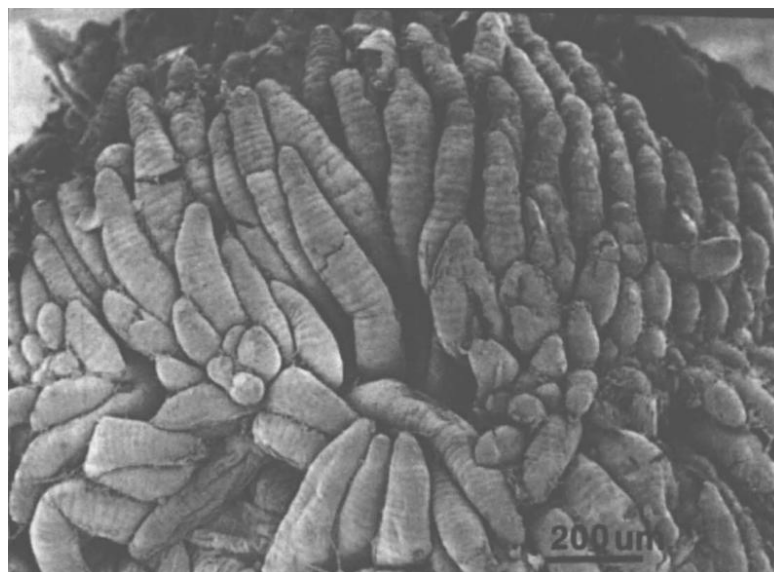


Figure 9. Low-power scanning electron micrograph of the shrimp hepatopancreas, showing the arrangement surface of the organ into a large number of blind-ended tubules (Caceci et al., 1988)

The tubule walls are composed of a simple epithelium, which contains five cell types including blasenzellen cells (B cells), embryonic cells (E cells), fibrillenzellen cells (F cells), restzellen cells (R cells) and midgut or basal cells (M cells). These cell types are not randomly distributed but show a clear

spatial organization along the longitudinal axis of the tubules. E cells are located at the narrow distal ends of the tubules and serve as the proliferative cell population. From these E cells, the differentiated B, F, and R cells are generated and subsequently migrate toward the medial and proximal regions of the tubules (Pathak et al., 2018). Young R cells and F cells are typically found near the distal region, whereas B cells are more abundant in the middle and proximal portions of the tubules (Pathak et al., 2018).

Each hepatopancreatic cell type is characterized by distinct cytological features that reflect its specialized function. E-cells are relatively featureless, and confined to the apical ends of the tubules, consistent with their role in cell renewal, as they are non-specialized cells which divide mitotically to replace other cell types that are worn out. F-cells are very large with a cytoplasm noticeably metachromatic, indicative of intense protein synthesis activity, and are responsible for the synthesis of digestive enzymes, which are stored in a single large vesicle before the cells differentiate into B-cells (Hu & Leung, 2007). B-cells are very prominent under the light microscope, with one or two very large vacuoles filled with flocculent material. These cells are involved in the release of digestive enzymes into the hepatopancreatic lumen through a holocrine secretion process. R cells contain multiple basal vacuoles rich in lipid material, reflecting their role in nutrient absorption and storage, and play a central role in the absorption of digested nutrients from the lumen and their storage within intracellular vesicles, thereby serving as major nutrient reservoirs for the shrimp. In contrast, M-cells are located at the basal part of the hepatopancreatic epithelium and have no direct contact with the hepatopancreatic lumen (Caceci et al., 1988; Nunes et al., 2014). The anatomical location and sectioning orientation of the hepatopancreas used for histological analysis are illustrated in Figure 10.

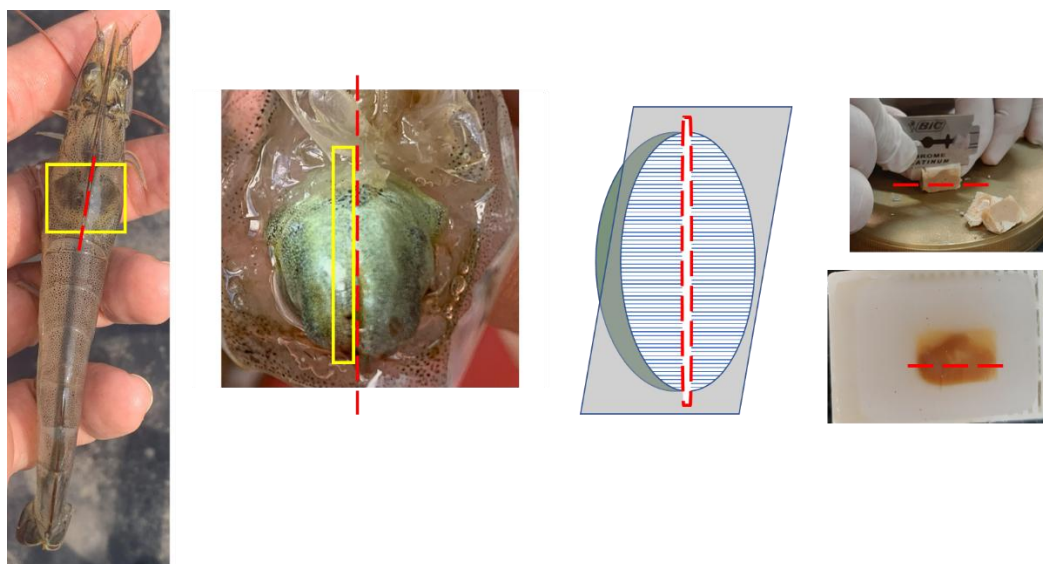


Figure 10. Anatomical location and sectioning of the hepatopancreas in whiteleg shrimp used for histological analysis in the present study. The red dashed line indicates the midgut position, while the yellow box represents the sectioning area used for hepatopancreas sampling and histological preparation

In general, the complex tubular organization and functional specialization of hepatopancreatic cells underpin the central role of this organ in shrimp physiology. Simultaneously, these characteristics explain why the hepatopancreas is particularly vulnerable to xenobiotics, including antibiotics (Vogt, 2019). Therefore, structural or functional alterations in specific hepatopancreatic cell types are commonly used as sensitive histopathological indicators of chemical and drug-induced stress in shrimp.

1.6.2 Effect of antibiotic/chemical on shrimp hepatopancreas histology

Hepatopancreas is widely regarded as a reliable indicator of shrimp health. Because of its central roles in digestion, nutrient storage, metabolism, and detoxification, histological alterations in the hepatopancreas are commonly used as indicators of stress, disease, and toxic exposure in shrimp (Manan et al., 2015). Because of its important functions, any damage to or cellular changes in this organ lead to negative effects, e.g., reduced growth, decreased immunological activity, or death.

Pathological alterations of the hepatopancreas are particularly evident in shrimp affected by AHPND, where this organ represents the main target tissue (Oanh et al., 2022). Infected shrimp lack mitotic activity in the E cells of the hepatopancreas, show dysfunction of the central hepatopancreatic B-, R-, and F-cells, sloughing of the central hepatopancreatic tubule epithelium and intertubular hemocytic aggregation followed by secondary bacterial infections (Lightner et al., 1996).

In addition to infectious diseases, several authors (Avunje et al., 2021, Maftuch et al., 2017, Romano et al., 2015, Bray et al., 2006) have demonstrated that the improper use of antibiotics or chemicals has caused serious adverse effects on shrimp health, especially on sensitive organs like hepatopancreas. The hepatopancreas is known as an organ of xenobiotic metabolism, where drugs and their metabolites are transformed before elimination from the shrimp body (Rewitz et al., 2006). However, this metabolic process is not instantaneous, and prolonged exposure or high doses may overwhelm cellular detoxification capacity, resulting in structural damage. Experimental studies have provided clear evidence of dose- and duration-dependent hepatopancreatic lesions following antibiotic administration. For example, Avunje et al. (2021) reported that feeding shrimp with feed containing oxytetracycline at 9 g/kg and 18 g/kg feed induced sloughing of hepatopancreatic tubular epithelial cells and degeneration, and hypertrophy of hepatopancreatocytes persisted until 14 days after treatment cessation, indicating that tissue recovery requires considerable time (Avunje et al., 2021). Similarly, Bray et al. (2006), when using 13.5 and 22.5 g oxytetracycline per kg of feed, reported reduced levels of lipid droplet storage in hepatopancreatic R-cells, as well as necrosis and sloughing of the hepatopancreatic tubule epithelium, the almost complete absence of hepatopancreatic lipids, and moderate atrophy of the hepatopancreas tubules in proximal portion. At the higher dose, the tubular atrophy in the hepatopancreatic cells was observed until 42 days, indicating the development of chronic pathological changes associated with prolonged antibiotic exposure. Comparable hepatopancreatic damage has also been reported for other

antibiotics. Destruction of hepatopancreatic cells through shrinkage of microvilli, atrophy, inflammatory cell infiltration and plasma leakage from blood vessels were observed when feeding whiteleg shrimp with enrofloxacin at a dose of 200 mg/kg body weight (Maftuch et al., 2017). These findings further support the notion that the higher the dose of antibiotics used, the more severe was the impact on the hepatopancreatic structure and function.

Besides the adverse effects of antibiotics, a decrease in the number of B cells in shrimp hepatopancreas was also found when feeding shrimp with other chemicals like an organic acids blend, which consisted of formic, lactic, malic and citric acids (Romano et al., 2015). Although such compounds are often applied to improve gut health or feed efficiency, these observations suggest that excessive or inappropriate use may negatively affect sensitive hepatopancreatic cells.

This evidence of negative effects on the hepatopancreas after prolonged treatment with excessive doses should be a strong caution to shrimp farmers who have overused antibiotics or chemicals in shrimp culture operation. Continuous or high-dose exposure not only compromises hepatopancreas integrity but may also impair immunity, reduce growth performance, and increase mortality risk, factors that collectively undermine sustainable aquaculture practices. In addition, these findings demonstrate that histological changes in the hepatopancreas are a sensitive indicator for evaluating antibiotic use in shrimp culture. Nevertheless, to date, detailed histopathological evidence demonstrating antibiotic-induced damage to the shrimp hepatopancreas remains limited. Among published studies, oxytetracycline and enrofloxacin are the antibiotics most consistently reported to cause significant hepatopancreatic damage, including epithelial sloughing, cellular degeneration, lipid depletion, and tubular atrophy. For many other antibiotics used in shrimp aquaculture, information on hepatopancreatic histological responses is scarce or has not been investigated, highlighting a significant gap in the current literature.

1.7 Antibiotic residues in shrimp and food safety concerns

1.7.1 Occurrence of antibiotic residues in Vietnamese shrimp products

Antibiotic residue occurrence in shrimp products imported from Vietnam into the European Union

Data extracted from the Rapid Alert System for Food and Feed (RASFF, 2025) for the period 2020-2025 show that the detection of antibiotic residues in shrimp products imported from Vietnam into the European Union occurred occasionally (Figure 11). From 2020 to 2022, notifications related to antibiotic residues in shrimp were rare and limited to isolated cases, mainly involving oxytetracycline (2020) and chloramphenicol (2020), as well as a single case of sulfamethoxazole detected in 2021 and ciprofloxacin in 2022. During this period, no detections were reported for tetracycline, DOX,

furazolidone, or sulfamethoxazole, suggesting either effective compliance with EU residue regulations or limited occurrence of these residues.

From 2023 onwards, a marked shift has been observed, characterized by a simultaneous increase in both the number of notifications and the diversity of antibiotic classes detected in shrimp products imported into the EU. In particular, antibiotics belonging to the tetracycline group, including tetracycline, oxytetracycline, and DOX, consistently appeared during the 2023–2025 period, indicating their frequent presence in monitored shipments. However, the temporary increase in DOX detections in 2023, followed by a subsequent decline, may reflect corrective measures by exporting producers or stricter regulatory oversight following initial non-compliance. In contrast, tetracycline detections remained stable in 2023–2024 and were no longer reported in 2025. Notably, furazolidone residues were repeatedly detected from 2023 to 2025, with a marked increase in notifications in 2025. Given that furazolidone belongs to the nitrofurans group, which is strictly prohibited in the EU due to its carcinogenic and genotoxic potential (EFSA, 2015), the repeated detection of furazolidone in shrimp products indicates that control of the use of banned antibiotics in shrimp farming is not entirely effective.

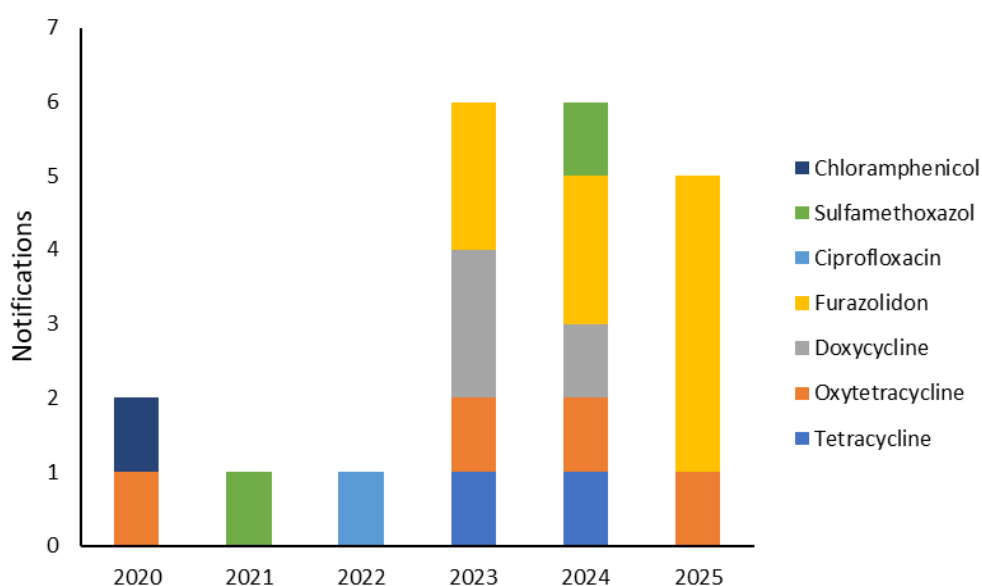


Figure 11. Rapid Alert System for Food and Feed notifications related to antibiotic residues detected in shrimp products imported from Vietnam into the European Union during 2020–2025 (RASFF, 2020–2025)

Antibiotic residue occurrence in shrimp products imported from Vietnam into Japan

In contrast to the EU, monitoring data from Japan between 2020 and 2025 reveal a different pattern in both the frequency and types of antibiotic residues detected in imported shrimp products (MHLW, 2025). Enrofloxacin and DOX dominated residue detections from 2020 to 2025 (Figure 12).

Enrofloxacin was detected throughout the entire study period. High numbers of notifications were observed between 2020 and 2022, followed by a decrease in 2023 and a subsequent increase in 2024, before declining again in 2025. This fluctuating pattern suggests variable reliance on fluoroquinolones over time rather than a sustained reduction, potentially reflecting episodic disease pressure. Nevertheless, the repeated detection of enrofloxacin across multiple years indicates that fluoroquinolone use remains an integral component of disease management in certain shrimp farming systems. Doxycycline detections were absent in 2020 and 2021 but appeared in 2022 and reached a pronounced peak in 2023, before decreasing in 2024 and rising again in 2025. The reappearance of DOX detections in 2025 suggests that control measures may not have been uniformly or sustainably implemented in shrimp culture. Furazolidone residues were rarely detected in Japan, with only one case reported in 2024. Despite the limited frequency, the presence of the banned nitrofurans compound is noteworthy.

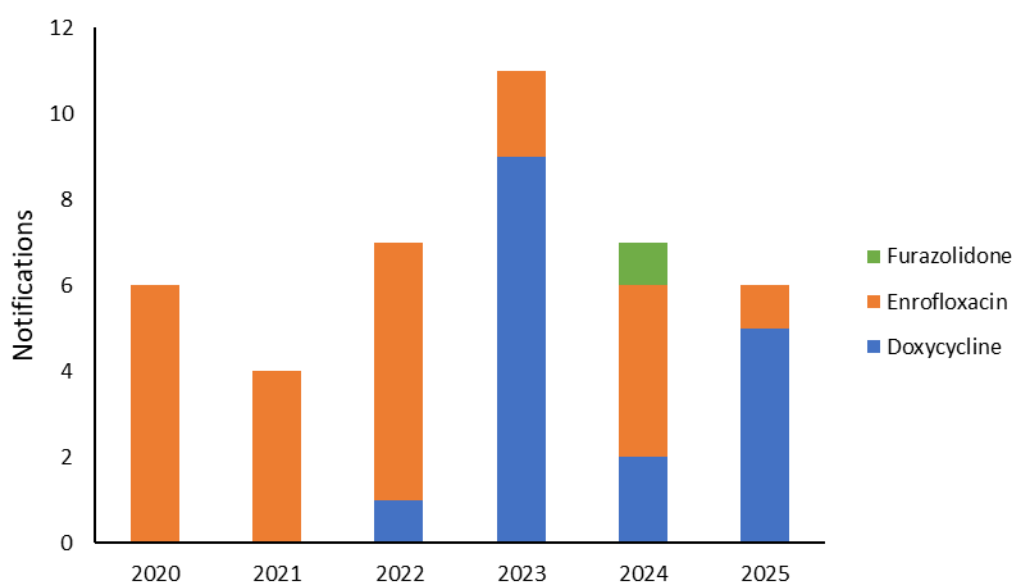


Figure 12. Ministry of Health, Labor and Welfare notifications related to antibiotic residues detected in shrimp products imported from Vietnam into Japan during 2020–2025 (MHLW, 2020–2025)

Comparative perspective between the European Union and Japan

Differences were observed in the patterns of antibiotic residue in Vietnamese shrimp products imported into the European Union and Japan from 2020 to 2025. In the EU, the number of reports was relatively low but showed an increase in both frequency and diversity of antibiotics after 2022, particularly concerning tetracyclines and furazolidone. In contrast, Japan reported a higher number of detections during the 2020–2025 period, mainly detecting two antibiotics: enrofloxacin and DOX. Instead of a continuous downward trend, these antibiotics showed fluctuations in frequency year by year, indicating

the continuous use and adaptation of antibiotic treatments in shrimp farming. In summary, the warnings from these markets underscore the need to combine prudent antibiotic use, effective withdrawal periods, and robust residue monitoring to ensure compliance with international food safety standards.

1.7.2 Maximum residue limits and international trade requirements

Maximum residue limits (MRLs) are regulatory thresholds established to protect consumers from unacceptable exposure to veterinary drug residues. An MRL is defined as the maximum concentration of residue legally permitted or considered acceptable in food under the regulations of a given jurisdiction or regulatory authority. Responsibility for ensuring compliance with MRLs lies with veterinarians and farmers through the appropriate use of licensed veterinary medicines. MRLs are derived from toxicological reference values, particularly the ADI, and are supported by residue depletion data obtained under controlled experimental conditions (Codex Alimentarius Commission, 2018).

In international trade, foods of animal origin in general, and aquaculture products in particular, must comply with the food safety standards of the importing country, usually national MRLs or Codex MRLs (Reeves, 2010). Although compliance with these standards is essential for international trade, their primary purpose is to protect public health by ensuring that consumers are not exposed to unsafe levels of veterinary drug residues. When no MRLs are established for a veterinary drug, this may indicate a regulatory ban or, more commonly, a lack of sufficient data to assess its safety, although in some cases certain substances may not require MRLs if they are considered of low concern for human health. In international shrimp trade, compliance with maximum residue limits (MRLs) is a fundamental requirement for market access. However, MRL values for the same antibiotic may vary between importing countries, reflecting differences in regulatory approaches and risk management decisions. For example, the MRL for DOX in shrimp muscle is set at 50 µg/kg in Japan (JFCRF, 2017), whereas a higher MRL of 100 µg/kg is established in the European Union (EMA, 2015). However, for FF, an MRL of 100 µg/kg for the sum of FF and its metabolite (FF amine) has been established by the European Commission (EC, 2010) and is also applied by the Korean regulation (Korean Ministry of Food and Drug Safety, 2019). These differences indicate that shrimp products compliant with residue limits in one market may not meet the requirements of another, leading to border rejection.

Differences in MRL requirements among importing countries show that residue limits are not only related to food safety but also directly affect access to international shrimp markets. For exporting countries, this creates practical challenges when the same antibiotic is regulated at different levels across markets. Whether these requirements can be met largely depends on how antibiotics are depleted in shrimp tissues following treatment. Withdrawal time refers to the minimum period required between the final administration of a veterinary drug and the harvest of aquatic animals intended for human

consumption. This period is established based on residue depletion studies and is used to ensure that antibiotic concentrations in edible tissues fall below the applicable MRLs before harvest (European Commission, 2010; Codex Alimentarius Commission, 2018). In practice, withdrawal times are important tools for supporting the responsible use of antibiotics, protecting consumer health, and reducing the risk of non-compliant shrimp products entering international markets. Experimental studies on antibiotic PK and depletion in whiteleg shrimp are thus important for supporting residue control and withdrawal time determination in commercial shrimp production.

1.8 Dietary exposure and risk assessment related to antibiotic residues

Risk assessment related to antibiotic residues in food is part of the broader framework of risk analysis, which also includes risk management and risk communication (Brimer, 2011). Risk assessment is a scientific framework used to estimate and evaluate the potential adverse effects of physical, biological, or chemical hazards on human health (Benford, 2013).

The process of **risk assessment** involves four main steps. First is hazard identification, which determines whether a chemical can cause adverse health effects. This is followed by hazard characterization, which includes both a qualitative evaluation of the effects caused by the hazard and a quantitative analysis of the relationship between dose and response. Next is the exposure assessment, which estimates the level of exposure to the hazard through food consumption. The final step, risk characterization, is conducted by comparing the estimated exposure level with toxicological reference values, most commonly the ADI.

For hazard characterization, toxicological studies are usually conducted in laboratory animals (rodents) and include the studies of acute toxicity, short-term toxicity (at least 90 days), long-term toxicity (up to 2 years), reproductive toxicity, and delayed neurotoxicity (Cabras, 2003). These studies are carried out for all chemicals for which authorization for use is requested. The outcomes of these toxicological studies allow determining the No Observed Effect Level (NOEL), defined as the highest dose at which no adverse effects are observed. The NOEL of long-term studies of the most sensitive species is used to determine the ADI with the application of a correction factor between 10 and 1000, the factor of 100 being usually used to calculate ADI value. The ADI is expressed as milligrams of substance per kilogram of body weight per day (mg/kg body weight/day).

Once the ADI is established through scientific **risk assessment**, risk management translates this information into practical guidelines or regulatory measures, such as establishing MRLs and withdrawal times for veterinary drugs in food products. Risk communication then plays a critical role in disseminating information about the risk and the decisions made, ensuring that all stakeholders, from policymakers to the public, understand the basis and implications of those decisions.

In the particular case of antibiotic residues in food of animal origin, one of the primary concerns for human health is the potential development of antimicrobial resistance, as continuous exposure to sub-therapeutic concentrations of antibiotics may promote the selection of resistant bacteria in the human microbiota (Marshall and Levy, 2011; Done et al., 2015). In addition, allergic or hypersensitivity reactions may occur in sensitive individuals (Dayan, 1993), and some antibiotics have been associated with effects on gut microbiota or, in rare cases, organ toxicity (EMA, 2015). These aspects highlight the importance of monitoring antibiotic residues in food products and evaluating their potential contribution to human health risks.

In dietary **risk assessment**, the exposure of consumers to chemical residues is commonly estimated using the Estimated Daily Intake (EDI), which reflects the amount of a compound ingested per kilogram of body weight per day. The EDI is calculated by multiplying the residue concentration of a compound by the estimated daily food intake and dividing by the average body weight (Juan et al., 2010). The risk can be assessed by calculating the Hazard Quotient (HQ) expressed as the ratio of EDI to ADI. A HQ value higher than or equal to 1 suggests that the particular compound has the potential to cause adverse effects on human health while a value lower than 1 indicates negligible effects, thus not posing any significant risk (Kumari et al., 2020 and Bishnoi et al., 2025).

In this thesis, dietary risk assessment was conducted using a deterministic approach based on measured antibiotic residue concentrations in shrimp, shrimp consumption data, body weight data, and comparison of EDI values with established ADIs through HQ calculations. In this study, the ADI values used for risk assessment were 3 µg/kg bw/day for DOX and oxytetracycline, based on microbiological effects on the human intestinal microbiota (a no-effect level of 2 mg per person per day was observed for the induction of resistant *Enterobacteriaceae* in humans, to which a safety factor of 10 was applied to establish an ADI of 3 µg/kg bw/day, taking into account a body weight of 70 kg) (EMA, 2015, Marshall and Levy, 2011). For both enrofloxacin and ciprofloxacin, the applied ADI was 2 µg/kg bw/day, also based on microbiological effects (JECFA, 1998, cited in Ji et al., 2010, Marshall and Levy, 2011). The ADI for sulfamethoxazole was considered within the sulfonamide group, for which an ADI of 50 µg/kg bw/day has been established based on toxicological effects, particularly thyroid-related alterations, observed in experimental studies using representative compounds such as sulfadimidine (JECFA, 1995).

1.9 Knowledge gaps and rationale of the PhD thesis

The Mekong Delta, located in southern Vietnam, is the country's most important aquaculture region and contributes substantially to national shrimp production and export value. Shrimp farming plays a crucial role in Vietnam's agricultural economy, particularly in the Mekong Delta, where production has increased rapidly over the past decade. Nevertheless, intensive and super-intensive shrimp farming has

led to increased vulnerability to bacterial diseases, which can significantly impact production yields and economic stability (Nguyen et al., 2020). To control bacteria in shrimp culture, effective disease management strategies used by farmers often involve the use of antibiotics (Rico et al., 2014; Phu et al., 2016; Chi et al., 2017). However, inappropriate or uncontrolled antibiotic use may result in antibiotic residues in shrimp products, raising concerns regarding food safety and consumer health. In practice, information on how these antibiotics are used, how long they persist in shrimp tissues, and how they may affect consumer exposure remains limited. This lack of scientific evidence makes it difficult for farmers and authorities to optimize antibiotic use while ensuring the safety of shrimp products. Therefore, the rationale of this PhD thesis is to address this gap by combining different but complementary approaches.

First, a survey on the use of antibiotics and chemicals in shrimp farming was conducted to identify commonly used antibiotics, thereby providing a basis for selecting commonly used antibiotics to represent subsequent experimental studies. Although antibiotics such as CFT, FF, and DOX are reported to be used in shrimp culture, data on their PK behavior, PD characteristics, tissue depletion, and withdrawal times in whiteleg shrimp are still lacking, whereas PK characteristics of antibiotics have mainly been investigated in terrestrial animals or fish species. According to Vinarov et al. (2021), shrimp differ markedly from terrestrial animals and fish in physiology and metabolism, and extrapolation of withdrawal periods from other species may therefore lead to inaccurate residue control. In addition, PK parameters within a single species may differ due to factors like geographic origin and genetic variation caused by selective breeding and artificial selection (Toutain et al., 2010; Ren et al., 2016).

Furthermore, scientific studies on the depletion of antibiotic residues and the calculation of withdrawal times are essential to ensure that antibiotic residues in shrimp tissue are reduced to acceptable levels before harvest and to comply with food safety requirements. The use of antibiotics in disease management may result in negative health effects on cultured shrimp, especially in the hepatopancreas, which is responsible for chemical metabolism and the normal growth and development of shrimps (Maftuch et al., 2017; Bray et al., 2006). Data on the potential effects of CFT, FF, and DOX on the hepatopancreas of whiteleg shrimp are currently scarce, despite their reported use in shrimp aquaculture.

At the consumer level, data on antibiotic residues in commercial shrimp products in Vietnam, particularly at the retail level, remains scarce. This lack of information on residues in the market limits the ability to link antibiotic use at farms to consumer exposure and underscores the need for systematic residue monitoring to manage food safety in shrimp farming.

The outcomes of this research will, on the one hand, provide essential knowledge on PK and withdrawal times of selected antibiotics (CFT, FF, and DOX) in whiteleg shrimp, which will facilitate the

establishment of guidelines for the optimal use of these antibiotics in shrimp and, on the other hand, provide an improved understanding of the impact of these antibiotics on hepatopancreas histology in whiteleg shrimp aquaculture during and after medication. In addition, monitoring antibiotic residues in markets will generate data to support dietary exposure and risk assessment for shrimp consumers in Vietnam.

2. Objectives

General objectives

The general objective of this research is to generate scientific evidence to support the reduction and optimization of antibiotic use in shrimp aquaculture, while minimizing the risk of antibiotic contamination in shrimp products, thereby contributing to the sustainable development of the shrimp industry in Vietnam. In addition, this research also aims to assess the potential dietary exposure and health risks associated with antibiotic residues in shrimp products, in order to support food safety management and consumer protection.

Specific objectives

1. To conduct a survey on the use of antibiotics and chemicals in super-intensive whiteleg shrimp aquaculture systems in the Mekong Delta, Vietnam, in order to identify the most commonly used antibiotics for subsequent experimental studies.
2. To investigate the PK, PD, residue depletion, and withdrawal times of selected antibiotics identified from the survey (CFT, FF, and DOX) in whiteleg shrimp (*Penaeus vannamei*) following oral administration.
3. To evaluate the effects of selected antibiotics on shrimp hepatopancreas histology under experimental conditions and assess potential impacts on shrimp health associated with antibiotic exposure.
4. To determine the occurrence of antibiotic residues in commercial whiteleg shrimp products and to conduct dietary exposure and risk assessment for shrimp consumers in Can Tho City, Vietnam.

3. Experimental section

This experimental section consists of three interconnected studies addressing antibiotic use, pharmacokinetics, pharmacodynamics, residue depletion, hepatopancreas histology, and consumer risk assessment in whiteleg shrimp aquaculture in the Mekong Delta, Vietnam. The overall study design is summarized in Figure 1.

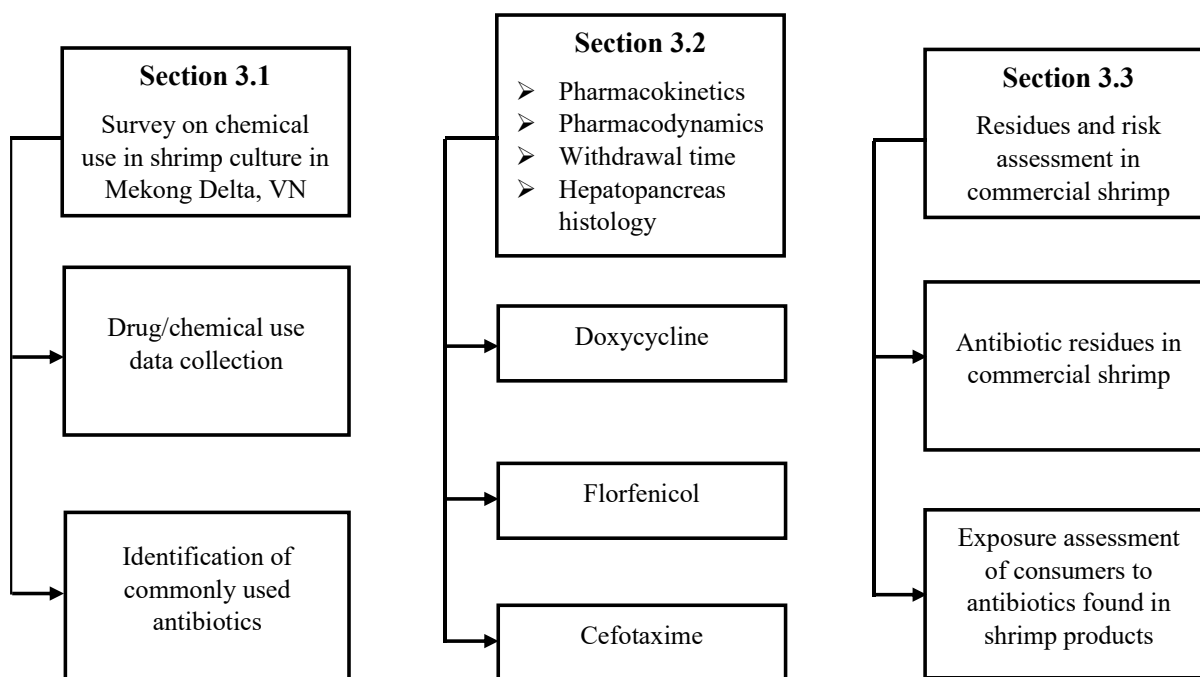


Figure 1. Overall study design and relationship between the experimental studies included in this PhD thesis

The first study (**Section 3.1**) investigated the use of chemicals and antibiotics in super-intensive whiteleg shrimp farming systems in the Mekong Delta. This survey study provided baseline information on farming practices and identified commonly used antibiotics in shrimp aquaculture, which served as the basis for selecting antibiotics for the subsequent experimental studies. This manuscript is currently in preparation for submission.

The second study (**Section 3.2**) focused on the pharmacokinetics, pharmacodynamics, residue depletion, withdrawal times, and hepatopancreas histology associated with doxycycline, florfenicol, and cefotaxime in whiteleg shrimp following oral administration. This section includes three scientific manuscripts: Section 3.2.1 (published in *Veterinary Research Communications*), Section 3.2.2 (published in *Fishes*), and Section 3.2.3 (published in *Fishes*).

The third study (**Section 3.3**) evaluated antibiotic residues in commercial whiteleg shrimp products collected in Can Tho city, Vietnam, and assessed the potential dietary exposure and consumer risk associated with antibiotic residues detected in shrimp products. This manuscript has been submitted to *Journal of Environmental Science and Health, Part B*. Reviewer comments have been received, and a revised version is currently being prepared.

3.1 THE USE OF DRUGS AND CHEMICALS IN SUPER INTENSIVE WHITELEG SHRIMP AQUACULTURE IN THE MEKONG DELTA, VIETNAM

Whiteleg shrimp (*Penaeus vannamei*) is a dominant species that has been intensively farmed in the Mekong Delta and widely exported to global seafood markets. This culture system is often associated with frequent disease outbreaks, which can significantly affect production efficiency and economic performance. To manage these issues, farmers commonly use a wide range of chemicals during shrimp production. Among these, antibiotics play an important role in the prevention and treatment of bacterial diseases. In addition, disinfectants, water treatment chemicals and probiotic products are also applied to improve water quality and feed utilization. However, information on the actual use of these products, particularly antibiotics, in super-intensive shrimp farming systems is still limited. Therefore, the objective of this study was to investigate the use of chemicals and antibiotics in super-intensive whiteleg shrimp farming systems in the Mekong Delta, Vietnam, with a particular focus on antibiotic use in order to provide background information for further experimental studies.

THE USE OF DRUGS AND CHEMICALS IN SUPER INTENSIVE WHITELEG SHRIMP AQUACULTURE IN THE MEKONG DELTA, VIETNAM

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Abstract

This study investigated the use of drugs and chemicals in super-intensive whiteleg shrimp (*Penaeus vannamei*) aquaculture systems in the Mekong Delta, Vietnam, in order to provide information on antibiotic and chemical use and to support aquaculture management authorities. A total of 210 whiteleg shrimp farmers (30 households per province), selected based on sample-size calculation, were interviewed. The results showed that most farmers used chlorine-containing products to disinfect preparation ponds, while iodine and benzalkonium chloride were commonly applied for disinfection during the grow-out period. Nearly all farmers used probiotic products to maintain water quality and improve feed digestibility. According to the farmers, the most common diseases in whiteleg shrimp were acute hepatopancreatic necrosis disease (60.0–96.7% of farmers reported), white feces disease (13.3–73.3%), *Enterocytozoon hepatopenaei* infection (3.3–56.7%), and white spot syndrome virus infection (6.7–50.0%). Farmers reported the use of 10 types of antibiotics to control bacterial infections. The most commonly used antibiotics were cefotaxime (10.0–40.0% of farmers reported), doxycycline (13.3–26.7%), and oxytetracycline (6.7–26.7%). Most antibiotics used by farmers were not listed as banned substances by the Ministry of Agriculture and Rural Development of Vietnam, except for enrofloxacin and ciprofloxacin. These findings indicate the need for improved farmer training on the appropriate use of drugs and chemicals to support food safety and sustainable shrimp production.

Keywords: antibiotics, chemicals, Mekong Delta, super-intensive shrimp farming, whiteleg shrimp.

1. Introduction

Aquaculture is an essential industrial sector that provides jobs, livelihoods, food and nutrition for millions of people around the world. Vietnam's shrimp industry has seen positive growth in both production and export between 2018 and 2024. The shrimp farming area expanded at an average annual rate of 5%, while the production gradually increased by 8.4% per year, largely due to advancements in whiteleg shrimp culture productivity. Whiteleg shrimp production consistently increased, showing an 82% growth over 6 years, with an average annual rise of 13% (VASEP, 2025). In 2025, Vietnamese's shrimp reached nearly 4.0 billion in export value, driven in part by sustained demand and expanded market access (VASEP, 2025).

As a consequence of the rapid growth and increase in stocking density in intensive model, the Vietnamese shrimp sector has faced major problems due to disease outbreak (Nguyen et al., 2020). Viral and bacterial diseases are among the most important health problems affecting shrimp aquaculture, including AHPND, WFD and WSSV (Oanh et al., 2008; Tran et al., 2013; Flegel and Sritunyaluucksana, 2018, Thinh et al., 2020). The increase in diseases leads to an increase in antibiotic and chemical use to prevent and control disease in shrimp aquaculture (Rico et al., 2014; Phu et al., 2016; Chi et al., 2017). According to Rico et al. (2013), shrimp farmers used fewer antibiotics and chemicals than those used in fish farming. Mai (2004) and Tai (2012) reported more than 30 types of antibiotics used in shrimp culture before 2004. In recent years, the trend of antibiotic use has been declining; shrimp farmers in Northern Vietnam mainly used oxytetracycline to control and prevent AHPND and WFD (Chi et al., 2017). In the South of Vietnam, antibiotics such as amoxicillin, cotrim, oxytetracycline, ciprofloxacin have been commonly used by shrimp farmers (Phu et al., 2019a; Thinh et al., 2020). However, the improper use of antibiotics may lead to residues in exported shrimp. Despite the importance of shrimp aquaculture in the Mekong Delta, information regarding antibiotic and chemical use in super-intensive whiteleg shrimp farming systems remain limited. In addition, updated information on commonly used antibiotics is necessary to support the selection of representative compounds for subsequent pharmacokinetic, residue depletion, and risk-assessment studies included in this thesis. Therefore, this study was conducted to investigate farming practices, disease occurrence, and the use of chemicals and antibiotics in super-intensive whiteleg shrimp aquaculture systems in the Mekong Delta, Vietnam, in order to provide baseline information for both farm management and subsequent experimental studies.

2. Methodology

The study was conducted between early 2023 and mid-2024 through face-to-face interviews with shrimp farmers in the seven shrimp-farming provinces of the Mekong Delta, Vietnam, including Ca Mau, Tra Vinh, Tien Giang, Ben Tre, Kien Giang, Soc Trang, and Bac Lieu. The target population consisted of whiteleg shrimp farms applying super-intensive farming systems with pond liners. Farms

were randomly selected from official farm lists provided by the provincial Departments of Agriculture and Rural Development. In cases where selected farmers declined participation or were unavailable, replacement farms were randomly selected from the same province to maintain the planned sample size and geographical distribution.

The sample size was estimated using the Yamane formula (Yamane, 1967):

$$n = \frac{N}{1 + N \times e^2}$$

where n is the required sample size

N is the total population size

e is the accepted margin of error

Based on an estimated population of approximately 230,000 whiteleg shrimp farming households in the surveyed provinces (VASEP, 2024), the calculated sample size ranged from approximately 100 to 400 farms at accepted margins of error between 10% and 5% ($e=0.1$ and $e=0.05$), respectively. A total of 210 farms were ultimately included in the survey, corresponding to 30 farms per province. This sample size was considered sufficient to capture variability in farming practices across the surveyed areas while still allowing detailed farm-level interviews and practical field implementation. In addition, the inclusion of approximately 30 participants per province is commonly considered adequate in mixed-methods field surveys to identify major patterns and practices while maintaining manageable data collection and interview depth.



Figure 1. Map of the seven selected study provinces in the Mekong Delta region, Vietnam, where whiteleg shrimp farming is practiced

The questionnaire was designed by the research team in consultation with specialists in shrimp aquaculture and included both quantitative and qualitative components. Prior to the main survey, the questionnaire was piloted with seven shrimp farming households and subsequently refined to improve clarity, consistency, and relevance to local farming practices. Interviews were conducted by trained interviewers under technical guidance from scientist specialized shrimp aquaculture.

Data collection focused on the most recent production cycle and included technical information such as farming duration, grow-out pond area, water input pond area, productivity, feed conversion ratio (FCR), harvest size, and participation in training programs. Disease-related information included the occurrence of common shrimp diseases. Data on chemical use covered antibiotics, chemicals, antiparasitic agents, probiotics, and nutritional supplements, together with information regarding their handling and management practices. Prior to analysis, survey data were reviewed for completeness and consistency, manually coded into predefined categories, checked for missing or inconsistent responses, and cleaned before statistical analysis.

3. Data analysis

Results are expressed in descriptive statistics, i.e., frequency of occurrence, mean value, and standard deviation. In addition, inferential statistical analyses, including independent sample T-tests and Chi-square tests, were performed where appropriate to evaluate differences and associations among selected variables.

4. Result and discussion

4.1 General information of whiteleg shrimp farms characteristics

General characteristics. General information on shrimp farms from seven provinces in the Mekong Delta is summarized in Table 1. The overall farming experience averaged 8.8 ± 6.3 years, ranging from 1 to 35 years. Longer experience in Tien Giang (12.8 ± 5.7 years) may reflect early adoption of super-intensive shrimp aquaculture compared to provinces such as Tra Vinh (5.5 ± 2.7 years) or Ben Tre (5.9 ± 3.1 years). Training participation varied among provinces, with an overall proportion of 40.5%. The highest training rate was observed in Bac Lieu (56.7%), while the lowest was recorded in Kien Giang (26.7%). Training plays a crucial role in adopting improved management practices and can impact farm productivity, disease management, and compliance with antibiotic checking procedures (Nair et al., 2025; Dandi et al., 2025).

Farm scale and pond characteristics. The number of grow-out ponds per farm ranged widely, with an overall mean of 3.8 ± 3.3 ponds (1–28 ponds). Farms in Kien Giang exhibited the largest scale (6.7 ± 5.8 ponds), whereas Ca Mau farms had the smallest number (2.1 ± 1.1 ponds). The mean area of grow-

out ponds across all farms was $5146 \pm 4974 \text{ m}^2$, with a broad range from 500 to 36,000 m^2 . Kien Giang farms had the largest average grow-out pond area ($10,186 \pm 8,807 \text{ m}^2$), while Ca Mau farms reported the smallest ($2,892 \pm 1,728 \text{ m}^2$). Similarly, the area of water input ponds varied considerably, with an overall mean of $9,034 \pm 7,965 \text{ m}^2$. This heterogeneity reflects the diversity of farming systems in the Mekong Delta, where both small-scale and larger intensive operations exist. Farms in Kien Giang had notably larger grow-out pond areas and number of ponds compared with other provinces, suggesting a degree of farm intensification or larger operational scale. Previous research on intensive whiteleg shrimp farming in the same region highlights the variability in farm structure and management practices across provinces, which is consistent with our findings of broad ranges in pond area and number of ponds per farm (Quyen et al., 2020).

Stocking density and productivity. Stocking density is a key driver of shrimp production and often differs by culture intensity. Our results showed an overall mean stocking density of $208 \pm 71 \text{ shrimp/m}^2$, ranging from 81 to 375 shrimp/m^2 . This is higher than densities commonly used in extensive aquaculture systems reported in previous studies in Vietnam but is consistent with intensive and super intensive production contexts for whiteleg shrimp reported in Southeast Asia. In Vietnam, emerging semi-biofloc systems have been shown to support densities up to 180–200 shrimp/m^2 with improved water quality and reduced antibiotic use (Kim & Dinh, 2024).

Average productivity across all farms was $1.9 \pm 1.3 \text{ tons/1000 m}^2/\text{crop}$ (equivalent to 19 tons/ha/crop), which suggests more productive culture systems than purely extensive models. According to Quyen et al. (2020), semi-intensive and intensive whiteleg shrimp systems in the Mekong Delta have reported yields ranging up to 8–10 tons/ha in intensive farms and lower yields in extensive systems. Farms in Ca Mau achieved the highest mean productivity, while Kien Giang farms recorded the lowest. Differences in stocking density and yield likely reflect variation between farming regions and farms, and depend on the level of investment in production equipment, the skill and management ability of farmers, environmental conditions, and disease situation (Tung et al., 2016).

Feed conversion ratio. The overall feed conversion ratio (FCR) was 1.2 ± 0.1 , which aligns with well-managed semi-intensive and intensive shrimp farms globally, where typical FCRs range from 1.1 to 1.5 under optimal feeding conditions. This result is consistent with the study of Cao et al. (2025) on intensive whiteleg shrimp farming in Vietnam, which also reported an effective feed conversion ratio (FCR) of approximately 1.21 under high production conditions. A lower FCR is indicative of efficient feed utilization and good pond management, including water quality control and appropriate feeding strategies, both of which are critical in maintaining growth performance, particularly at elevated stocking densities (Bajracharya et al., 2025).

Harvest size and crop cycles. Harvest size varied widely among provinces, with Soc Trang showing the largest mean harvest size ($44.6 \pm 23.9 \text{ shrimp/kg}$) and Bac Lieu the smallest ($27.5 \pm 5.9 \text{ shrimp/kg}$).

Such variation may reflect differences in grow-out duration, feed type, stocking density, and environmental conditions, all of which are known to affect shrimp growth performance. Seasonal variations and differential management practices among provinces may also contribute to this variability (Quyen et al., 2020).

Farms operated an average of 3.0 ± 0.7 crops per year. The relatively stable number of months per crop (~3 months) reflects the adoption of a fast production cycle to maximize annual yields.

Antibiotic checking. A relatively high proportion of farmers (68.1%) reported antibiotic residue checking before selling shrimp across all farms, with the highest rate in Bac Lieu (83.3%). This reflects increasing awareness and regulatory pressure, particularly due to concerns over antibiotic residues in export products. Experimental evidence in Vietnam demonstrates that quantifying antibiotics and providing residue feedback can significantly reduce antibiotic use, which is crucial given global concerns about antimicrobial resistance and food safety (Suzuki et al., 2023). Furthermore, Li et al. (2024) studied the distribution of antibiotic residues in *Penaeus vannamei* ponds, confirming that antibiotics can accumulate in pond components, with sediments acting as long-term storage sites; therefore, residue monitoring is a crucial aspect for both product safety and environmental protection.

In summary, the farm-level results of this study align with contemporary trends in shrimp aquaculture, where incremental technological adoption, feed optimization, and targeted training are pushing production toward higher efficiencies.

Table 1. General characteristics of shrimp farms in the surveyed provinces of the Mekong Delta region, Vietnam

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Year of farming (years)	9.5±6.0 (2-23)	5.5±2.7 (2-10)	12.8±5.7 (3-21)	5.9±3.1 (2-10)	8.7±4.9 (1-20)	8.1±8.6 (1-30)	11.0±7.8 (1-35)	8.8±6.3 (1-35)
Training (%)	43.3	33.3	40.0	43.3	26.7	40.0	56.7	40.5
Number of grow-out ponds (m ²)	2.1±1.1 (1-5)	3.1±2.7 (1-15)	3.7±3.1 (1-15)	3.4±1.7 (2-10)	6.7±5.8 (1-28)	3.3±2.5 (1-15)	4.4±2.5 (2- 16)	3.8±3.3 (1-28)
Area of grow-out pond (m ²)	2892±1728 (1000- 8000)	4500±4028 (800-21000)	4743±4865 (1000-22500)	4230±2115 (2000-13000)	10186±8807 (500-36000)	4730±4180 (1200-25500)	4879±2087 (2000- 10000)	5146±4974 (500-36000)
Area of water input pond (m ²)	5197±2956 (2350- 14500)	6400±6335 (2500- 25000)	6717±5224 (1000-26500)	5337±1576 (3200-9500)	12790±9817 (2700-40000)	10843±7146 (2800-29000)	16321±110 26 (3500- 45000)	9034±7965 (1000-45000)
Stocking density (shrimp/m ²)	185±52 (100-300)	254±56 (150-333)	212±70 (81-350)	276±53 (167-375)	188±62 (100-375)	184±64 (107-333)	158±60 (100-333)	208±71 (81-375)
Productivity (tons/1000m ² /crop)	2.5±1.2 (0.5-5)	2.3±1.8 (0.2-7.5)	2.0±1.1 (0.6-5)	2.1±0.8 (0.5-3.5)	1.0±1.2 (0.1-4)	1.8±1.3 (0.1-5)	1.4 ± 0.9 (0.3-4)	1.9±1.3 (0.1-7.5)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Harvest size (g/shrimp)	25.9±8.3 (13-48)	29.3±9.9 (13-50)	31.4±9.2 (17-50)	31.0±6.3 (22-40)	26.5±6.1 (20-40)	27.3±10.7 (10-48)	37.8±7.5 (22-50)	29.9±9.1 (10-50)
Survival rate (%)	58 (17-96)	35 (2-85)	33 (8-80)	26 (9-43)	21 (2-70)	44 (3-63)	24 (5-64)	34 (2-96)
Feed Conversion Ratio (FCR)	1.2±0.1 (1-1.4)	1.3±0.1 (1-1.8)	1.3±0.1 (1.1-1.6)	1.2±0.1 (1.1-1.4)	1.2±0.1 (1-1.5)	1.2±0.1 (1-1.6)	1.3±0.2 (1-2)	1.2±0.1 (1-2)
Number of crop/year	2.7±0.6 (2-4)	3.0±0.9 (2-6)	3.2±0.6 (2-5)	3.4±0.8 (3-6)	2.9±0.8 (1-6)	3.1±0.4 (2-4)	3.0±0.6 (2-5)	3.0±0.7 (1-6)
Number of month/crop	3.0±0.3 (2.5-4)	3.0±0.5 (2-4)	3.2±0.4 (3-4)	3.3±0.4 (2.5-4)	3.2±0.3 (3-4)	3.1±0.5 (2-4)	3.3±0.3 (3-4)	3.1±0.4 (2-4)
Nursery pond applied (%)	50.0	23.3	63.3	66.7	90.0	10	86.7	55.7
Nursery time (day)	30.9±12.1 (15-60)	27.7±9.2 (19-45)	17.3±3.8 (10-24)	25.9±4.3 (10-30)	21.8±5.0 (10-35)	21.7±2.9 (20-25)	19.8±5.4 (10-30)	22.8±7.6 (10-60)
Checking antibiotic before selling (%)	46.7	63.3	40.0	76.7	63.3	80.0	83.3	68.1

4.2 Clinical signs of diseases reported by shrimp farmers

Disease occurrence reported by shrimp farmers varied among provinces (Table 2). The AHPND was the most commonly reported disease among farmers in the 7 surveyed provinces, with the highest rate in Ben Tre (96.7%) and the lowest in Soc Trang (60%). Other provinces also showed very high rates of this disease, above 60%. The high overall reporting of AHPND (76.2%) among surveyed farmers reflects its status as a major constraint in shrimp aquaculture (Figure 2). Flegel & Sritunyaluucksana (2018) confirmed that AHPND was caused by *V. parahaemolyticus* that carries a pVA plasmid containing genes for PirA^{VP} and PirB^{VP} toxins. The common clinical signs found in AHPND-infected shrimp include lethargic motility, empty midgut, pale coloration and shrinking of the hepatopancreas. These clinical signs are the most commonly observed in diseased shrimp (Zorriehzahra & Banaederakhshan, 2015). In addition, AHPND normally occurred between 20 and 40 days after stocking post-larvae (Phu et al., 2019a).

White feces disease was reported by 31.4% of farmers overall (Figure 2). The highest was in Tien Giang at 73.3%, followed by Soc Trang (40%), and Kien Giang and Bac Lieu at 30%. WFD is named after the typical clinical sign of white fecal strings (Tangprasittipap et al., 2013; Sriurairatana et al., 2014). It was identified as a multi-factor disease caused by various microorganisms such as parasites, bacteria or viruses, or by the microsporidian parasite *Enterocytozoon hepatopenaei*, which is also a pathogen (Ha et al., 2011; Flegel & Sritunyaluucksana, 2018). Besides, dissected midguts are distended and full of white to yellow contents. Sriurairatana et al. (2014) reported that WFD in shrimp farms led to enormous economic losses worldwide each year. According to Hou et al. (2018), the mortality rate of WFD in the summer may be as high as 50% in ponds; however, the specific reasons for WFD outbreaks remain unclear.

Enterocytozoon hepatopenaei (EHP) infection was reported by nearly one quarter of surveyed farmers, with a pronounced prevalence in Kien Giang. This result is consistent with field surveys and disease-risk assessments showing that microsporidian infections are relatively common in Southeast Asian shrimp farms, contributing to growth retardation, production losses and may increase susceptibility to other disease pressures (Oanh et al., 2022).

Although less prevalent than AHPND, WSSV was still reported by a considerable proportion of farmers, with the highest provincial reporting in Tien Giang (50.0%). WSSV is a highly contagious viral pathogen of shrimp that causes white spots on the exoskeleton and extensive mortalities when outbreaks occur, often leading to abrupt collapses of shrimp ponds. Recent reviews on WSSV highlight its importance as one of the most devastating viral diseases affecting penaeid shrimp worldwide, with no widely effective therapeutic option currently available (Paul et al., 2025). The virus causing WSD is highly virulent and may cause up to 100% mortality within 3–7 days after infection (Loy, 2014). Walker

& Mohan (2009) also reported that WSSV can infect several aquatic crustacean and invertebrate species.

In addition, three provinces reported the newly recognized disease translucent post-larvae disease (TPD), Bac Lieu has the highest rate of 40%, followed by Kien Giang (16.7%) and then Tra Vinh (3.3%). TPD is an emerging condition affecting early life stages of whiteleg shrimp. While fewer peer-reviewed journal articles have been published specifically on TPD in Vietnam, government and industry monitoring data indicate its emergence and the need for improved disease surveillance at early production stages, especially in nurseries and hatcheries (Chanh, 2023).

Yellow head disease (YHD) used to be common in shrimp culture, in current survey only Tien Giang recorded the disease at low rate (0.5%). YHD is a highly pathogenic viral disease known to cause rapid mortality in shrimp farms in parts of Southeast Asia.

Table 2. Shrimp diseases reported by farmer in the surveyed provinces of the Mekong Delta region, Vietnam (%)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Acute								
hepatopancreatic necrosis disease (AHPND)	63.3	76.7	83.3	96.7	76.7	60.0	76.7	76.2
White feces disease (WFD)	20.0	13.3	73.3	13.3	30.0	40.0	30.0	31.4
Enterocytozoon hepatopenaei (EHP)	3.3	16.7	30.0	20.0	56.7	10.0	23.3	22.9
White spot syndrome virus (WSSV)	6.7	10.0	50.0	16.7	23.3	13.3	13.3	19.0
Translucent post- larvae disease (TPD)	0.0	3.3	0.0	0.0	16.7	0.0	40.0	8.6
Yellow Head Disease (YHD)	0.0	0.0	3.3	0.0	0.0	0.0	0.0	0.5

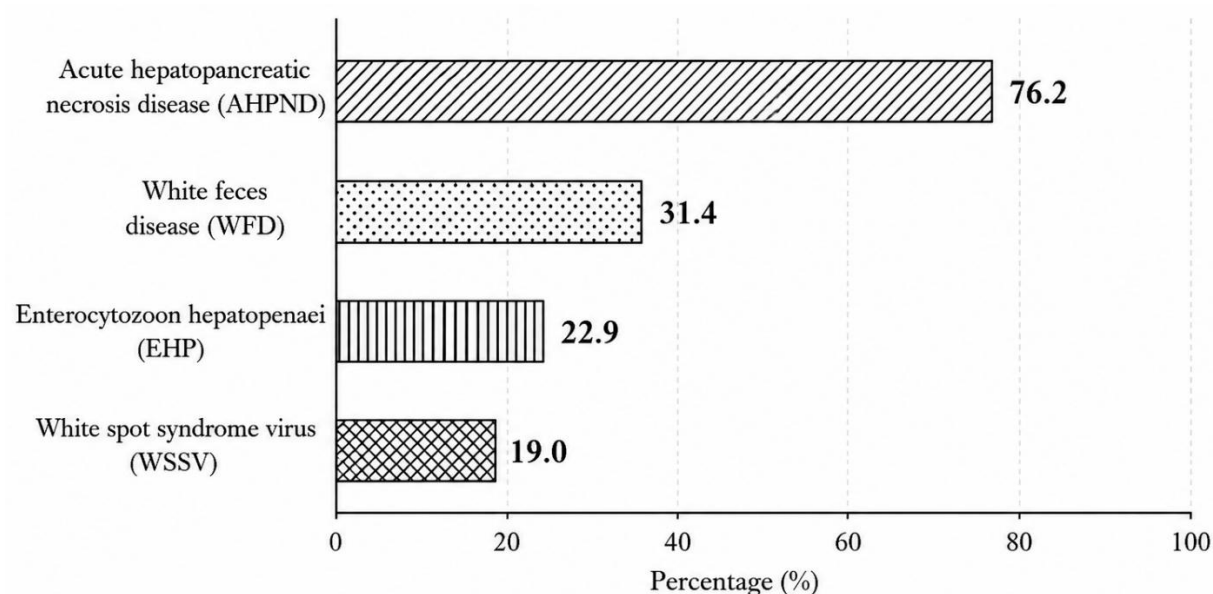


Figure 2. Overall percentage of the four most commonly reported shrimp diseases by farmers in the surveyed provinces of the Mekong Delta, Vietnam (n = 210 farms)

Overall, the range of diseases reported underscores the complex disease landscape facing shrimp farmers in the Mekong Delta. AHPND and WFD stand out as the most frequently observed syndromes, aligning with the survey by Thinh & Phu (2021), which showed that AHPND and WFD have been still common diseases in the Mekong Delta reported by farmers. The simultaneous emergence of multiple disease syndromes also suggests that integrated health management approaches, including water quality control, probiotic use, and reduced reliance on antibiotics, may become increasingly important.

4.3 The use of antibiotics in shrimp farming

The situation of antibiotic use and non-use among surveyed shrimp farms is summarized in Table 3. Overall, 51.9% of farms reported no antibiotic use, whereas 48.1% reported using antibiotics during cultivation. Luu et al. (2021) reported that 83% of shrimp farms in Vietnam use antibiotics to treat shrimp diseases, a result much higher than our study (48.1%), reflecting the recent increase in the adoption of alternative management strategies, such as improved biosecurity, the use of probiotics and water quality control, as well as increased awareness of food safety requirements for export markets. Antibiotic use in aquaculture is often driven by high stocking density, disease prevalence, and limited access to rapid diagnostics, leading farmers to rely on antimicrobials as a preventive or curative measure rather than targeted treatment (Rico et al., 2014; Phu et al., 2016; Chi et al., 2017). In Vietnam, antibiotics used in aquaculture must be registered and approved by the Ministry of Agriculture and Environment before commercialization. In practice, shrimp farmers commonly access antibiotics

through veterinary drug companies, aquaculture product retailers, and feed or chemical suppliers. Although veterinary prescription is not always required in conventional small-scale farming systems, farms applying international certification standards such as Global GAP, or Best Aquaculture Practices (BAP) generally require antibiotics to be prescribed by veterinarians, and supporting documentation has to be in place. Among farms reporting antibiotic use, 21.9% used a single antibiotic, 19.5% used two antibiotics, and 6.7% reported using three antibiotics during a crop. Farms in Kien Giang showed the highest proportion of multiple-antibiotic use, with 30.0% using two antibiotics and 10.0% using three antibiotics per crop. The observation that a subset of farms used two or more antibiotics within a single production cycle raises concerns regarding selection pressure for antimicrobial resistance. Recent study emphasizes that repeated or combined antibiotic use in aquaculture environments can promote the persistence of antibiotic residues and resistant bacteria in pond water and sediments, posing risks to both aquatic ecosystems and public health (Cabello et al., 2016). Moreover, antibiotics should not be used to prevent disease or to treat viral diseases (Te, 2003).

Table 3. Proportion of shrimp farms using and not using antibiotics in the surveyed provinces of the Mekong Delta region, Vietnam (%)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Shrimp farming not using antibiotic	50.0	60.0	56.7	56.7	33.3	56.7	50.0	51.9
Shrimp farming using antibiotics	50.0	40.0	43.3	43.3	66.7	43.3	50.0	48.1
Use 1 antibiotic	30.0	20.0	16.7	20.0	26.7	20.0	20.0	21.9
Use 2 antibiotics	16.7	13.3	20.0	16.7	30.0	16.7	23.3	19.5
Use 3 antibiotics	3.3	6.7	6.7	6.7	10.0	6.7	6.7	6.7

Ten antibiotics were reported to be used in whiteleg shrimp farming across the seven surveyed provinces of the Mekong Delta (Table 4). The most frequently reported antibiotics overall were CFT (25.2%), followed by DOX (16.7%) and oxytetracycline (15.7%) (Figure 3), and Kien Giang had the highest rate of use of these three antibiotics. The results of this study are consistent a survey conducted in five

provinces of the Mekong Delta performed by Thinh & Phu (2021), farmers used 10 types of antibiotics during cultivation of shrimp in order to prevent and control disease, i.e., FF, enrofloxacin, ciprofloxacin, tetracycline, oxytetracycline, DOX, CFT, cotrim (containing sulfamethoxazole and trimethoprim), amoxicillin, and chloramphenicol. The frequent reporting of CFT, a third-generation cephalosporin, is notable given its classification as a critically important antimicrobial for human medicine by the World Health Organization. The use of cephalosporins and fluoroquinolones (e.g., ciprofloxacin and enrofloxacin) in aquaculture has been highlighted as a particular concern due to their association with clinically relevant resistance in human pathogens (WHO, 2019; FAO, 2020). The continued detection of tetracyclines (DOX and oxytetracycline) and FF aligns with previous reports identifying these compounds as among the most commonly used antibiotics in shrimp aquaculture in Southeast Asia (Pham et al., 2015; Phu et al., 2019a; Thinh et al., 2020).

The reported number of antibiotic applications per crop ranged from 0 to 4 times, depending on province and farm (Table 4). The diversity of antibiotics and the repeated use of antibiotics per crop reported in this study suggests a potential risk for antibiotic residue occurrence in shrimp products, especially when withdrawal times are not strictly followed. Specifically, the Rapid Alert System for Food and Feed (RASFF) from the EU reported that shrimp products from several regions in Vietnam occasionally contain antibiotic residues exceeding permitted levels, e.g., from 2020 to 2024, the presence of residues of ciprofloxacin, oxytetracycline, DOX, tetracycline, sulfamethoxazole and furazolidone in shrimp frozen products was reported (RASFF, 2025). Generally, given that shrimp from the Mekong Delta are primarily produced for export markets, continued surveillance of antibiotic use patterns and residue levels is essential to ensure compliance with international food safety standards and to reduce the risk of trade rejections.

Table 4. Antibiotics reportedly used by farmers in the surveyed provinces of the Mekong Delta region, Vietnam (%)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Enrofloxacin	0.0	6.7	3.3	10.0	6.7	3.3	3.3	4.8
Ciprofloxacin	3.3	6.7	3.3	13.3	0.0	20.0	3.3	7.1
Amoxicillin	3.3	0.0	3.3	0.0	0.0	0.0	3.3	1.4
Florfenicol	3.3	3.3	6.7	10.0	13.3	3.3	3.3	6.2
Doxycycline	13.3	13.3	16.7	16.7	26.7	16.7	13.3	16.7
Oxytetracycline	13.3	10.0	6.7	6.7	26.7	20.0	26.7	15.7
Cotrim	3.3	0.0	0.0	0.0	3.3	0.0	6.7	1.9
Cefotaxime	33.3	16.7	33.3	16.7	40.0	10.0	26.7	25.2
Gentamycin	0.0	3.3	3.3	0.0	0.0	0.0	0.0	1.0
Fosfomycin	0.0	3.3	0.0	0.0	0.0	0.0	0.0	0.5
Number of times of antibiotic used per crop of a farm	0 - 3	0 - 2	0 - 3	0 - 3	0 - 3	0 - 3	0 - 4	0 - 4

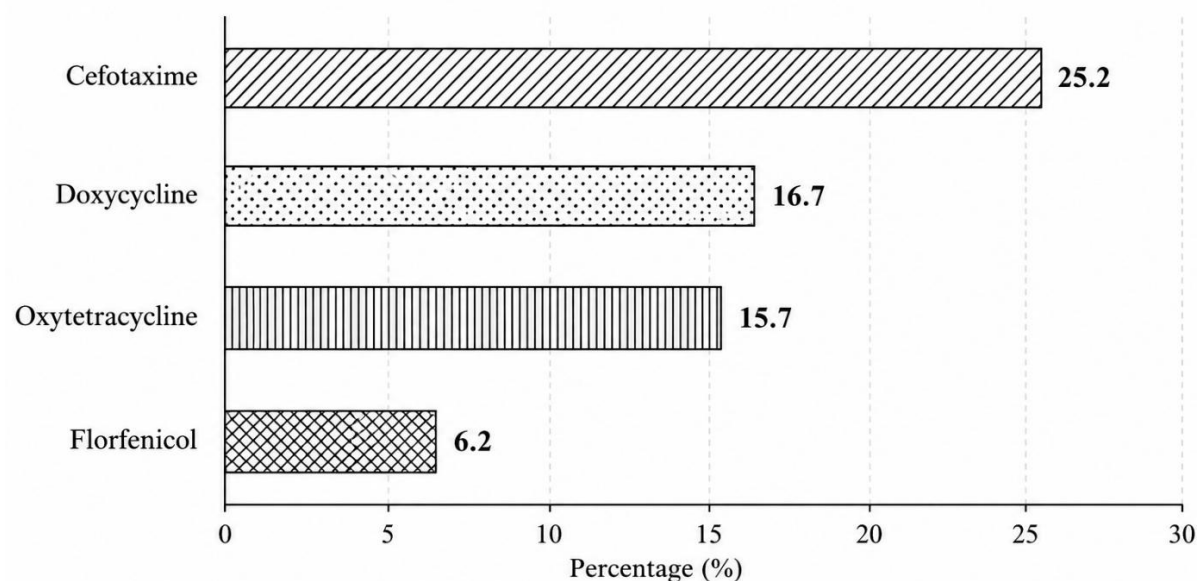


Figure 3. Overall percentage of the four most commonly reported antibiotics used by shrimp farmers in the surveyed provinces of the Mekong Delta, Vietnam (n = 210 farms)

4.4 The use of chemicals, probiotics, and nutritional supply products in shrimp farming

The survey results show that shrimp farmers mainly use chemicals to manage ponds and water (Table 5). A high proportion of farmers reported using chlorine (89.0%) and potassium permanganate (KMnO_4 , 76.2%) for pond sanitation and water preparation, consistent with common practices in shrimp farming to disinfect equipment and inhibit contaminating bacteria in shrimp pond water before stocking (Nadella et al., 2025). The use of CaO and CaCO_3 was also widespread, reflecting their role in pH buffering, alkalinity adjustment, and pond treatment to improve water quality and promote plankton growth in the initial farming stage (Chi et al., 2017). Nevertheless, reliance on strong chemicals can have drawbacks, including disrupting beneficial microbial communities and affecting the chemical composition of water if not carefully dosed and managed.

Regarding chemicals used for water treatment during the grow-out stage, iodine was used by 70.0% of farmers, followed by CaCO_3 (60.5%) and benzalkonium chloride (20.0%). Thinh & Phu (2021) showed that most farmers use CaCO_3 , iodine, or BKC every 10 or 15 days to disinfect aquaculture water for controlling pathogens. The relatively limited use of compounds such as glutaraldehyde and CuSO_4 (<1.0%) suggests increased awareness of their potential toxicity and environmental impacts. The types of chemicals found in this survey were similar to those reported by Phu et al. (2019a) for Southern Vietnam and Chi et al. (2017) for Northern Vietnam.

Notably, the widespread use of probiotics in most farms (96.7%) reflects a trend towards more advanced pond management. During the shrimp farming process, shrimp farmers used probiotics as a feed

additive to improve shrimp digestibility, and as water quality improvers (Chi et al., 2017; Thinh & Phu, 2021; Noman et al., 2024). Recent studies in the Mekong Delta and specialized literature emphasize that probiotics (e.g., *Bacillus* spp., *Lactobacillus* spp.) are considered a biological alternative to chemicals and antibiotics, helping to balance the microbial ecosystem in pond water, promote the decomposition of organic matter, reduce toxins such as NH_3 and H_2S , and enhance shrimp health through interaction with the gut microbiota (Balcázar et al., 2006; Wang et al., 2008; Newaj-Fyzul et al., 2014).

Furthermore, the widespread application of mineral and vitamin supplements (95.7%) in the survey shows farmers' awareness of micronutrient supplementation to improve shrimp nutritional status and help them withstand environmental stress and reduce disease rates when faced with unfavorable conditions (Agriterro Report, 2024). In addition, herbs in aquaculture are used fresh or as extracts; these extracts contain various natural compounds such as phenolics, polyphenolics, alkaloids, quinones, terpenoids, lectins, and polypeptides that have antioxidant, antibacterial, and immune-boosting activities for livestock, which aligns with current trends in biological enhancement in aquaculture (Harikrishnan et al., 2011a, 2011b, 2011c). Blumenthal et al. (2000) and Logambal et al. (2000) also showed that the use of plant extracts can reduce treatment costs and be environmentally friendly with a tendency towards biodegradability; the high diversity of plant extracts makes parasites less likely to develop resistance. Although shrimp farmers use many natural products and probiotics, this survey also reveals the presence of products such as antiparasitic drugs (like ivermectin and praziquantel). The use of antiparasitic drugs indicates that farmers still face challenges in treating internal and intestinal diseases in shrimp.

Overall, along with the use of traditional chemicals to control bacteria and improve water quality, the increasing adoption of probiotics and nutritional supplements indicates a shift towards more sustainable pond management solutions, reducing reliance on chemicals.

Table 5. The use of chemicals, probiotics, and nutritional supplied products reported by famers in the surveyed provinces of the Mekong Delta region, Vietnam (%)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Chemical use during pond preparation and water preparation								
Chlorine	96.7	93.3	76.7	96.7	83.3	86.7	90.0	89.0
KMnO₄	70.0	90.0	70.0	83.3	90.0	63.3	66.7	76.2
CaO	73.3	56.7	70.0	56.7	60.0	46.7	53.3	59.5
CaCO₃	40.0	33.3	26.7	40.0	36.7	33.3	30.0	34.3
CuSO ₄	10.0	6.7	26.7	0.0	33.3	0.0	10.0	12.9
H ₂ O ₂	6.7	13.3	16.7	0.0	0.0	6.7	20.0	9.0
BKC	3.3	13.3	6.7	0.0	3.3	23.3	13.3	9.0
Iodine	10.0	3.3	6.7	10.0	0.0	26.7	3.3	8.6
TCCA	3.3	0.0	6.7	0.0	20.0	6.7	3.3	5.7
Chemical use for water treatment during grow-out stage								
Iodine	83.3	86.7	83.3	93.3	53.3	36.7	53.3	70.0
CaCO₃	73.3	66.7	53.3	56.7	50.0	73.3	50.0	60.5
BKC	13.3	20.0	40.0	0.0	20.0	23.3	23.3	20.0
Chlorine	6.7	13.3	20.0	26.7	3.3	23.3	13.3	15.2
KMnO ₄	6.7	16.7	13.3	16.7	3.3	13.3	6.7	11.0
Virkon	12.9	3.3	6.7	0.0	17.9	0.0	3.3	6.2
CuSO ₄	0.0	3.3	0.0	0.0	0.0	0.0	3.3	1.0

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
Glutaraldehyde	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.5
Internal parasite control								
Praziquantel	22.6	18.3	15.0	23.3	17.9	20.0	26.7	20.5
Ivermectin	33.8	29.3	40.3	39.3	29.9	33.0	43.7	35.6
Others	11.4	7.8	9.9	15.6	8.5	10.1	7.3	10.1
Probiotic	93.3	100	100	100	96.7	90.0	96.7	96.7
Nutritional supplied products (mineral and vitamin)								
	90.0	100	100	100	96.7	90.0	93.3	95.7
Natural herbs and herbal products								
Natural herbs (garlic)	3.3	13.3	6.7	20.0	6.7	16.7	26.7	13.3
Herbal products	23.3	20.0	40.0	33.3	20.0	36.7	26.7	28.6

KMnO₄: potassium permanganate; CaO: calcium oxide; CaCO₃: calcium carbonate; CuSO₄: copper sulfate; H₂O₂: hydrogen peroxide; BKC: benzalkonium chloride; TCCA: trichloroisocyanuric acid; Virkon: potassium peroxymonosulfate

4.5 Handling of chemicals, guidance, and safety practices in the surveyed provinces of the Mekong Delta, Vietnam

The survey revealed that shrimp farmers' decisions on chemical use were mainly based on personal experience, with nearly 100% of interviewed farmers across the seven provinces reporting this practice (Table 6). In contrast, relatively few farmers followed label instructions (8.1%) or received guidance from technicians employed by chemical suppliers (11.4%). Support from authorities, such as extension

services and local aquaculture departments, was reported by only 2.4% of farmers, likely due to the limited number of officers available in the region.

Record-keeping practices were relatively limited, with only 16.7% of farmers maintaining records of chemical use. Inadequate record keeping has been identified as a major constraint for traceability and food safety assurance in Vietnamese aquaculture, particularly among small-scale producers (FAO, 2023).

In terms of safety practices, nearly 65% of farmers purchased chemicals and stored them in storage areas located next to ponds and away from living areas, this proportion reached 100% in Tien Giang and Ben Tre. However, these storage areas are also used by farmers and workers for resting during working hours, so storing chemicals there could affect the health of the workers.

While a relatively high proportion of farmers (71.4%) were aware of banned chemicals, the use of personal protective equipment such as gloves and masks during chemical handling was reported by only 38.6% of respondents. However, none of the farmers reported being aware of the potential health effects of chemical exposure, such as skin irritation or burning. Moreover, strong oxidizing disinfectants like chlorine or formalin can cause skin burns and respiratory irritation during handling. Therefore, training courses or workshops should be organized to guide farmers in the correct and safe use of these chemicals. In addition, chemicals should be stored in covered cabinets or containers away from cooking and resting areas. After mixing chemicals, farmers should wash thoroughly after handling chemicals and clean or replace their protective equipment as appropriate.

Table 6. Handling of chemical, guidance and safety (%)

	Ca Mau (n=30)	Tra Vinh (n=30)	Tien Giang (n=30)	Ben Tre (n=30)	Kien Giang (n=30)	Soc Trang (n=30)	Bac Lieu (n=30)	Overall (n=210)
<i><u>Making decision on chemical use</u></i>								
Following label instruction	3.3	6.7	16.7	3.3	16.7	3.3	6.7	8.1
Support from technician from chemical company	3.3	3.3	6.7	23.3	23.3	13.3	6.7	11.4
By their own experience	100	100	100	100	80.0	100	100	97.1
Local authority support	3.3	3.3	0.0	0.0	10.0	0.0	0.0	2.4
<i><u>Record chemicals and others</u></i>	3.3	0.0	46.7	46.7	3.3	10.0	6.7	16.7
<i><u>Safety on handling chemicals</u></i>								
Available chemical storage cabinet	10.0	0.0	40.0	70.0	20.0	6.7	6.7	21.9
Purchase and store the chemicals in storage	80.0	96.7	100	100	13.3	13.3	43.3	63.8
Having knowledge of banned chemicals	56.7	43.3	53.3	96.7	86.7	63.3	100	71.4
Using gloves, masks during handling	73.3	40.0	50.0	43.3	23.3	23.3	16.7	38.6
Effects of chemicals on health (itching, burning,...)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4.6 Production and antiparasitic use in shrimp farms using and not using antibiotics

Production in shrimp farms using antibiotics (2.00 ± 1.38 tons/1000 m²/crop) was slightly higher than that observed in farms not using antibiotics (1.82 ± 1.22 tons/1000 m²/crop). However, the difference was not statistically significant ($p > 0.05$). This result suggests that antibiotic use alone may not be clearly associated with improved production performance under the surveyed farming conditions. Production outcomes in shrimp farming are likely influenced by multiple factors related to farm management and culture conditions, which may contribute to the variability observed among farms.

Similarly, the proportion of farms using antiparasitic agents did not differ significantly between shrimp farms using antibiotics (34.7%) and those not using antibiotics (40.4%) ($p > 0.05$). These findings indicate that the use of antibiotics was not clearly associated with antiparasitic application practices among the surveyed farms.

Table 7. Production and antiparasitic use in shrimp farms using and not using antibiotics

Variable	Shrimp farming not using antibiotic (n=109)	Shrimp farming using antibiotic (n=101)	p-value
Production (tons/1000 m ² /crop)	1.82±1.22	2.00±1.38	0.324
Antiparasitic use, n (%)	44 (40.4)	35 (34.7)	0.393

Values are presented as mean ± standard deviation (SD) or number (%). Independent samples t-test was used for comparison of production, while Chi-square test was used for comparison of antiparasitic use.

5. Conclusion

Research revealed the extensive use of chemicals and supportive products in whiteleg shrimp farming in the Mekong Delta, particularly during pond preparation and grow-out stages. AHPND and WFD were two common diseases reported by shrimp farmers. The commonly used antibiotics were CFT, DOX and oxytetracycline to prevent and control disease during farming. Most farmers used probiotic products to improve water quality and feed digestibility, reflecting farmers' efforts to enhance shrimp health. In general, enhanced farmer training, improved extension services, and stricter guidance on responsible chemical use and safety practices are necessary to promote more sustainable and safer shrimp aquaculture.

6. Limitations of the study

This survey study relied partly on self-reported information provided by shrimp farmers during interviews, which may be influenced by recall bias or incomplete farm records in some cases. Although interviewers conducted field observations during farm visits and reviewed farm records when available, the study primarily focused on farmer interviews and did not systematically include additional data sources such as veterinary prescription records, antimicrobial sales records, or interviews with other stakeholders. Furthermore, residue monitoring and environmental sampling at farm level were beyond the scope of the present survey. Therefore, the findings should be interpreted as an overview of antibiotic-use practices and farmer-reported management strategies in the surveyed shrimp farming systems.

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3.2 PHARMACOKINETICS, PHARMACODYNAMICS, WITHDRAWAL TIME OF DOXYCYCLINE, FLORFENICOL AND CEFOTAXIME IN WHITELEG SHRIMP (*PENAEUS VANNAMEI*) AND IMPACT ON HEPATOPANCREAS HISTOLOGY

Previous investigation of whiteleg shrimp farming in the Mekong Delta showed that AHPND is commonly reported by farmers, and different antibiotics have been used in practice to deal with these disease problems. Among them, cefotaxime (CFT) was the most frequently reported, while doxycycline (DOX) is also widely applied, and florfenicol (FF) has been reported in several farms. Although oxytetracycline was also commonly used, it was not selected for further investigation in this thesis because more pharmacokinetics (PK) data are already available for this compound. In contrast, FF was included to provide additional information under local farming conditions in Vietnam. However, antibiotic use in shrimp aquaculture remains largely based on farmers' experience, and information on appropriate dosages and efficacy is still limited. In addition, data on the PK, PK/PD relationship, residue depletion, and withdrawal times of these antibiotics in whiteleg shrimp are still scarce, which makes their effectiveness against bacterial pathogens such as *V. parahaemolyticus* in shrimp unclear. The hepatopancreas, which plays an important role in metabolism and detoxification, may be affected by antibiotic administration, but this aspect has not been clearly investigated so far. Therefore, the present study was conducted to investigate the PK, PK/PD relationship, withdrawal time, and hepatopancreas histological effects of DOX, FF, and CFT in whiteleg shrimp under experimental conditions using doses relevant to farming practices.

3.2.1 PHARMACOKINETICS, PHARMACODYNAMICS AND WITHDRAWAL TIME OF DOXYCYCLINE IN WHITELEG SHRIMP (*PENAEUS VANNAMEI*) AFTER ORAL ADMINISTRATION

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Abstract

This research aimed to investigate the pharmacokinetics (PK), withdrawal time and hepatopancreas histological impact of doxycycline (DOX) in whiteleg shrimp (*Penaeus vannamei*). To determine PK parameters in hemolymph, hepatopancreas, and muscle, a single oral dose of 20 mg DOX/kg body weight was administered, following by interval sampling during 24 hours. DOX concentrations were quantified by high-performance liquid chromatography coupled with tandem mass spectrometry, and PK parameter estimation was done using a one-compartmental model. The maximum concentrations in shrimp hemolymph, hepatopancreas and muscle were 1.09 µg/mL, 2.37 µg/g and 0.46 µg/g at 1.6 h, 0.23 h and 2 h, respectively. Pharmacokinetics/pharmacodynamics (PK/PD) properties were integrated based on the concentration-time curves of DOX in the hepatopancreas over the MIC of DOX against *V. parahaemolyticus*. It was indicated that the time during which the DOX concentration in the hepatopancreas was above the MIC (T>MIC) was approximately 4 hours, corresponding theoretically to an 8-hour dosing interval. However, this PK/PD interpretation is presented as supportive information and should be interpreted with caution. A depletion study was conducted by administering DOX-medicated feed once and twice daily for three consecutive days at the same dose. Doxycycline residues in shrimp muscle dropped below the limit of detection after 14 days of stopping medication. Based on a maximum residue limit of 50 µg/kg, the estimated withdrawal time at 26.5°C ranged from 8 to 10 days, depending on the dosing regimen (once or twice a day). Regarding histological analysis, the effect of DOX on hepatopancreas was significant during the twice a day dosing treatment, but recovery of hepatopancreatic cells was observed within seven days after medication.

Keywords: Doxycycline, hepatopancreas, pharmacokinetics, whiteleg shrimp, withdrawal time.

1. Introduction

Aquaculture industry plays a critical role in global food security, with whiteleg shrimp (*Penaeus vannamei*) being one of the most widely cultivated species in Vietnam with an annual production of 1.26 million tons in 2024 (VASEP, 2025). Nevertheless, the intensive and super-intensive shrimp farming has led to increased vulnerability to bacterial diseases, which can significantly impact production yields and economic stability (Nguyen et al., 2020). Regarding bacterial disease, *Vibrio* spp., particularly *V. parahaemolyticus*, is a prevalent pathogen responsible for AHPND, which has been firstly reported in Vietnam since 2010, with massive mortality of farmed shrimp in the Mekong Delta (Tran et al., 2013; Nghia et al., 2015). To control *Vibrio* pathogen and other bacteria in aquaculture, effective disease management strategies used by farmers often involve the use of antibiotics, including DOX (Phu et al., 2016; Chi et al., 2017; Dao et al., 2022).

Doxycycline (α -6-deoxy-5-hydroxytetracycline) is a second-generation, broad-spectrum tetracycline antibiotic with bacteriostatic activity, its antimicrobial activity targets bacterial ribosomes, where it inhibits protein synthesis by preventing the addition of amino acids to polypeptide chains within this cellular organelle (Etebu et al., 2016). Doxycycline is highly effective against Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). This antibiotic is permitted for use in aquaculture in several countries under national regulatory frameworks. In Vietnam, DOX is included in the list of veterinary drugs allowed for circulation and use (Ministry of Agriculture and Rural Development, 2024). However, improper use of antibiotics such as high doses and non-compliance with antibiotic withdrawal periods has resulted in the presence of antibiotic residues found in exported shrimp products. Between 2020 and 2024, the Rapid Alert System for Food and Feed (RASFF) from the European Union reported that exceeding residue limits of antibiotics including DOX, oxytetracycline, tetracycline, sulfamethoxazole, furazolidone and ciprofloxacin were detected in frozen shrimp products imported from many countries including Vietnam (RASFF, 2025). Moreover, residual levels of antibiotics that exceed the maximum residue limit (MRL) pose potential risks to human health (Quesada et al., 2013; Guidi et al., 2018), and contribute to the issue of antimicrobial resistance (Chopra and Roberts, 2001). Nevertheless, to our knowledge, no data are available about the pharmacokinetics (PK) and tissue residue depletion of DOX in whiteleg shrimp.

Research on the PK of DOX in crustaceans remains limited. To date, only one PK study of DOX has been reported in a freshwater crustacean, the crayfish (*Procambarus clarkii*) (Xu et al., 2022), in which oral administration at 20 mg/kg resulted in a maximal concentration ($C_{\max}$) of 17.58 $\mu\text{g/g}$ and a time to reach maximal concentration ($T_{\max}$) of 6 h in the hepatopancreas. While, no comprehensive PK data are available for penaeid shrimp. In contrast, PK studies on tetracycline-class antibiotics, particularly oxytetracycline, have been conducted in several shrimp species, including white shrimp (*Litopenaeus setiferus*) (Reed et al., 2004), black tiger shrimp (*Penaeus monodon*) (Uno et al., 2006), and Pacific white shrimp (*Penaeus vannamei*) following single and multiple oral administrations (Uno et al., 2010;

Ma et al., 2019). These studies reported measurable oxytetracycline concentrations in shrimp hemolymph, with peak hemolymph concentrations ($C_{\max}$) generally ranging from approximately 3 to 28 $\mu\text{g/mL}$, and $T_{\max}$ values occurring within approximately 4 to 10 h, depending on shrimp species, dosage, and experimental conditions. In addition, tissue-specific distribution kinetics of oxytetracycline in shrimp, such as accumulation in the exoskeleton, have also been reported (Faroongsarn et al., 2013). Whereas, PK characteristics of DOX have mainly been investigated in fish species such as rainbow trout (Altan et al., 2024), striped catfish (Vinh et al., 2024), channel catfish (Xu et al., 2020; Ai et al., 2011), African catfish (Ibrahim et al., 2019), tilapia (Yang et al., 2014). In addition, PK data for DOX in terrestrial animals have been widely reported and were summarized by Yang et al. (2014). Despite the availability of these oxytetracycline PK studies, the distribution, tissue exposure, and elimination kinetics of DOX in penaeid shrimp remain largely unexplored. Therefore, information on shrimp-specific PK/PD relationships for DOX is still lacking. Establishing appropriate PK/PD indices for antibacterial drugs in aquaculture is essential for optimizing dosing strategies against various bacterial infections, which helps ensuring effective treatment while reducing the risk of antimicrobial resistance in aquatic species (Mothadaka et al., 2023). Additionally, integrating PK parameters such as $C_{\max}$ and $\text{AUC}_{0-24\text{h}}$ along with a suitable PD parameter, such as the MIC to figure out the appropriate therapeutic dose and treatment interval when using antibiotics, ensures their effective application in controlling bacterial infections in aquatic species (Mothadaka et al., 2023).

The impact of antibiotics on shrimp hepatopancreas histology is another key aspect that needs to be evaluated. The hepatopancreas is a multifunctional organ responsible for digestion, metabolism, and immune responses, it plays a central role in shrimp health and overall growth (Manan et al., 2015). Adverse histological changes in this organ can occur with exposure to improperly used antibiotics or chemicals (Maftuch et al., 2017; Bray et al., 2006). A study on the hepatopancreas histology of whiteleg shrimp revealed that CFT administration had no negative impact on hepatopancreatic structure or health (Huynh et al., 2024). Despite this, there has been no research specifically examining the adverse effects of DOX on the hepatopancreas of whiteleg shrimp.

In general, due to the common use of DOX in whiteleg shrimp aquaculture, its PK/PD properties and effect on shrimp hepatopancreas as well as muscle residue depletion should be investigated to rationalize DOX use in aquaculture and ensure food safety for consumers.

2. Materials and Methods

2.1 Chemicals

A commercial product “Doxy 10%”, labeled to contain 10% (w/w) of DOX, purchased from a veterinary medicine supplier in Vietnam (Vemedim Co Ltd). Prior to the experiment, the absence of DOX residues in hemolymph and tissues (hepatopancreas and muscle) of whiteleg shrimp was confirmed. In addition,

the actual concentration of DOX in the commercial product was quantitatively verified by LC–MS/MS using an analytical-grade DOX standard for calibration. C18 Bondesil powder with a particle size of 40 µm was supplied by Agilent (Santa Clara, CA, USA). High-purity chemicals above 98%, including the DOX standard obtained from Dr. Ehrenstorfer® (Augsburg, Germany) and DOX D3 (internal standard, IS) obtained from TLC Pharmaceutical Standards Ltd. (Ontario, Canada). In addition, methanol, acetonitrile, distilled water and other chemicals were sourced from Merck (Darmstadt, Germany).

2.2 *Vibrio parahaemolyticus* strains

The *V. parahaemolyticus* strain associated with AHPND on whiteleg shrimp was isolated and obtained from the culture collection of the Faculty of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University. Sample collection included 42 bacterial strains isolated from shrimp disease cases recorded between 2022 and 2024 across seven provinces including Ca Mau, Kien Giang, Soc Trang, Can Tho, Dong Thap, Tra Vinh, and Tien Giang of the Mekong Delta, Vietnam. The bacterial strains were reactivated prior to the experiment following the procedure described in Section 2.2.1.

2.2.1 Antibiotic susceptibility testing

Vibrio parahaemolyticus was cultured in nutrient broth supplemented with NB+ (1.5% NaCl) and incubated at 28°C for 18 hours. The bacteria were then transferred to tryptic soy agar plates supplemented with TSA+ (1.5% NaCl) and incubated for an additional 18 hours. The colony color and shape were recorded, and Gram staining was performed to confirm the purity of the bacterial culture. To increase biomass, pure colonies were cultured in NB+ at 28°C for 24 hours. Afterward, the bacterial concentration was adjusted to approximately 10⁸ CFU/mL using McFarland's 0.5 Barium Sulfate Standard Solution (Sotomayor et al., 2019).

The antibiotic resistance of *V. parahaemolyticus* was assessed using the agar disk diffusion method, based on the protocols of Balouiri et al. (2016) and Jiang et al. (2020). Commercial DOX disks (30 µg; Oxoid, UK) was applied in this test. One hundred microliters of the bacterial suspension were evenly spread on the surface of a TSA+ plate. After 15-min, the DOX disk was carefully placed on the agar surface with sterile fine-pointed forceps to ensure proper contact. The plates were then incubated at 28°C for 24 hours. The inhibition zone diameters (including the disk diameter) were visually measured and the size of the clear zone was used to categorize the isolates as susceptible, intermediate, or resistant (CLSI M100, 2018).

2.2.2 Minimum inhibitory concentration

Vibrio parahaemolyticus was activated following the method previously described. The MIC of DOX was determined using the broth macro dilution method with modifications as described by Ali et al. (2019). A 128 mg DOX standard solution was prepared by dissolving with 10 mL of sterile distilled

water to reach a final concentration of 1280 µg/100 µL. This solution was then subjected to a series of 12 successive dilutions.

For the test, 1 mL of the bacterial solution was diluted 200-fold with TSB+ medium. Fourteen sterilized test tubes, each with screw caps, were labeled and filled with 4.9 mL of the diluted bacterial solution. Then, to each test tube, 100 µL of the prepared DOX standard solution was added to achieve final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL. In addition, a positive control (TSB+ solution with bacteria) and a negative control tube (TSB+ solution without bacteria) were prepared as well. All test tubes were incubated at 28°C for 24 hours. The MIC was determined as the lowest concentration of DOX where no visible bacterial growth was observed. MIC values are interpreted using the interpretive criteria for *Vibrio* spp. from Clinical and Laboratory Standards Institute (CLSI M45, 2015). The percentile function from Excel software (Microsoft Office 2019) was utilized to calculate the MIC50 and MIC90 from the MIC dataset.

2.3 Whiteleg shrimp

Healthy whiteleg shrimp (15.4 ± 2.3 g/shrimp) were supplied by the Faculty of Marine Science and Technology, College of Aquaculture and Fisheries, Can Tho University. The shrimp were confirmed to be negative for AHPND, which is primarily caused by *Vibrio* spp., particularly *V. parahaemolyticus* and *Enterocytozoon hepatopenaei* (EHP) based on molecular diagnostic methods (PCR). The shrimp were reared from the post-larval stage without the use of antibiotics for 45 days before conducting the experiment. Shrimp were stocked into 2 m³ tanks at a density of 100 shrimp/m³ and acclimated for 1-week. During the acclimatisation period, shrimp were fed a commercial pellet feed containing 40% crude protein (Proconco, Vietnam) four times a day at 6:00 am, 10:00 am, 2:00 pm, and 6:00 pm, with the feed amount corresponding to 3% of body weight of shrimp. Any leftover feed was removed by siphoning after 30 minutes of feeding (lower than 5% of the total amount). The water temperature was recorded at $26.5 \pm 0.9^\circ\text{C}$. To maintain proper oxygen levels above 5 mg/L, the experimental tanks were fully equipped with an aeration system. Additionally, water quality parameters such as alkalinity (adjusted to 140-160 mg HCO₃⁻/L), salinity (adjusted to 10‰), temperature, pH, and nitrite (NO₂⁻), were regularly monitored and controlled to ensure optimal conditions for shrimp health and normal development throughout the entire experiment. This salinity level falls within the typical range (10–15‰) commonly used in whiteleg shrimp farming systems in Vietnam.

2.4 Pharmacokinetics of doxycycline after oral administration

Whiteleg shrimp were stocked and acclimated in 6 tanks, with each tank representing one replicate (2 kg shrimp per tank). A single dose of 20 mg DOX/kg body weight was administered to the shrimp, and this dosage was chosen based on the standard therapeutic dose recommended by the manufacturers of commercial products, and in previous studies where the same dose was applied to treat bacterial infections in fish and shrimp via oral administration (Ibrahim et al., 2019; Xu et al., 2019a, b and 2021;

Vinh et al., 2024). The DOX commercial product used in this study contained 10% active ingredient. Shrimp were fed at 3% body weight per day, corresponding to 60 g feed per tank per day, divided into four feedings. For the PK experiment, medicated feed was administered only during the first feeding (6:00 am), while non-medicated feed was provided at subsequent feeding times, i.e., at 10:00 am, 2:00 pm, and 6:00 pm. Based on a shrimp biomass of 2 kg per tank, a total of 40 mg active DOX per tank was required to achieve the target dose. Since only 15 g feed was administered during the medicated feeding, the medicated feed was prepared to contain 2667 mg active DOX/kg feed, corresponding to 26.67 g commercial product/kg feed. The medicated feed was prepared by dissolving the calculated amount of DOX product in tap water, thoroughly mixing it with pelleted feed. After that, the medicated feed was coated with 2% squid oil, and then the feed was left for 15 minutes to allow the drug and oil to be fully absorbed into the pellets before being administered to the shrimp. The concentration of DOX in medicated feed was 1502 mg/kg feed, analyzed by LC-MS/MS.

Following administration, hemolymph, muscle, and hepatopancreas of shrimp were collected at 0.5, 1, 2, 4, 8, 12, and 24 hours post-medication. In this experiment, the tank was considered the experimental unit. At each time point, three shrimp from each tank were collected. For hemolymph collection, approximately 0.5 mL hemolymph per shrimp was collected from the pericardial cavity using a 1 mL syringe pre-filled with an anticoagulant solution (EDTA 0.01M, NaCl 0.338M, glucose 0.115M, trisodium citrate dihydrate 0.03M), corresponding to a 1:1 (v/v) ratio of anticoagulant to hemolymph. Hemolymph from three shrimp from the same tank was pooled to form one composite sample per tank at each sampling time, transferred to 1.5 mL centrifuge tubes, and centrifuged at 9500 g for 5 minutes. After centrifugation, the supernatant was collected as cell-free hemolymph (Lorenzon et al., 1999). Simultaneously, the hepatopancreas and muscle tissues from those shrimps were collected and pooled into marked plastic bags to obtain one pooled tissue sample for each tank, forming one replicate. Thus, each sampling time included six independent pooled samples corresponding to six replicate tanks ($n = 6$). The selected sample size was based on previously published pharmacokinetic studies in shrimp and other aquatic species, where similar sample sizes were commonly applied (Ma et al., 2017; Yang et al., 2014; Xu et al., 2022). All hemolymph, muscle, and hepatopancreas samples were then frozen and stored at -80°C for subsequent analysis.

2.5 Doxycycline muscle tissue depletion and hepatopancreas histological examination

The experiment was carried out with two groups, each group consisting of three replicate tanks with the density of 100 shrimp/ m^3 . In group 1, shrimp were administered DOX-medicated feed at a dose of 20 mg DOX/kg body weight per day, delivered as a single feeding at 6:00 am for three consecutive days (medicated feed was prepared as described earlier). In group 2, the medicated feed was provided to the shrimp twice daily, at 6:00 am and 6:00 pm, for three consecutive days. In this group, the dose of 20 mg DOX/kg body weight was administered at each feeding, resulting in a total daily dose of 40 mg DOX/kg body weight. After the three-day period of DOX-medicated feed administration, non-DOX

medicated feed was given for the next 21 days, four times daily at 6:00 am, 10:00 am, 2:00 pm, and 6:00 pm. Shrimp were sampled on days 1 and 3, at 1 hour after feeding DOX medicated feed. Further sampling was carried out on days 1, 3, 7, 14, and 21 after the cessation of medicated feed. At each sampling time, 10 shrimp were randomly collected from each tank, and shrimp muscles were pooled, minced, and stored at -80°C for later analysis.

Additionally, to assess the impact of DOX on the shrimp's hepatopancreas, histological examination was also performed in this experiment. Samples were collected at three time points: one day before medication, one hour after the last dose of medication, and seven days after the cessation of medication. At each sampling time, three individual shrimp per tank were collected (three replicate tanks per treatment group), resulting in a total of nine shrimp ($n = 9$) per treatment group, which were separately processed for histological analysis.

2.6 Quantification of doxycycline in shrimp hemolymph and tissues (hepatopancreas and muscle)

Doxycycline quantification was performed using a Waters ACQUITY Ultra-High-Performance Liquid Chromatography system (Waters, Milford, MA, USA) coupled to a Xevo™ TQ-S triple quadrupole mass spectrometer combined with electrospray ionization (ESI) operated in the positive ion mode (Waters). The system was equipped with an Acquity UPLC CSH C18 column (2.1×50 mm, $1.7 \mu\text{m}$) (Waters, Milford, MA, USA). Mass spectrometry parameters, including capillary voltage (2.8 kV), ion source temperature (150°C), cone gas flow (50 L/h), desolvation gas flow (800 L/h), and desolvation temperature (400°C), were optimized. Argon was used as the collision gas at a pressure of 4×10^{-3} mbar. The following ion transitions (precursor > product ion, m/z) were monitored for quantification and confirmation:

- DOX:
 - Quantification: m/z 445.08 > 428.1 (collision energy: 18 eV)
 - Confirmation: m/z 445.08 > 154.06 (collision energy: 28 eV)
- DOX D3 (internal standard):
 - Quantification: m/z 448.2 > 431.1 (collision energy: 18 eV)

Liquid chromatographic separation was performed using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 0.25 mL/min. The initial mobile phase composition (90% A and 10% B) was maintained for 0.6 min. A linear gradient was then applied to reach 10% A and 90% B at 2.5 min. This composition was held for 0.5 min, after which the system was returned to the initial conditions (90% A and 10% B) over 0.5 min and maintained for an additional 1 min for column equilibration.

2.6.1 Hemolymph sample preparation

Doxycycline extraction from whiteleg shrimp hemolymph was performed using a modified method of Vinh et al. (2024). In brief, 0.5 mL of hemolymph was spiked with 10 μ L of 1 μ g/mL DOX D3 as an internal standard and vortexed after addition of 3 mL of acetonitrile. After centrifugation at 9500 g for 10 min, the supernatant was collected and evaporated to dryness under a gentle nitrogen stream at $40 \pm 1^\circ\text{C}$ using a nitrogen evaporator. The residue was reconstituted in 1 mL of mobile phase in a ratio of 95% A: 5% B. The mobile phase included two solvents: solvent A (50% acetonitrile + 50% methanol containing 0.1% formic acid) and solvent B (0.1% formic acid in water). Before LC–MS/MS injection, the reconstituted sample was filtered through a 0.22 μ m nylon membrane filter (Advantec MFS, CA, USA) and an aliquot of 10 μ L was injected into the LC–MS/MS system.

2.6.2 Tissue and feed sample preparation

Doxycycline extraction from feed and shrimp tissues (hepatopancreas and muscle) was performed after mincing using an Ultra Turrax homogenizer (T-25, IKA, China). Samples (3.00 ± 0.1 g) were transferred into a 50 mL tube, fortified with 60 μ L of IS (DOX D3, 1000 ng/mL), and left at room temperature for 15 min. The extraction process involved adding 1 mL of 0.1 M phosphate buffer at pH of 8.0, vortexing for 1 min, and then 20 mL of acetonitrile was introduced, followed by vortexing for 30 sec and sonication for 15 min in an Elma Ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany). To collect the supernatant, the tubes were then centrifuged at 3501 g for 5 min. Acetonitrile was adjusted to a final volume of 24 mL and 8 mL of this was transferred into a 15 mL glass test tube containing 40 mg of Bondesil C18. After centrifugation again at 3501 g for 4 min, the supernatant was collected in a 10 mL glass test tube and evaporated at $40 \pm 1^\circ\text{C}$ in a water bath under nitrogen flow. The dried residues were reconstituted in 1 mL of mobile phase before injection into the LC–MS/MS system. If DOX concentrations exceeded the calibration range, samples were diluted with a blank sample extract containing IS at the same concentration as the samples before reinjection.

2.6.3 Method validation

The analytical methods were validated in accordance with European Commission Decision 2002/657/EC (EC, 2002) and met the required acceptance criteria to ensure accurate and reliable DOX quantification in shrimp muscle tissue. Key validation parameters are presented in Luy (2017). The limits of detection (LOD) and limits of quantification (LOQ) of the analytical methods were 2.5 and 5 μ g/kg or μ g/L in tissue and hemolymph, respectively. The standard calibration curve, prepared using blank samples, exhibited a linear range of 5–100 μ g/kg. The extraction recovery ranged from 94% to 100%, ensuring reliable quantification. The method demonstrated strong linearity with coefficients of determination (R^2) above 0.99 and high specificity with interference levels below 2.5%. Precision was also confirmed through repeatability (relative standard deviation of 3.05%–4.59%) and within-laboratory reproducibility (relative standard deviation of 2.06%–4.47%). The apparent recovery varied

between 97% and 100%. The decision limit (CC_α) and detection capability (CC_β) were calculated to be 53.8 $\mu\text{g/kg}$ and 57.6 $\mu\text{g/kg}$, respectively. To minimize potential matrix effects, quantification was performed using matrix-matched calibration curves prepared from blank shrimp samples, e.g., shrimp muscle, hepatopancreas and hemolymph.

2.7 Pharmacokinetic estimation

The concentration of DOX in hemolymph, hepatopancreas, and muscle was modelled using a naïve pooled population approach based on a one-compartmental model with first-order absorption and elimination. This approach was applied because the destructive sampling design and limited number of samples obtained from individual shrimp prevented repeated measurements from the same individuals, making standard non-compartmental analysis (NCA) unreliable and mixed-effects modeling unsuitable. However, the naïve pooled method does not allow quantification of inter-individual variability, and the resulting PK estimates should therefore be interpreted as descriptive or approximate typical PK rather than a true population model.

For hemolymph, the following PK parameters were calculated: absorption rate constant (k_a), absorption half-life ($T_{1/2a}$), maximal hemolymph concentration ($C_{\max}$), time to reach maximal hemolymph concentration ($T_{\max}$), area under the hemolymph concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (CL/F) and apparent volume of distribution after oral administration (V_d/F).

For the hepatopancreas and muscle tissues, the PK parameters calculated included maximal tissue concentration ($C_{\max}$), time to reach maximal tissue concentration ($T_{\max}$), and area under the tissue concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$), which was determined using the linear-up log-down trapezoidal method. All data were processed using Phoenix 8 (Certara, Princeton, NJ, USA).

2.8 Withdrawal times estimation

The withdrawal time for DOX was estimated following the European Medicines Agency guidelines (EMA, 2022), utilizing the statistical software WT 1.4 developed by Hekman (2004). For withdrawal time estimation, residue depletion data used for withdrawal time calculation were obtained at 1 h, 1, 3, and 7 days after the final dose. Doxycycline concentrations in muscle were measured at multiple time points after treatment and analyzed using linear regression based on time. The used unit was degree-days, which were determined by multiplying the average daily water temperature ($^{\circ}\text{C}$) by the total number of days the temperature was recorded. The withdrawal period was defined as the time when the upper one-sided 95% tolerance limit for DOX residue concentration dropped below the MRL, with 95% confidence level. For ease of interpretation, withdrawal times were also expressed in calendar days based on the mean rearing temperature.

2.9 Hepatopancreas histological analysis

The histology of shrimp hepatopancreas was examined using the methods as described in Huynh et al. (2024). Hepatopancreas histological sections were examined using a microscope (Novex, Arnhem, the Netherlands). The counts of B and F cells were performed according to Nima et al. (2022) with slight modifications. Both cell types were counted in the same microscopic fields, with different fields randomly selected, and a total of 40 tubules were counted per individual shrimp sample (Huynh et al., 2024).

To assess the effects of dosing and/or sampling on the hepatopancreas, statistical analyses on histopathology results were carried out using one-way and two-way ANOVA with SPSS software (version 20.0). When significant differences were detected, Duncan's multiple range test was employed as a post hoc analysis. A significance level of $p < 0.05$ was used to evaluate statistical differences.

3. Results

3.1 Minimum inhibitory concentration of doxycycline and its susceptibility to *Vibrio parahaemolyticus*

The MIC values of DOX against 42 *V. parahaemolyticus* isolates—collected from diseased shrimp in seven provinces of the Mekong Delta—ranged between 2 and over 256 $\mu\text{g/mL}$ (Table 1). For 15/42 isolates MIC values of $\leq 4 \mu\text{g/mL}$ were noted, a smaller portion (only 8 isolates) clustered at 8 $\mu\text{g/mL}$, and the most common group showed MIC values $\geq 16 \mu\text{g/mL}$ as observed in 19 isolates. Generally, regarding strains showed to be inhibited in a specific DOX concentration (29 strains) the calculation showed that 50% and 90% of tested strains had the MIC smaller or equal to 4 $\mu\text{g/mL}$ and 19.2 $\mu\text{g/mL}$ (corresponding to MIC₅₀ and MIC₉₀), respectively.

Regarding the disk diffusion assay, the inhibition zone diameters for the strains from various regions of the Mekong Delta were as follows: Ca Mau (isolates 1–17, ranging from 0–21 mm), Kien Giang (isolates 18–19, ranging from 8–20 mm), Soc Trang (isolates 20–30, ranging from 10–25 mm), Can Tho (isolates 31–33, ranging from 11–19 mm), Dong Thap (isolate 34, 19 mm), Tra Vinh (isolates 35–39, ranging from 6–20 mm), and Tien Giang (isolates 40–42, ranging from 0–17 mm) (Table 1).

Table 1. Minimum inhibitory concentration and inhibition zone diameters of DOX against *V. parahaemolyticus* isolates associated with shrimp disease

Isolate no.	MIC (µg/mL)	MIC interpretation	Inhibition zone (mm)	Zone interpretation
1	4	S	21	S
2	16	R	13	I
3	32	R	15	S
4	16	R	16	S
5	2	S	18	S
6	4	S	19	S
7	4	S	16	S
8	2	S	18	S
9	2	S	16	S
10	8	I	17	S
11	8	I	19	S
12	16	R	20	S
13	32	R	13	I
14	>256	R	0	R
15	>256	R	0	R
16	>256	R	6	R
17	>256	R	6	R
18	2	S	20	S
19	>256	R	8	R

Isolate no.	MIC (µg/mL)	MIC interpretation	Inhibition zone (mm)	Zone interpretation
20	8	I	25	S
21	8	I	20	S
22	8	I	19	S
23	2	S	17	S
24	2	S	18	S
25	2	S	17	S
26	8	I	14	S
27	>256	R	12	I
28	>256	R	10	R
29	2	S	18	S
30	64	R	14	S
31	8	I	19	S
32	>256	R	11	I
33	8	I	19	S
34	2	S	19	S
35	2	S	20	S
36	2	S	20	S
37	>256	R	6	R
38	>256	R	6	R
39	4	S	18	S

Isolate no.	MIC ($\mu\text{g/mL}$)	MIC interpretation	Inhibition zone (mm)	Zone interpretation
40	>256	R	17	S
41	>256	R	0	R
42	>256	R	7	R

Isolate number from 1 to 17 in Ca Mau regions, 18-19 in Kien Giang regions, 20-30 in Soc Trang regions, 31-33 in Can Tho regions, 34 in Dong Thap region, 35-39 in Tra Vinh regions, 40-42 in Tien Giang regions

S – susceptible, I – intermediate, R – resistant. MIC interpretations were assigned according to CLSI M45 (2015) interpretive criteria for *Vibrio* spp. Zone diameter interpretations were applied according to CLSI M100 (2018)

3.2 Pharmacokinetics of doxycycline after oral administration

Figure 1 illustrates the concentration-time curves of DOX in hemolymph, hepatopancreas, and muscle of whiteleg shrimp following an oral administration of a single dose at 20 mg DOX/kg body weight. The concentration of DOX over time was highest in shrimp hepatopancreas, followed by shrimp hemolymph, and then shrimp muscle.

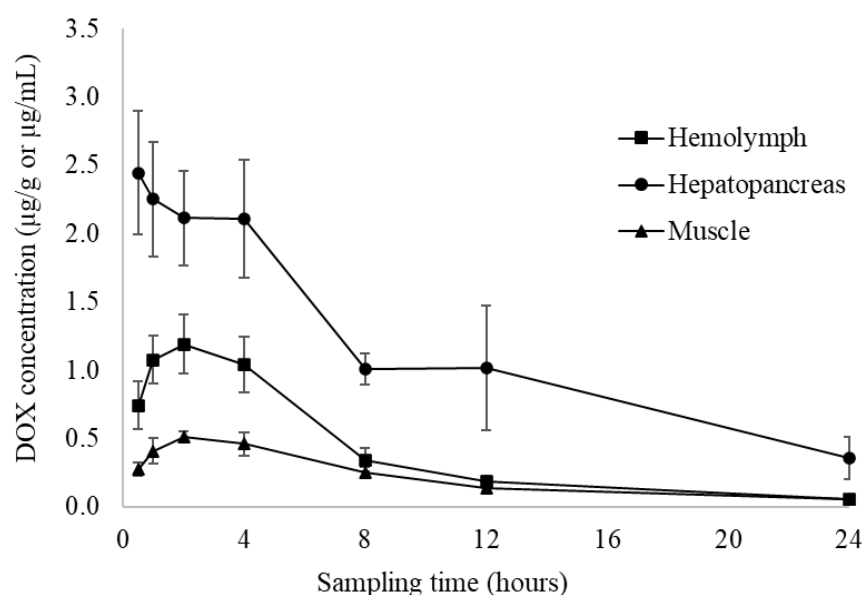


Figure 1. Doxycycline concentrations in hemolymph, hepatopancreas and muscle of whiteleg shrimp after oral administration of a single dose at 20 mg/kg body weight, the data are presented as mean $\pm$ standard deviation (n = 6)

The PK parameters of DOX in shrimp hemolymph (Table 2) include the absorption rate constant (k_a) of 1.68 h^{-1} , absorption half-life ($T_{1/2a}$) of 0.41 h, peak hemolymph concentration ($C_{\max}$) of $1.09 \text{ } \mu\text{g/mL}$, and the time to reach maximum concentration ($T_{\max}$) at 1.6 h and area under the concentration-time curve ($\text{AUC}_{0-\infty}$) of $9.45 \text{ } \mu\text{g.h/mL}$ (Table 2). The apparent total body clearance (CL/F) was 2.12 L/kg/h , apparent volume of distribution (V_d/F) of 14.61 L/kg , elimination rate constant (k_{el}) of 0.15 h^{-1} , and elimination half-life ($T_{1/2el}$) of 4.78 h (Table 2).

Table 2. Pharmacokinetic parameters of DOX in whiteleg shrimp hemolymph after a single oral administration at a dose of 20 mg DOX/kg body weight

PK parameter	Unit	Hemolymph estimate
k_a	h^{-1}	1.68
$T_{1/2a}$	h	0.41
$C_{\max}$	$\mu\text{g/mL}$	1.09
$T_{\max}$	h	1.60
CL/F	L/kg/h	2.12
V_d/F	L/kg	14.61
$\text{AUC}_{0-\infty}$	$\mu\text{g.h/mL}$	9.45
k_{el}	h^{-1}	0.15
$T_{1/2el}$	h	4.78

Values represent population PK parameter estimates derived from six independent tank-based concentration–time profiles ($n = 6$). Absorption rate constant (k_a), absorption half-life ($T_{1/2a}$), maximal hemolymph concentration ($C_{\max}$), time to maximal hemolymph concentration ($T_{\max}$), area under the hemolymph concentration–time curve from time 0 to infinity ($\text{AUC}_{0-\infty}$), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (CL/F) and apparent volume of distribution after oral administration (V_d/F).

When examining the PK of DOX in shrimp tissues, i.e., hepatopancreas and muscle, as shown in Table 3, it is clear that the $C_{\max}$ in the hepatopancreas ($2.37 \mu\text{g/g}$) obtained after 0.23 h ($T_{\max}$) were markedly five times higher compared to that in the muscle which was only $0.46 \mu\text{g/g}$ after 2 h of dosing. In terms of the $AUC_{0-\infty}$ parameter, the value of hepatopancreas ($31.72 \mu\text{g.h/g}$) was significantly higher than that in muscle ($5.55 \mu\text{g.h/g}$), i.e., 5.7 times.

Table 3. Main PK parameters of DOX in hepatopancreas and muscle tissues of whiteleg shrimp after a single oral administration at a dose of 20 mg DOX/kg body weight

PK parameter	Unit	Hepatopancreas estimate	Muscle estimate
$C_{\max}$	$\mu\text{g/g}$	2.37	0.46
$T_{\max}$	h	0.23	2.00
$AUC_{0-\infty}$	$\mu\text{g.h/g}$	31.72	5.55

Values represent population PK parameter estimates derived from six independent tank-based concentration–time profiles ($n = 6$). Maximal tissue concentration ($C_{\max}$), time to maximal tissue concentration ($T_{\max}$) and area under the tissue concentration–time curve from time 0 to inf ($AUC_{0-\infty}$)

3.3 Residue depletion and withdrawal time estimation of doxycycline

The DOX concentrations in the shrimp muscle when feeding once and twice a day for 3 consecutive days are presented in Table 4. On the first day of treatment, the DOX levels in shrimp muscle were $226 \mu\text{g/kg}$ for the once-a-day regimen and $559 \mu\text{g/kg}$ for the twice-a-day regimen. After that, these concentrations reached the highest concentration at day 3 of medication for both treatments, peaking at $323 \mu\text{g/kg}$ for the once-a-day treatment and $599 \mu\text{g/kg}$ for the twice-a-day treatment. After stopping medication, DOX concentrations in shrimp muscle gradually decreased, the values declined to $7.27 \mu\text{g/kg}$ in once-a-day feeding and $10.33 \mu\text{g/kg}$ for twice-a-day feeding at day 7 post medication. The DOX levels were below the LOD ($2.5 \mu\text{g/kg}$) after 14 days onwards in both feeding regimes.

Table 4. The concentration of DOX in whiteleg shrimp muscle during and after medication at a dose of 20 mg DOX/kg body weight when feeding shrimp with DOX-medicated feed once daily and twice daily for 3 consecutive days

Period	Sampling time (days)	DOX concentrations (µg/kg)	
		Once daily treatment	Twice daily treatment
Medication	1	226 ± 62.2	559 ± 16.8
	3	323 ± 52.0	599 ± 35.6
After medication	1	54.33 ± 22.50	57.00 ± 13.08
	3	20.67 ± 7.64	28.33 ± 7.09
	7	7.27 ± 2.41	10.33 ± 4.04
	14	< LOD	< LOD
	21	<LOD	<LOD

Values are expressed as mean ± standard deviation, n = 3. LOD=limit of detection of 2.5 µg/kg

The withdrawal time of DOX in whiteleg shrimp muscle was estimated in this experiment after feeding shrimp with DOX medicated feed once-a-day and twice-a-day for three consecutive days at 20 mg/kg body weight and an ambient temperature of 26.5°C. Based on an MRL of 50 µg/kg set by Japan (JFCRF, 2017), the results for the withdrawal time of DOX in shrimp muscle were 186 degree-days (corresponding to 8 days at 26.5°C) for feeding once-a-day treatment (Figure 2A), and 241 degree-days (equating to 10 days) for twice-a-day treatment (Figure 2B). withdrawal time of DOX in shrimp muscle were shorter when applying the MRL of 100 µg/kg as set by the European Union (EMA, 2015), namely 140 degree-days (equating to 6 days) and 190 degree-days (approximately 8 days) in once-a-day (Figure 2C) and twice-a-day regimens (Figure 2D), respectively.

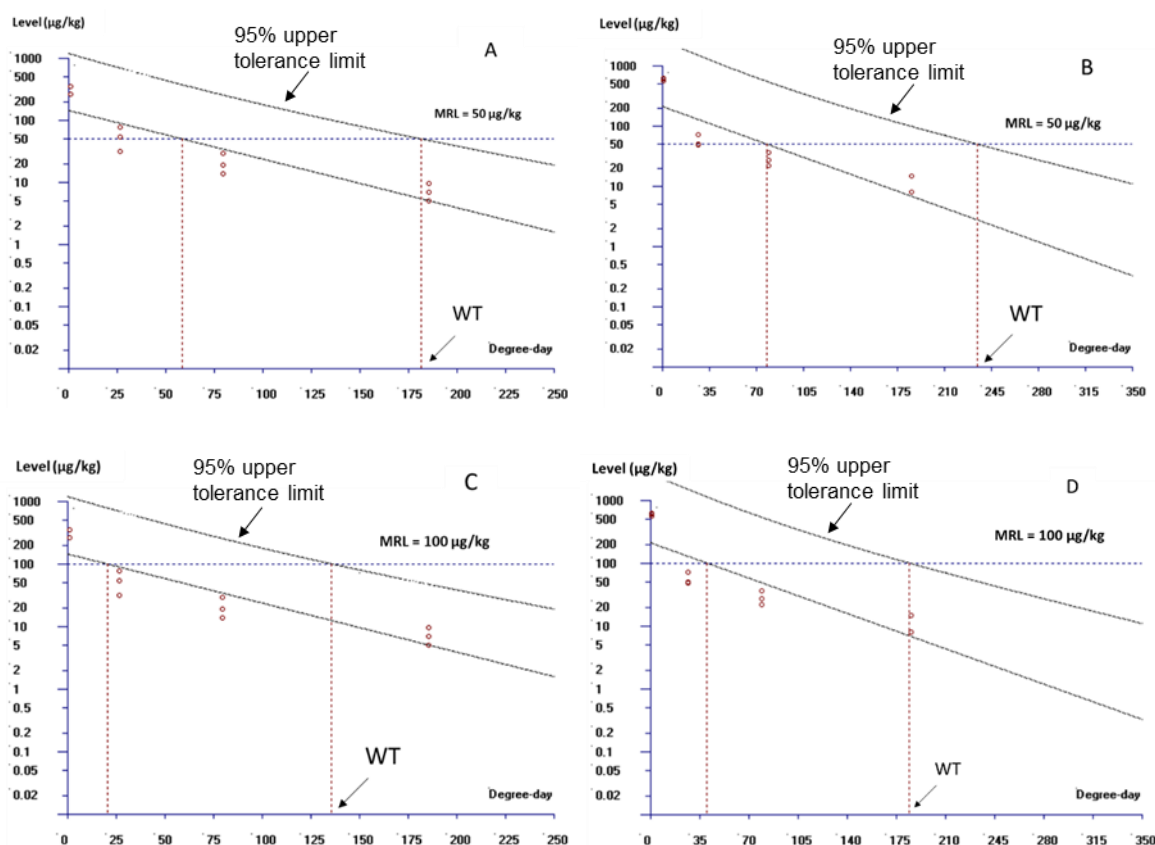


Figure 2. Plot of DOX residual levels in whiteleg shrimp muscle recorded at 1 h, 1, 3, and 7 days, corresponding to 1.1, 26.5, 79.5, and 185.5 degree-days, respectively, following the cessation of treatment as a function of degree-days. The WTs were calculated as the time when the one-sided 95% upper tolerance limit was below the MRL (50 µg/kg and 100 µg/kg) when feeding shrimp with DOX-medicated feed once daily (A, C) and twice daily (B, D) during 3 consecutive days at a dose of 20 mg/kg body weight, at an average ambient temperature of 26.5°C

3.4 Hepatopancreas histology of whiteleg shrimp

Before antibiotic application, the histological examination of the hepatopancreas revealed no abnormalities, with the tissue structure displaying the normal presence of both B and F cells (Figure 3A). Following DOX administration, histological changes in the hepatopancreas were observed in both once daily and twice daily dosing regimens. On day 3 of medication, shrimp receiving DOX once daily showed mild structural alterations in the hepatopancreatic tubules, including changes in the distribution and morphology of epithelial cells (Figure 3B). Whereas, more pronounced alterations were observed in shrimp receiving DOX twice daily on day 3, with apoptosis-like morphological changes were observed in the hepatopancreatic tubule epithelial cells, characterized by cell shrinkage and nuclear condensation (Figure 3C). By day 7 after stopping medication, the recovery of the hepatopancreatic structure was evident in both treatment groups, with improved tubule organization and cellular

appearance in the once-daily (Figure 3D) and twice-daily (Figure 3E) regimens, approaching the normal histological architecture observed before treatment.

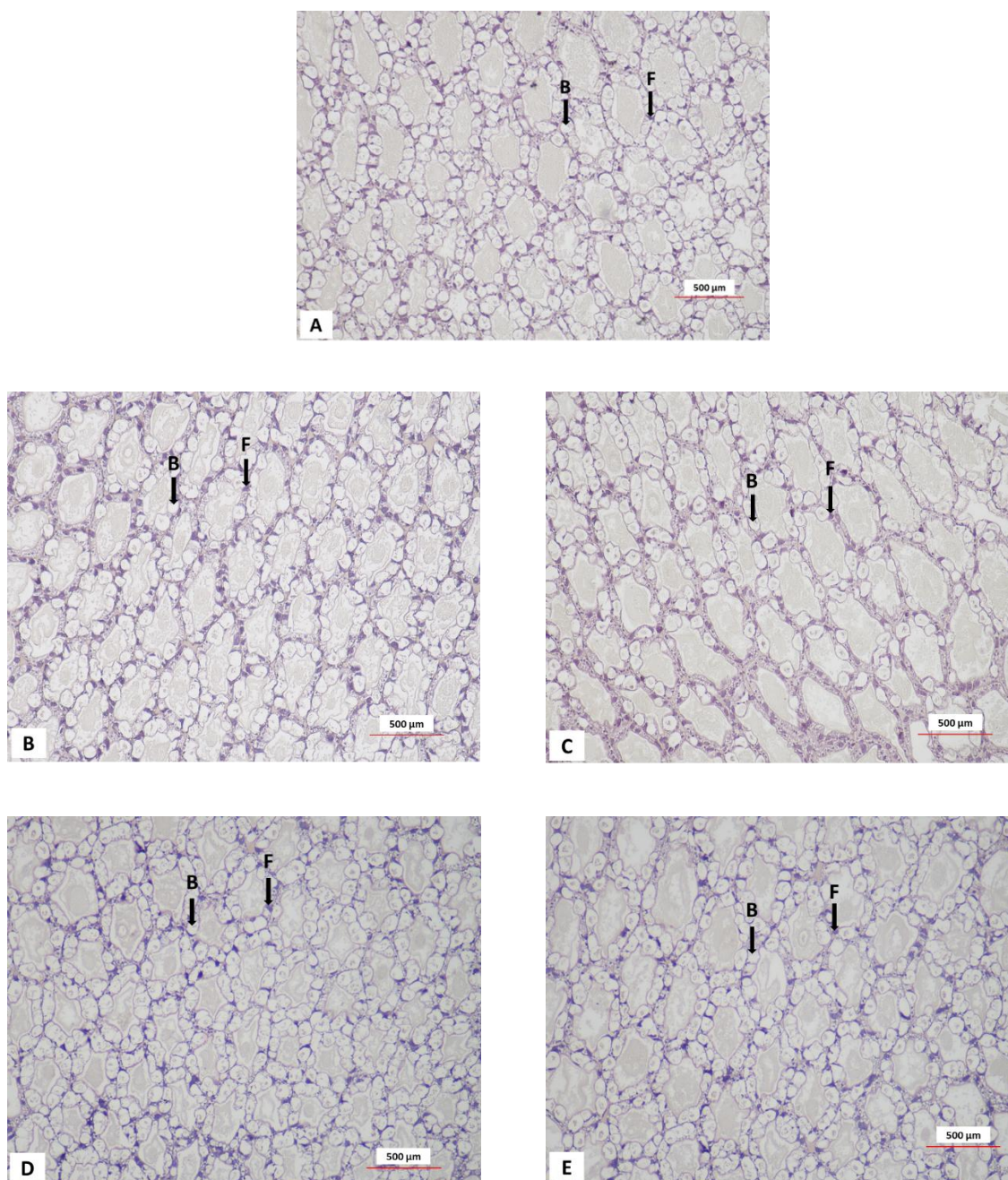


Figure 3. Representative histological sections of hepatopancreas of whiteleg shrimp before treatment (A) and after administering DOX-mediated feed once daily (B) and twice daily (C) on day 3 of medication, and at 7 days post-treatment for once daily (D) and twice daily (E) groups (100× magnification). B means blister cells, F means fibrillar cells

Following the administration of medicated feed once daily and twice daily for 3 consecutive days at a dose of 20 mg/kg body weight, the results concerning the count of B and F cells in the hepatopancreas are presented in Figure 4 and Figure 5. Upon statistical evaluation using two-way ANOVA, no significant difference in the number of B cells ($p > 0.05$) in the shrimp hepatopancreas was found between the two doses applied (Figure 4). However, a significant variation in B cell counts was observed over experimental duration ($p < 0.05$), i.e., by day 3 of medication, the number of B cells in the shrimp hepatopancreas was significantly reduced compared to prior to medication ($p < 0.05$). By day 7, following the cessation of the treatment, the B cell count had rebounded and returned to levels comparable to those observed before treatment with no significant difference noted when compared to before medication ($p > 0.05$). Furthermore, no interaction between the dosage regimen and experimental duration was detected with regard to B cell counts ($p > 0.05$).

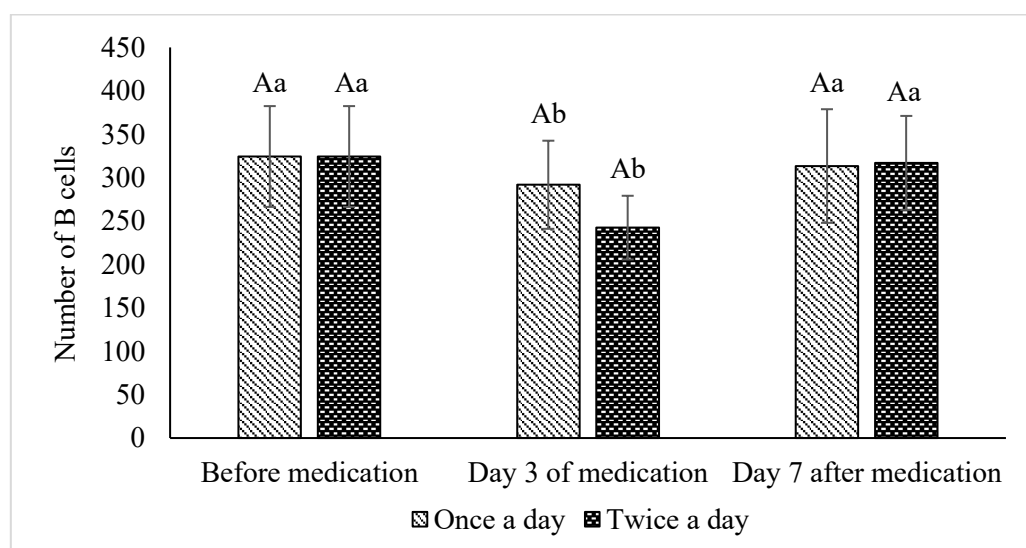


Figure 4. The number of B cells of shrimp hepatopancreas (mean $\pm$ SD, $n = 9$) before medication, on day 3 of medication and on day 7 post-medication for once daily (left column) and twice daily (right column) feeding regimens.

Value with identical capital letters indicate no significant different between the applied doses ($p > 0.05$).

Value with matching lowercase letters denote no significant different between sampling time ($p > 0.05$).

Regarding the F cell counts in the shrimp hepatopancreas (Figure 5), as two-way ANOVA revealed a significant interaction between dosing regimen and experimental duration ($p < 0.05$), interpreted the two factors were accessed by one-way ANOVA. The result showed that on day 3 of medication, the F cell number of shrimp in the twice daily treatment was significantly lower than all other groups ($p < 0.05$). However, after 7 days stopping medication, the number of F cells of twice daily treatment were

increased and recovered and no significant difference was found when comparing with the counts of F cells of shrimp sampled before medication ($p > 0.05$).

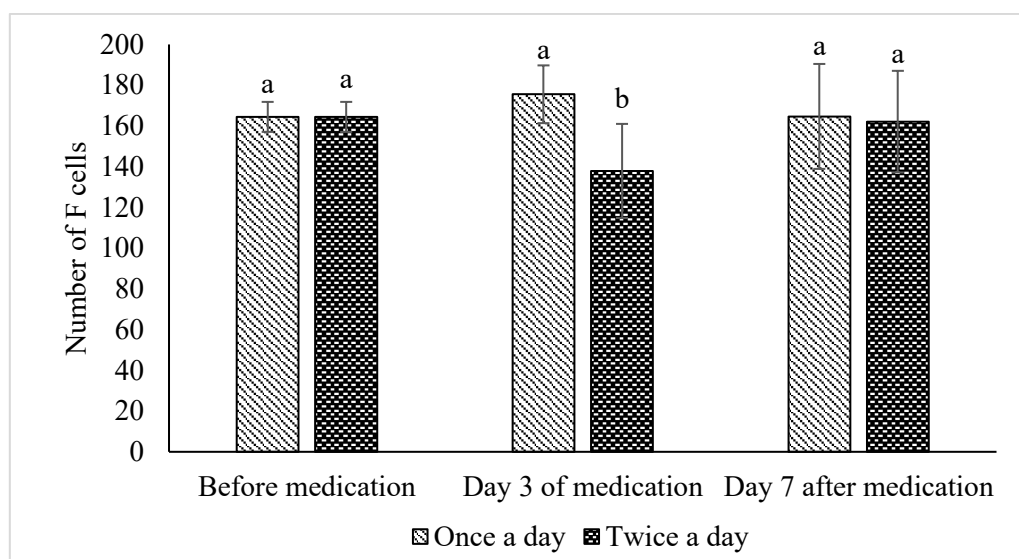


Figure 5. The number of F cells of shrimp hepatopancreas (mean $\pm$ SD, $n = 9$) before medication, on day 3 of medication and on day 7 post-medication for once daily (left column) and twice daily (right column) feeding regimens.

Values with different lowercase letters indicate significant differences among groups based on simple-effects comparisons due to a significant interaction between dosing regimen and sampling time (two-way ANOVA, $p < 0.05$)

4. Discussion

4.1 Antimicrobial activity of doxycycline against *Vibrio parahaemolyticus* strains

Based on Clinical and Laboratory Standards Institute classification (CLSI, 2018), fifteen isolates were susceptible to DOX with a sensitivity rate of 35.7% ($\text{MIC} \leq 4 \mu\text{g/mL}$), while 19% (8/42) of strains were intermediately resistant ($\text{MIC} = 8 \mu\text{g/mL}$). Additionally, 19 isolates showed resistance to DOX at a rate of approximately 45% ($\text{MIC} \geq 16 \mu\text{g/mL}$). In the disk diffusion assay, according to CLSI guidelines (2018), the classification of the antimicrobial susceptibility of bacterial strains includes three categories based on the diameters of the inhibition zones, i.e., ≥ 14 mm indicates susceptibility, 11–13 mm denotes intermediate susceptibility, and ≤ 10 mm indicates resistance. Based on these criteria, *V. parahaemolyticus* strains examined in this study were classified as susceptible, intermediately resistant and resistant to DOX with the proportions of 66.7% (28/42), 9.5% (4/42) and 23.8% (10/42), respectively. Several differences were noted between susceptibility classification based on MIC and disk diffusion. According to Balouiri et al. (2016), MIC determination provides a quantitative measure

of antibacterial activity under standardized broth conditions, whereas disk diffusion results are influenced by antibiotic diffusion properties, agar composition, and bacterial growth characteristics. Furthermore, a standard disk diffusion breakpoint for *Vibrio* spp. has not been formally established by CLSI, and the inhibition zone diameter criterion applied in this study was taken from published literature. Moreover, MIC values in current study were considered for calculating the PK/PD index and interpreting dosage, while disk diffusion was used as a supportive susceptibility screening method. These findings underscore the varying levels of antibiotic susceptibility among *V. parahaemolyticus* strains collected from different geographical locations. These results were aligned with previous finding from *V. parahaemolyticus* isolated from shrimp, i.e., Letchumanan et al. (2015) indicating that *V. parahaemolyticus* isolates from shrimp in Malaysia exhibited high susceptibility to tetracycline (82%), meanwhile, the investigation on *V. parahaemolyticus* showed 36.6% of resistance (Saifedden et al., 2016).

The sensitivity rate of *V. parahaemolyticus* to DOX in present study (66.7%) was consistent with the report of Thi et al. (2024), the disk diffusion results of 32 strains of *V. parahaemolyticus* isolated from farmed shrimp in Ben Tre and Soc Trang provinces in the Mekong Delta, Vietnam exhibited that these strains were sensitive to DOX at a proportion of 68%. However, another study showed that *V. parahaemolyticus* isolated from AHPND shrimp cultured in the Bac Lieu province of Mekong Delta was highly sensitive to DOX, with a rate of 94% (Luan et al., 2023). Besides, the results of the inhibition zone diameter in this investigation also aligns with a previous study on *V. parahaemolyticus* isolated from shrimp diseases, the diameter was 16.05 ± 1.3 mm and showed sensitivity to DOX (Yudiati et al., 2021). Results from the antibiotic sensitivity test and MIC determination in current research were however higher when compared with the study of Dang et al. (2022) evaluating the susceptibility of 58 *V. parahaemolyticus* bacteria isolated from diseased shrimp in Mekong Delta, Vietnam towards DOX, with MIC values ranging from 2 to 4 µg/mL, and a resistance rate of 3.5% for isolates based on disk diffusion test result. According to Nikaido (2009) and Prescott et al. (2000), plasmids play an important role in the dissemination of antibiotic resistance in bacteria, as they can carry genes encoding resistance to various classes of antibiotics, including tetracyclines, β-lactams, macrolides, trimethoprim/sulfamethoxazole, aminoglycosides, and phenicols. The spread of antibiotic resistance may be enhanced when plasmids carrying resistance genes are transferred between bacteria of the same or different species through conjugation. This horizontal gene transfer contributes to the dissemination of resistance traits and may partly explain the variation in susceptibility rates among isolates of the same bacterial species observed across different geographical regions (Thi et al., 2014). The observation that approximately 50% of *V. parahaemolyticus* isolates showed resistance to DOX has important practical implications for shrimp disease management in the Mekong Delta. Resistance patterns may vary considerably among farming regions due to differences in antibiotic-use practices, which are often based on farmer experience rather than antimicrobial susceptibility testing. Consequently, the

effectiveness of DOX treatment may differ between provinces or farms, potentially leading to treatment failure when antibiotics are applied empirically. In practice, antimicrobial susceptibility testing is commonly performed by veterinary chemical companies or aquaculture service providers to support antibiotic selection for farmers. Therefore, establishing and regularly updating regional antimicrobial susceptibility testing databases for shrimp pathogens would be important to support prudent antibiotic use and improve the effectiveness of disease control strategies in shrimp farming areas.

Overall, the findings from the current investigation demonstrated that the susceptibility of *V. parahaemolyticus* strains toward DOX varied depending on regions. Therefore, it is crucial that the antimicrobial effectiveness of DOX continues to be monitored regularly, particularly with its increasing usage and its extensive application in treating shrimp diseases, to ensure sustained effectiveness and minimize the risk of resistance development over time.

From a practical perspective, alternative strategies for controlling *V. parahaemolyticus* infections should be strengthened in shrimp aquaculture systems to reduce selection pressure for antimicrobial resistance. Improved farm management and biosecurity measures, including better water-quality control, appropriate stocking density, pond hygiene, and early disease prevention, may help reduce disease outbreaks. The application of probiotics, immunostimulants, and herbal products has also been increasingly considered as supportive approaches in shrimp health management. Furthermore, the adoption of advanced aquaculture systems such as recirculating aquaculture systems (RAS), biofloc technology, and integrated farming models including shrimp–seaweed, shrimp–rice, and shrimp–mangrove systems may contribute to reducing antibiotic dependence in shrimp culture. In parallel, strengthening farmer education and improving access to technical guidance on responsible antibiotic use remain essential for sustainable disease management in shrimp farming.

4.2 Pharmacokinetics of doxycycline in whiteleg shrimp

Pharmacokinetic studies provide essential information on the absorption, distribution, metabolism, and excretion of drugs in aquatic species (Rodgers and Rowland 2006; Jambhekar and Bree 2009). These parameters are crucial to optimize treatment regimens as well as minimize residual risk. In the current research, the maximum DOX concentration in the hepatopancreas of shrimp was approximately twice as high as that in hemolymph and about five times higher than that in muscle. This trend aligns with findings of Xu et al. (2022), who investigated the PK of DOX in crayfish (*Procambarus clarkii*) administered orally at a dose of 20 mg/kg, where DOX accumulation in the crayfish hepatopancreas was observed to be the highest, nevertheless, the maximal concentrations in hepatopancreas (17.58 µg/g), muscle (3.13 µg/g) and hemolymph (3.07 µg/mL) found in crayfish were higher than those in our study. A similar distribution pattern of tetracycline-class antibiotics has been reported in shrimp species. For example, PK studies of oxytetracycline in Pacific white shrimp (*Penaeus vannamei*)

demonstrated that drug concentrations were highest in the hepatopancreas, followed by hemolymph and muscle after oral administration (Ma et al., 2019). In that study, peak oxytetracycline concentrations reached 27.77 $\mu\text{g/mL}$ in hemolymph and 149.52 $\mu\text{g/g}$ in the hepatopancreas following a single oral dose, which were markedly higher than the concentrations observed for DOX in the present study. Similarly, oxytetracycline PK in black tiger shrimp (*Penaeus monodon*) showed a C_{max} of 21.1 $\mu\text{g/mL}$ following oral administration (Uno et al., 2006). In Pacific white shrimp (*Penaeus vannamei*), hemolymph C_{max} values of 3.37 and 17.4 $\mu\text{g/mL}$ with T_{max} values of 7 and 10 h were reported at doses of 10 and 50 mg/kg bw, respectively (Uno et al., 2010). These values are much higher than the peak hemolymph concentration observed in the present study (1.09 $\mu\text{g/mL}$). Such differences may be attributed to variations in antibiotic compound, dosage, and experimental conditions, as well as differences in PK properties between DOX and oxytetracycline in shrimp.

The hepatopancreas in crustaceans plays a vital role not only for metabolism and excretion but also for the substance absorption (Verri et al., 2001; Faroongsarng et al., 2007). In this study, the T_{max} values in whiteleg shrimp hepatopancreas (0.23 h) and muscle (2 h) were significantly shorter (6 h in hepatopancreas and 4 h in muscle) compared to crayfish, but the C_{max} and $\text{AUC}_{0-\text{inf}}$ values of shrimp hepatopancreas, muscle and hemolymph were largely lower than those in crayfish with the same dose of application as in our study (Xu et al., 2022). The persistent high DOX concentration in those crayfish organs resulted in a notably longer elimination half-life ($T_{1/2\text{el}}$), with a value of 69.5 h for hemolymph compared with whiteleg shrimp (4.78 h) in our study.

When comparing the PK of DOX following a single dose of 20 mg/kg body weight after oral administration with other aquatic animals, particularly in the plasma of several fish species, the observed C_{max} value in this study (1.09 $\mu\text{g/mL}$) was lower than the findings in rainbow trout, striped catfish, African catfish and tilapia which exhibited values of 1.12, 5.06, 2.29 and 2.27 $\mu\text{g/mL}$, respectively (Vinh et al., 2024, Altan et al., 2024, Ibrahim et al., 2019, Yang et al., 2014). In addition, the T_{max} value in whiteleg shrimp was shorter than that reported for these fish species. Such interspecies differences in PK parameters are consistent with recognized physiological and anatomical variation among taxa, which can influence drug absorption, distribution, metabolism and elimination (Toutain et al., 2010). In particular, oral drug absorption is highly dependent on gastrointestinal tract structure and function. Shrimp lack a true vertebrate stomach and have a relatively short digestive tract, which likely contributes to more rapid but less extensive systemic uptake compared to fish (Vinarov et al., 2021). In addition, this apparent discrepancy due to differences in circulatory systems between species, in the case of an animal with an open circulatory system, all of the organs and internal structures are constantly bathed in hemolymph (Fang et al., 2018). Concerning the value of $\text{AUC}_{0-\text{inf}}$, the DOX concentration distribution in shrimp hemolymph (9.45 $\mu\text{g.h/mL}$) was significantly lower than that observed in rainbow trout and tilapia which applied at the same dose (20 mg/kg bw), which had values of 242.25 and 113.45 $\mu\text{g.h/mL}$, respectively, reported by Altan et al. (2024) and Yang et al. (2014), suggesting lower systemic

exposure. These differences may reflect combine effects of reduced oral bioavailability and more rapid elimination mechanisms in shrimp. The apparent volume of distribution (V_d/F) for DOX in shrimp was 14.61 L/kg, indicating efficient DOX distribution from hemolymph, and the value was higher than that reported in rainbow trout (4.74 L/kg) and striped catfish (6.55 L/kg) albeit possible differences in oral bioavailability need to be accounted for (Altan et al., 2024; Vinh et al., 2024). It is noteworthy that research on PK of DOX in African catfish (Ibrahim et al., 2019) and tilapia (Yang et al., 2014) presented a biphasic hemolymph concentration profile, characterized by two C_{max} peaks after oral administration at a single dose of 20 mg/kg body weight, indicating enterohepatic recirculation, however, this phenomenon was neither observed in whiteleg shrimp in our study nor in crayfish (Xu et al., 2022), and may be attributed to fundamental anatomical and physiological differences between crustaceans and vertebrates. Following absorption and distribution, DOX is eliminated from the shrimp's body. In the current study, DOX was eliminated relatively rapidly from the hemolymph of whiteleg shrimp, with an elimination half-life ($T_{1/2el}$) of 4.78 h. The elimination was faster when compared to fish, where $T_{1/2el}$ values were reported as 18.5 h, 39.78 h, 5.81 h, and 77.2 h in striped catfish, rainbow trout, African catfish, and tilapia, respectively (Vinh et al., 2024; Altan et al., 2024; Ibrahim et al., 2019; Yang et al., 2014). Additionally, the apparent total body clearance (CL/F) in this study was 2.12 L/kg/h, which was higher than the 0.8 L/kg/h reported in striped catfish by Vinh et al. (2024). The $T_{1/2el}$ value was markedly shorter than those reported in fish species, which is in agreement with the higher apparent total body clearance observed in shrimp. This indicates a more rapid elimination of DOX in whiteleg shrimp compared to striped catfish because clearance reflects the elimination of drug from the body. Furthermore, PK parameters can differ significantly between or within species, influenced by factors such as species specificity, health status, geographical location, age, size, salinity, water temperature, drug administration routes, and other experimental conditions (Björklund and Bylund, 1991; Zhang and Li, 2007). Generally, these physiological differences provide a mechanistic basis for the observed PK parameter variations between shrimp and fish species.

Toutain et al. (2010) reported that PK parameters may vary due to several factors like geographical region, genetic diversity resulting from artificial selection and breeding activities even within the same aquatic species. Additionally, drug metabolism in ectothermic animals is considerably affected by environmental factors, particularly water temperature (Vinh et al., 2024). Luo et al. (2019) also suggested that different routes of drug administration may influence dosage regimens. For example, Xu et al. (2022) presented that the time to maximal concentration of DOX in crayfish hepatopancreas differed based on the administration route, occurring at 6 h, 8 h and 12 h via oral administration, intramuscular injection and intrasinus injection, respectively. These findings highlight the importance of administration routes in influencing PK parameters in aquatic species. In shrimp aquaculture in Vietnam, the most common method applied by shrimp farmers is feeding shrimp orally.

Regarding PK/PD indices, according to Mothadaka et al. (2023), the critical breakpoints for PK/PD surrogate markers vary across different antimicrobial groups, the minimum required values should be $C_{\max}/MIC > 8-10$, $AUC_{0-24h}/MIC > 125$, and $T > MIC > 50\%$ which depend on whether concentration-dependent or time-dependent antibiotics are considered to achieve optimal therapeutic outcomes. It is worth noting that the PK/PD guidelines regarding therapeutic performance for various antibacterial drugs are quite different. Doxycycline is considered as both a concentration-dependent and time-dependent antibiotic, in the case of concentration-dependent, the ratio of $C_{\max}/MIC$ or AUC_{0-24h}/MIC is used for dosage calculation (Toutain et al., 2002). Mothadaka et al., 2023; Altan et al., 2024). However, in the present study, the DOX concentrations achieved in shrimp tissues resulted in relatively low $C_{\max}$ and AUC values, which limited the applicability of AUC/MIC-based PK/PD modeling for robust dose calculation. In addition, Cunha (2003) and Xu et al. (2020) reported that DOX is also classified as a time-dependent antibiotic, meaning that $T > MIC$ a suitable parameter for determining appropriate dosing intervals. However, in this study, $T > MIC$ was included to illustrate the relationship between tissue concentrations and the MIC, without implying its use as a primary PK/PD index for dose optimization, which is more commonly based on AUC/MIC for tetracyclines. In the current study, taking the MIC of DOX towards *V. parahaemolyticus* strains of 2 µg/mL (Table 1), the time during which the DOX concentration in the hepatopancreas was above the MIC ($T > MIC$) was 4 h. This MIC value corresponded to the lowest observed MIC among the tested isolates and was selected because DOX concentrations achieved in shrimp tissues were relatively low; therefore, PK/PD indices in the present study could only be meaningfully evaluated for susceptible strains within the range of attainable tissue concentrations. For time-dependent antimicrobials such as DOX, antibacterial efficacy is generally associated with $T > MIC$ covering approximately 40–50% of the dosing interval (AliAbadi and Lees, 2000). Based on this theoretical criterion, a $T > MIC$ of approximately 4 h would correspond to a dosing interval of approximately 8 h; however, according to the calculation, DOX showed efficiency in only 10% of the collected strain, and both MIC₅₀ and MIC₉₀ were higher than the maximum concentration of DOX in the investigated organs. These findings suggest that the administered dose may be insufficient to achieve adequate therapeutic exposure against many field isolates. Therefore, the dosage should be increased to prolong the interval time but the dose must be lower than the safety threshold which needs further investigation. However, increasing the DOX dose should be carefully evaluated because higher antibiotic exposure may increase residue accumulation in shrimp tissues and prolong withdrawal times. In addition, excessive or inappropriate antibiotic use in aquaculture environments may contribute to increased selection pressure for resistant bacteria in pond ecosystems (Cabello, 2006; Done et al., 2015). Residual DOX released into pond water and sediments may also affect environmental microbial communities and contribute to the persistence of antimicrobial resistance genes (Kümmerer, 2009). Therefore, dose optimization should balance therapeutic effectiveness with food safety, environmental protection, and prudent antibiotic use principles. Furthermore, this interpretation should be approached with caution, as $T > MIC$ is not considered the

principal PK/PD index for DOX, and other indices such as AUC/MIC may provide a more robust basis for dose optimization. In addition, in this study, the use of hepatopancreas concentrations for PK/PD interpretation was considered biologically relevant, as this organ is the main target of AHPND and the primary site of pathological damage caused by *V. parahaemolyticus* in shrimp. The application of DOX in practice should be based on the MIC database in the region and dose regimes should be optimized.

4.3 Depletion and withdrawal time of doxycycline in whiteleg shrimp

The elimination of DOX residues in the muscle of shrimp was relative quick, with concentrations dropping below the LOD (2.5 µg/kg) from 14 days onwards after stopping administration of medicated feed, whether given once or twice daily. In comparison to previous studies, the DOX residual concentrations in whiteleg shrimp muscle were significantly lower than those reported for fish species. For instance, after stopping DOX-medicated feed for 3 consecutive days at the same dosage used in this study (20 mg DOX/kg body weight), residual levels in grass carp muscle were found to be 49.9 µg/kg on day 42 (Xu et al., 2019b) and 20.9 µg/kg in yellow catfish muscle on day 28 (Xu et al., 2021).

According to Avunje et al. (2021), the withdrawal time refers to the period needed for drug residues in edible tissues to decrease to a safe level for consumer consumption. Applying a MRL of 50 µg/kg, the corresponding withdrawal time for this study was estimated at approximately 8 days and 10 days at 26.5°C for feeding shrimp with DOX at the given dose in once and twice-a-day treatments, respectively. The results demonstrated that the withdrawal times in this study were shorter than those of other species at this MRL. For example, in yellow catfish, the withdrawal time was 27 days in muscle after 3 daily oral administrations at 20 mg DOX/kg body weight at 24°C (Xu et al., 2021). Similarly, Xu et al. (2019b) observed that the withdrawal time of DOX in grass carp was longer (50 days) at 24°C after oral administrations for 3 consecutive days at a dose of 20 mg DOX/kg body weight. In contrast, Vinh et al. (2024) reported a short withdrawal time of 7 days at 29.4°C when stripped catfish were fed DOX-medicated feed for 5 consecutive days at the same dosage regimen. The varying values of withdrawal times among species may be due to different species, size, dosage regimen and temperature. Regarding the temperature, Corum et al. (2023) found that at an MRL of 50 µg/kg, the withdrawal times of DOX in rainbow trout muscle were 43 days and 35 days following oral administration at 20 mg/kg for 5 consecutive days at 10 and 17°C, respectively. The result indicated that higher temperatures facilitate faster elimination of antibiotic residues, resulting in a shortened WT. Additionally, the depletion and withdrawal time of DOX in whiteleg shrimp in this study were longer than those of other antibiotics commonly used in shrimp farming, such as CFT (Huynh et al., 2024), oxytetracycline (Wang et al., 2004; Avunje et al., 2021), sulphamethoxazole (Wang et al., 2004).

4.4 Impact of doxycycline on hepatopancreas histology of whiteleg shrimp

Hepatopancreas of shrimp plays a pivotal role in digestion and metabolism and is one of the indicators which help to predict the health status of shrimp (Caceci et al., 1988; Manan et al., 2015). Due to its vital functions, any cellular damage or structural alteration in this organ can have detrimental effects, including reduced growth and immunological activity, and even increased mortality. Caceci et al. (1988) identified that there are four types of epithelial cells in hepatopancreases, each with specific functions, which include embryonic cells (E cells), responsible for mitotic division; fibrillar cells (F cells), responsible for extracellular digestion; resorptive cells (R cells), which store glycogen and reserve lipid droplets, and blister cells (B cells), which contain digestive enzymes. For the purpose of the current study, a relatively detailed histological examination of the hepatopancreas was conducted, focusing specifically on B and F cells. The selection of these cell types was based on the previous research, Nima et al. (2022) found that the proportion of R cells in the hepatopancreas remains relatively unchanged in both normal and growth-retarded shrimp, whereas the number of B cells are significantly reduced in shrimp with slowed growth compared with normal shrimp. Another reason was the observations from shrimp farmers, who noted that shrimp growth tends to slow when medicated feed containing antibiotics is used. This growth retardation is often linked to compromised digestive functions in the hepatopancreas. Both B and F cells are integral to digestion - F cells synthesize and store digestive enzymes in large vesicles, which is later transformed into B cells (Hu and Leung, 2007). These enzymes are then released into the hepatopancreatic lumen via a holocrine secretion process facilitated by B-cells (Nunes et al., 2014).

In the present study, the hepatopancreatic cell numbers fully recovered after seven days of stopping DOX-medicated feed at a dose of 20 mg/kg body weight, administered either once or twice daily for 3 consecutive days. This reversible and transient histological alteration is consistent with the PK characteristics observed in the present study, namely the relatively low tissue exposure and rapid elimination of DOX from the shrimp body. The low C_{max} and short elimination half-life in hemolymph and hepatopancreas suggest limited systemic accumulation of DOX, which may explain the absence of severe or persistent histopathological damage. This outcome is consistent with findings from previous research on CFT in whiteleg shrimp, which similarly showed no histopathological effects on hepatopancreatic cells at a dose of 25 mg/kg body weight (Huynh et al., 2024). In contrast, studies on other antibiotics or other veterinary medicines, such as enrofloxacin, oxytetracycline, and maduramicin, have reported significant adverse effects on shrimp health, particularly on sensitive organs like the hepatopancreas (Avunje et al., 2021, Gao et al., 2021, Maftuch et al., 2017, Bray et al., 2006). The hepatopancreas is known as a primary site of xenobiotic metabolism, where the drug and its residual compounds are catabolized to be eliminated from the shrimp body (Rewitz et al., 2006). Therefore, the mild and reversible histological changes observed in this study likely reflect the role of the hepatopancreas in drug processing rather than toxic injury, further supporting the PK findings of low

tissue exposure and rapid drug clearance at the applied dose. Several research reports have demonstrated that higher doses of drugs correlate with more severe histological damage to the hepatopancreas. For instance, Gao et al. (2021) reported that exposure to 0.7 mg/L of maduramicin in crayfish (*Procambarus clarkii*) reduced the number of hepatic epithelial cells, while a higher concentration of 7.0 mg/L induced more severe pathological changes in crayfish hepatopancreas. The finding highlights the risks associated with excessive antibiotic use and serves as a warning to shrimp farmers. Whiteleg shrimp are typically cultured from post-larvae to harvest within approximately 4–5 months, during which repeated antibiotic treatments may affect shrimp health, particularly hepatopancreatic function, and may also contribute to antibiotic residues in edible tissues. Previous studies have demonstrated that some antibiotics, such as enrofloxacin, can induce histopathological damage in shrimp hepatopancreas (Maftuch et al., 2017). Therefore, evaluating hepatopancreatic health following antibiotic administration is important for assessing the biological safety of antibiotic use in shrimp culture. In the present study, DOX administration at the investigated dose did not induce severe or persistent hepatopancreatic damage. However, the potential effects of higher doses on hepatopancreatic tissues still require further investigation.

5. Conclusion

The findings of this study have provided valuable insights into the PK of DOX as well as its optimal use in shrimp to control pathogens while ensuring consumer safety. The results indicate that after a single dose of 20 mg/kg body weight, DOX is quite rapidly absorbed and eliminated in hemolymph, hepatopancreas, and muscle of whiteleg shrimp. Regarding food safety, after oral administration, DOX requires 8 days and 10 days to deplete in shrimp muscle at 26.5°C for once and twice daily regimens, respectively. In addition, DOX oral medication caused slight effect on hepatopancreas but the recovery was found after 7 days of cessation of medication. For managing *V. parahaemolyticus* infections, however, the dose should be increased to prolong the interval time in future studies while taking possible adverse effects on the hepatopancreas into account. Optimizing dosage is essential not only for effective disease control but also for minimizing antibiotic resistance risks.

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Competing Interests

The authors declare that they have no competing interests to declare that are relevant to the content of this article.

Author Contributions:

All authors contributed to the study conception, visualization, design and methodology. Material preparation, data collection and curation, validation, analysis and software were performed by Huynh Thi Kim Duyen, Nguyen Quoc Thinh, Tran Minh Phu, Dang Thi Hoang Oanh, Marie-Louise Scippo, Mathias Devreese, Siska Croubels. The first draft of the manuscript was written by Huynh Thi Kim Duyen and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Animal welfare and Ethics statement

The experiments and animals used in this work were followed and treated according to Decision No. 3965/QD-DHCT Date October 15 2021 “Can Tho University Regulation on Ethics in animal experimentation” ULR: <https://dra.ctu.edu.vn/images/upload/news/246.pdf>.

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3.2.2 PHARMACOKINETICS, PHARMACODYNAMICS AND DEPLETION OF FLORFENICOL APPLIED IN WHITELEG SHRIMP (*PENAEUS VANNAMEI*) AQUACULTURE AND IMPACT ON SHRIMP HEPATOPANCREAS HISTOLOGY

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Abstract

Florfenicol (FF) is one of the common antimicrobials used to control bacterial disease in shrimp aquaculture. This study aimed to determine the pharmacokinetics (PK) parameters of FF in whiteleg shrimp plasma, hepatopancreas and muscle as well as its residue depletion in shrimp muscle and the impact on shrimp hepatopancreas histology during and after FF medication. In the PK experiment, shrimp were fed once at 10 mg FF/kg body weight (bw) via oral in-feed administration to determine PK parameters in plasma, hepatopancreas and muscle. The maximum concentration ($C_{\max}$) of 60.56 $\mu\text{g/L}$ in plasma was observed after 1.77 h ($T_{\max}$). In muscle, a $C_{\max}$ of 11.76 $\mu\text{g/kg}$ was attained after 0.20 h, while in hepatopancreas, the $C_{\max}$ was higher (386.92 $\mu\text{g/kg}$) and was rapidly obtained ($T_{\max} = 0.19$ h). The $C_{\max}$ values in shrimp plasma were below the MIC against *Vibrio parahaemolyticus*, known to cause AHPND in shrimp. Therefore, it can be concluded that to ensure the effectiveness of this treatment, the dose should be higher than 10 mg FF/kg bw. Florfenicol depletion in whiteleg shrimp muscle and its histological impact on hepatopancreas were determined after feeding FF-medicated feed once-a-day or twice-a-day for 3 consecutive days with a dose of 10 mg FF/kg bw. The residues in shrimp muscle were rapidly eliminated and fell below the limit of quantification at 24 h after stopping medication. The withdrawal time of FF in shrimp muscle was 27.9 degree-days (2 days at 26.5°C) according to the maximum residue limit of 100 $\mu\text{g/kg}$ set by the European Commission and Korean Ministry and when feeding FF twice-a-day for 3 days. The results from histological analysis showed that there was no negative effect on shrimp hepatopancreas after stopping medication in both once- and twice-a-day treatments.

Keywords: depletion; florfenicol; hepatopancreas; pharmacokinetics; whiteleg shrimp.

Key Contribution: This study clarifies how FF is distributed in whiteleg shrimp and its PK properties as well as the impact on hepatopancreas; and a suitable withdrawal time of FF is defined ensuring the safety of shrimp products.

1. Introduction

Whiteleg shrimp (*Penaeus vannamei*) is being cultured in Vietnam with an annual production of 1.12 million tons in 2023 (VASEP, 2024a). Since the development of intensive and super-intensive farming systems, the Vietnamese shrimp sector is facing issues such as disease outbreaks and increase in chemical use related to the increase in stocking density (Nguyen et al., 2020). Shrimp diseases are mainly due to pathogens such as viruses and bacteria, causing, e.g., AHPND, white feces disease and white spot syndrome (Tran et al., 2013; Flegel and Sritunyaluucksana, 2018, Thinh et al., 2020). In particular, diseases caused by *Vibrio* spp. result in serious economic losses (Chanratchakool & Phillips, 2002, Zhou et al., 2012). Several researchers (Rico et al., 2014; Phu et al., 2016; Chi et al., 2017) have reported that shrimp farmers normally use antibiotics to manage shrimp health as well as prevent and treat bacterial infections.

Florfenicol (FF) is one of the antibiotics commonly used for the treatment of bacterial diseases in whiteleg shrimp and other aquaculture species (Ren et al., 2016; Phu et al., 2016; 2019a; Lillehaug et al., 2018). Florfenicol is a broad-spectrum synthetic bacteriostatic antibiotic belonging to the phenicol group. The mode of action of FF is similar to that of chloramphenicol, acting through the inhibition of protein synthesis via binding to the 50S ribosomal subunit of the target microorganism, resulting in the prevention of protein chain elongation in bacterial cells, and the inhibition of bacterial growth (Wang et al., 2009; Lambert 2012; Nejad et al., 2017). Since chloramphenicol is prohibited for use in aquaculture, FF is used as an alternative because FF does not have the potential side-effects of chloramphenicol (Dowling, 2006). Florfenicol is one of the antibiotics approved by the US Food and Drug Administration for aquaculture that has been shown to be effective in treating diseases caused by Gram-positive and Gram-negative bacteria in aquaculture and livestock (Samuelsen & Bergh, 2004; Caipang et al., 2009; Huang et al., 2015).

In aquaculture, FF pharmacokinetics have mostly been studied in fish, e.g., catfish (Park et al., 2006; Gaunt et al., 2012; Yang et al., 2013; Pham et al., 2023), Atlantic salmon (Martinsen et al., 1993; Horsberg et al., 1996), lumpfish (Kverme et al., 2019), orange-spotted grouper (Feng et al., 2016), cod (Samuelsen et al., 2003), crucian car (Yang et al., 2019), olive flounder (Lim et al., 2011), rainbow trout (Pourmolaie et al., 2015; Mallik et al., 2023), Asian tilapia (Feng et al., 2009) and seabass (Rairat et al., 2019). While several PK studies have been published on fish, such studies on shrimp are limited. For instance, FF PK studies on whiteleg shrimp were investigated by Fang et al. (2013) and Ren et al. (2016) in China, and in black tiger shrimp by Tipmongkolsilp et al. (2006) in Thailand. These studies found that the values of $C_{\max}$ ($\mu\text{g/g}$) and $T_{\max}$ (h) in the hepatopancreas of shrimp treated with FF by oral administration were as follows: whiteleg shrimp 164 and 0.5 (at a dose of 10 mg FF/kg bw), black tiger shrimp 0.7 and 1 (at 0.8 g FF/kg feed), respectively (Fang et al., 2013; Tipmongkolsilp et al., 2006). However, PK parameters vary among shrimp species living in different locations or environments (Fang et al., 2013; Ren et al., 2016). For instance, after oral administration at a dose of 30 mg/kg bw, the $T_{\max}$

value of FF in the plasma of shrimp (*Exopalaemon carinicauda*) at 28°C ($T_{\max}$ of 0.5 h) was faster than that at 22°C ($T_{\max}$ of 1 h); the corresponding values for shrimp hepatopancreas were 1 h and 2 h, respectively [38]. In addition, PK parameters within a single fish species may differ due to factors like geographic origin and genetic variation caused by selective breeding and artificial selection (Toutain et al., 2010). To determine an effective therapeutic dose as well as interval time when using FF in shrimp aquaculture to be effective in treating bacterial infections, PK parameters are essential to establish and to compare with a PD parameter, namely, the MIC against the bacteria causing the disease, which is called the PK/PD index. This may help to reduce antibiotic resistance of pathogens in shrimp farming.

Hepatopancreas (also called the midgut gland) health is one of the indicators which may be used to predict the health status of shrimp (Manan et al., 2015). The hepatopancreas of shrimp and other crustaceans is the most sensitive organ to drug and chemical exposure. Several studies have revealed that the improper use of antibiotics or chemicals has caused serious adverse effects on shrimp health, especially on sensitive organs like the hepatopancreas. The higher the dose of antibiotics used, the more severe is the negative impact on shrimp hepatopancreas (Bray et al., 2006; Romano et al., 2015; Maftuch et al., 2017; Avunje et al., 2021). This evidence of negative effects on the hepatopancreas after extensive duration of treatments with excessive doses should be a strong caution to shrimp farmers who have overused antibiotics or chemicals in shrimp aquaculture. Although FF is commonly used in shrimp culture, there has been no study on the side-effects of FF on whiteleg shrimp hepatopancreas.

Based on the above evidence, research on the PK, PD and withdrawal time of FF in shrimp should be conducted to evaluate how FF is processed by shrimp, as well as to study the impact of using FF medicated feed on the shrimp hepatopancreas to monitor shrimp health and to ensure the safety of shrimp food products.

2. Materials and methods

2.1. Chemicals and equipment

High purity chemicals (>98%) meeting the FF standard and internal standard (penicillin V) were provided by Dr. Ehrenstorfer® (Augsburg, Germany). The instrument used for FF quantification was a Waters ACQUITY Ultra-High-Performance Liquid Chromatograph (UHPLC) (Waters, Milford, MA, USA) with an Acquity UPLC CSH C18 column, 2.1 mm × 50 mm, 1.7 µm (Waters, Milford, MA, USA). C18 Bondesil powder with a particle size of 40 µm was obtained from Agilent (Santa Clara, CA, USA). Other chemicals, including acetonitrile, methanol and distilled water, were purchased from Merck (Darmstadt, Germany).

2.2. Bacterial strains

A total of 42 bacterial strains were used for antimicrobial tests. These strains were isolated from AHPND shrimps which were collected from shrimp farms located in 7 provinces of the Mekong Delta, including Ca Mau, Kien Giang, Soc Trang, Can Tho, Dong Thap, Tra Vinh and Tien Giang from 2022 and 2024. Strains were identified as *V. parahaemolyticus* and stored at -80°C in tryptone soy broth (TSB, Merck) containing 25% glycerol and supplemented with 1.5% (w/v) sodium chloride.

2.2.1. Antibiotic susceptibility testing

Vibrio parahaemolyticus strains from glycerol stock were recovered by inoculating into tryptic soy agar supplemented with 1.5% NaCl (TSA^+) and incubating at 28°C for 18 h. The color and shape of colonies were recorded. Gram stain was applied to check the purity of the bacteria. Pure bacterial colonies were cultured to increase the biomass in nutrient broth at 28°C for 24 h and the concentration of bacteria was adjusted to an approximate density of 10^6 CFU/mL using McFarland's 0.5 Barium Sulfate Standard Solution (Sotomayor et al., 2019).

The resistance of *V. parahaemolyticus* to FF was determined by an agar disk diffusion method as described by Balouiri et al. (2016) and Jiang et al. (2020), with some modifications. Disks containing 30 micrograms of FF were used. One hundred microliters of bacterial suspension were evenly spread over the surface of the TSA^+ plate. After 15 min, an FF disk was gently fixed onto the agar surface using a sterile fine-pointed forceps to ensure complete contact and the plate was incubated at 28°C for 24 h. The diameters of the inhibition zones (as judged by the unaided eye), including the diameter of the disk, were measured and the size of the clear zone of inhibition was used to classify isolates as susceptible, intermediate, or resistant according to the guideline of the Clinical and Laboratory Standards Institute (CLSI, 2017).

2.2.2. Determination of the minimum inhibitory concentration of florfenicol

The bacteria were activated as described above and the bacterial initial concentration was 10^6 CFU/mL. The MIC of FF was determined for each *V. parahaemolyticus* strain using the broth macro-dilution method with some modifications (Ali et al., 2019). An FF standard solution with a final concentration of $1280\text{ }\mu\text{g}/100\text{ }\mu\text{L}$ was prepared using sterile distilled water. Serial dilutions were then prepared to obtain 12 decreasing concentrations. The bacterial suspension (1 mL) was 200-fold diluted with TSB^+ medium. Fourteen sterilized screwed capped test tubes were prepared and labeled, which were then loaded with 4.9 mL of diluted bacterial suspension. After that, 100 μL of the prepared FF standard solutions was added to achieve a final concentration of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 $\mu\text{g}/\text{mL}$. Simultaneously, one positive control tube (TSB^+ solution with bacteria) and one negative control tube (TSB^+ solution without bacteria) were also prepared. All these test tubes were incubated for 24 h at 28°C . MIC was defined as the lowest concentration of FF that showed no visible bacterial growth.

2.3. Shrimp

Healthy whiteleg shrimp (*Penaeus vannamei*), weighing 10–15 g each, were obtained from the Faculty of Marine Science and Technology, College of Aquaculture and Fisheries, Can Tho University. The shrimp had been reared from the post-larval stage without the use of antibiotics prior to the start of the experiment. They were stocked in 2 m³ tanks at a density of 100 shrimp/m³ and acclimated for one week.

During the acclimation period, shrimp were fed a commercial pelleted feed (Proconco, Can Tho City, Vietnam) containing 40% crude protein, administered four times daily (06:00, 10:00, 14:00, and 18:00 h) at a feeding rate equivalent to 3% of the shrimp's body weight. Uneaten feed, which was consistently less than 5% of the total offered, was removed 30 min post-feeding via siphoning.

Experimental tanks were equipped with a recirculating water system and aeration to maintain dissolved oxygen levels above 5 mg/L. Key water quality parameters were regularly monitored and maintained within optimal ranges for shrimp culture: salinity was adjusted to 10‰, alkalinity was maintained at 140–160 mg HCO₃⁻/L, and temperature, pH, and nitrite levels were also closely controlled. All these environmental parameters were monitored throughout the study.

Vime-fenfish 2000, a product containing 20% FF, was sourced from the Vemedim Company (Can Tho City, Vietnam). Prior to the experiment, the absence of FF residues in shrimp tissues and the actual concentration of FF in the product were verified using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS).

2.4. Pharmacokinetics experiment

2.4.1. Experimental design

The experimental procedure followed the protocol described by Huynh et al. (2024). Whiteleg shrimp were distributed and acclimated in six separate tanks, corresponding to six biological replicates. Each group received a single oral dose of FF at 10 mg/kg body weight (bw). This dosage was chosen based on standard therapeutic recommendations provided by commercial product manufacturers. Additionally, a national survey on antibiotic usage in Vietnamese shrimp farming reported the same dosage (Phu et al., 2019). Previous studies have also employed this dose effectively to manage bacterial infections in aquatic animals (Fang et al., 2013; Pham et al., 2023).

The required FF concentration in the feed was calculated based on the estimated feed intake for 1 kg of shrimp biomass. To prepare the medicated feed, FF 20% (Vime-fenfish 2000) was manually mixed with commercial pellets to achieve a final concentration of 1333 mg FF/kg feed. The antibiotic and feed were thoroughly blended in a cylindrical container for 5 min. Subsequently, the mixture was coated with 2% fish oil and air-dried for 15 min before administration at 06:00 h. For the remaining daily feedings (10:00, 14:00, and 18:00 h), shrimp received non-medicated feed.

2.4.2. Sample collection

Sampling was conducted at 0.5, 1, 2, 4, 8, 12, and 24 h post-treatment. At each time point, three shrimp were collected from one tank (one replicate). The sample size used in this study ($n = 6$) was selected based on previously published pharmacokinetic studies in shrimp and other aquatic species, where similar experimental designs and replicate numbers were commonly applied (Ma et al., 2017; Yang et al., 2014; Xu et al., 2022). Approximately 0.5 mL of blood was drawn from the pericardial cavity of each shrimp using a 1 mL syringe pre-filled with an anticoagulant solution (0.01 M EDTA, 0.338 M NaCl, 0.115 M glucose, and 0.03 M trisodium citrate dihydrate). Blood samples from three shrimp within a tank were pooled into a 1.5 mL centrifuge tube and centrifuged at $9500\times g$ for 5 min to separate the plasma.

Simultaneously, hepatopancreas and muscle tissues were excised from the same three shrimp and pooled into labeled plastic bags to constitute one replicate. All collected plasma, muscle, and hepatopancreas samples were immediately frozen and stored at -80°C until further analysis.

2.5. Depletion of florfenicol in muscle tissue and hepatopancreas histological observation

2.5.1. Depletion of florfenicol in shrimp muscle

Florfenicol depletion in whiteleg shrimp was assessed following oral administration. The study was conducted using two treatment groups, each consisting of three replicate tanks. Shrimp in Group 1 received FF-medicated feed (prepared as previously described) once daily at 06:00 h, whereas shrimp in Group 2 were fed the same medicated feed twice daily at 06:00 and 18:00 h. The treatment lasted for three consecutive days at a dosage of 10 mg FF/kg body weight.

Following the treatment period, all shrimp were fed non-medicated feed for 21 days, administered four times daily at 06:00, 10:00, 14:00, and 18:00 h. Muscle samples were collected from shrimp on days 1 and 3, one hour after administration of the medicated feed. Subsequent samplings were conducted on days 1, 3, 7, 14, and 21 post-treatment. At each time point, ten shrimp were randomly collected from each tank. Muscle tissues were excised, homogenized, pooled, and stored at -80°C until extraction and analysis.

2.5.2. Shrimp hepatopancreas histological examination

In addition to FF residue analysis, hepatopancreas samples were collected for histological examination to assess potential impacts of FF exposure. For this purpose, three shrimp per tank were sampled at three time points: one day prior to FF administration (baseline), one hour after the final FF dose, and seven days after treatment cessation. Hepatopancreatic tissues were preserved and processed into histological sections following the procedure described by Huynh et al. (2024). Histological examinations were conducted using a Novex microscope (Arnhem, the Netherlands). The numbers of B- and F-cells were counted according to the modified method of Nima et al. (2022). Cell counts were

performed within a consistent microscopic field, randomly selecting different fields across 40 tubules per shrimp.

Statistical analyses of dosing and/or sampling effects were conducted using one-way and two-way ANOVA with SPSS 20.0 software. Duncan's multiple range test was employed for post hoc comparisons. Differences were considered statistically significant at $p < 0.05$.

2.6. Florfenicol and florfenicol amine quantification

Florfenicol and florfenicol amine (FFA) in shrimp plasma were extracted and analyzed using a modified version of the procedure described by Pham et al. (2023). In brief, 0.5 mL of plasma was spiked with penicillin V solution as an internal standard and mixed with 3 mL of acetonitrile. The mixture was then centrifuged at 9503 g for 10 min, the supernatant was collected, dried under nitrogen, and the dry residue was dissolved in the mobile phase. Before LC–MS/MS injection, the extract was filtered through a 0.22 µm nylon membrane filter (Advantec MFS, Dublin, CA, USA).

Hepatopancreas and muscle samples were separately homogenized using an Ultra Turrax homogenizer (T-25, IKA, China). The extraction method was adapted from the United States Department of Agriculture (USDA, 2010) with some modifications. Briefly, 3.00 ± 0.1 g of homogenized sample was transferred into a 50 mL tube, spiked with 60 µL of 1 µg/mL IS, vortexed, and incubated at room temperature for 15 min. After incubation, 1 mL of 0.1 M phosphate buffer (pH 8.0) was added to the tube, which was vortexed for 1 min, followed by the addition of 20 mL of acetonitrile. The mixture was vortexed for 30 sec and sonicated for 15 min in an Elma Ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany). Samples were then centrifuged (22R, Andreas Hettich, Tuttlinggen, Germany) at 3501 g for 5 min to collect the supernatant. Acetonitrile was added to adjust the final volume to 24 mL, then 8 mL was transferred to a 15 mL glass test tube containing 40 mg Bondesil C18 and vortexed for 20 min. After centrifugation at 3501 g for 4 min, the supernatant was collected into a 10 mL glass test tube and then evaporated at $40 \pm 1^\circ\text{C}$ in a water bath under nitrogen flow. The dried residues were reconstituted in 1 mL of mobile phase [acetonitrile and methanol (1:1) with 0.1% formic acid in water], filtered through a 0.22 µm nylon membrane filter (Advantec MFS, CA, USA) and injected into the LC–MS/MS system.

Detection and quantification of FFA and FF were performed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) equipped with an electrospray ionization (ESI) probe. The ESI probe operated in positive ion mode for FFA detection and in negative ion mode for FF. Optimization of the mass spectrometry parameters included capillary voltage, ion source temperature, cone gas flow, desolvation gas flow, and desolvation temperature. Argon was used as the collision gas, and the collision cell pressure was 4×10^{-3} mBar. For quantitation (shown in bold) and confirmation, the following two specific fragmentation transitions (precursor > product ion, m/z) were used, with the collision energy shown in brackets: FF **355.97** > **184.94** (24 eV) and 355.97 > 335.99 (14 eV); FFA **248.07** > **130.21** (24

eV) and $248.07 > 91.07$ (44 eV); penicillin V (IS) **351.04 > 229.09** (18 eV) and $351.04.1 > 257.04$ (14 eV).

The limits of detection (LOD) were 2.5 µg/kg for FF and 5 µg/kg for FFA in all matrices, while the limits of quantification (LOQ) were 5 and 10 µg/kg, respectively. Both LOD and LOQ were determined following AOAC guidelines (AOAC, 2002). The working ranges of the standard calibration curve, prepared using blank samples, exhibited a linear range of 5–80 µg/kg for FF and 10–80 µg/kg for FFA. Extraction recovery ranged from 92% to 99%. Linearity, specificity, precision (repeatability and within-laboratory reproducibility), apparent recovery, decision limit (CC_α) and detection capability (CC_β) were evaluated and validated according to Commission Decision 2002/657/EC [54]. All parameters fell within the acceptance criteria.

2.7. Pharmacokinetic modelling and data analysis

Pharmacokinetics modelling of FF concentrations in plasma, hepatopancreas and muscle tissues was performed using a naïve pooled population approach, applying a one-compartmental model with first-order absorption and elimination. In plasma, the following PK parameters were calculated: absorption rate constant (k_a), absorption half-life ($T_{1/2abs}$), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), area under the plasma concentration–time curve from time 0 to infinity (AUC_{0-inf}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (Cl) and apparent volume of distribution after oral administration (V_d).

For hepatopancreas and muscle tissues, the calculated parameters included the maximal tissue concentration (C_{max}), time to maximal tissue concentration (T_{max}), and area under the tissue concentration–time curve from time 0 to infinity (AUC_{0-inf}), which was computed using the linear-up log-down trapezoidal method. All data analysis and PK modeling were conducted using Phoenix 8 (Certara, Princeton, NJ, USA).

2.8. Withdrawal time estimation and data analysis

To estimate the withdrawal time of FF, the withdrawal time was determined using the WT 1.4 software, developed by Hekman (2004), in accordance with the guidelines issued by the European Medicines Agency (EMA, 2022). The FF residue concentrations in shrimp muscle tissue were measured at multiple post-treatment time points. These concentrations were analyzed using linear regression against time expressed in degree-days, which were calculated by multiplying the average daily water temperature (°C) by the total number of days the temperature was measured. The withdrawal time was defined as the time point at which the upper one-sided 95% tolerance limit for FF residues dropped below the maximum residue limit (MRL) of 100 µg/kg—established by both the EU and Korea—with 95% confidence.

3. Results

3.1. Antimicrobial activity

Disk diffusion tests. The antimicrobial activity of FF towards 42 *V. parahaemolyticus* strains, evaluated by the disk diffusion method (30 µg FF disk), expressed as inhibition zone diameters, were 6 to 36 mm for strains collected in Ca Mau, 22 to 26 mm for strains coming from Kien Giang, 18–36 mm (Soc Trang), 24–25 mm (Can Tho), 30 mm (Dong Thap), 25–28 mm (Tra Vinh), 25–28 mm (Tien Giang) (Table 1).

Table 1. The MIC (µg/mL) and inhibition zone diameters (mm) of FF for *V. parahaemolyticus* strains

Isolate Number	Inhibition Zone Diameter	MIC	Isolate Number	Inhibition Zone Diameter	MIC
1	36	8	22	34	8
2	22	1	23	24	4
3	21	1	24	26	4
4	24	1	25	22	1
5	28	2	26	21	8
6	30	2	27	18	256
7	31	8	28	24	8
8	6	>256	29	23	16
9	22	4	30	22	4
10	24	2	31	25	8
11	20	4	32	25	4
12	30	4	33	24	4
13	18	256	34	30	4

Isolate Number	Inhibition Zone Diameter	MIC	Isolate Number	Inhibition Zone Diameter	MIC
14	24	8	35	25	4
15	31	16	36	25	4
16	18	256	37	26	4
17	25	16	38	28	2
18	26	4	39	27	8
19	22	2	40	27	8
20	36	4	41	25	8
21	31	8	42	28	8

Isolates numbered 1 to 17 collected in Ca Mau province, 18–19 from Kien Giang province, 20–30 from Soc Trang province, 31–33 from Can Tho University, 34 from Dong Thap province, 35–39 from Tra Vinh province, 40–42 from Tien Giang province.

Minimum inhibitory concentration. Figure 1 shows the repartition of MIC values of FF for 42 *V. parahaemolyticus* strains isolated from 7 provinces of the Mekong Delta, Vietnam. For thirty-five strains of *V. parahaemolyticus* (83.3%), the FF MIC ranged between 1 and 8 µg/mL. For approximately 7% (3/42 strains) of the *V. parahaemolyticus* tested strains, the FF MIC was 16 µg/mL and for four strains (about 10%), FF MIC was above 256 µg/mL (Figure 1). In Ca Mau province (isolates 1–17), 5/18 isolates had MIC values ranging above 8 µg/mL while 2/10 isolates from Soc Trang province had (Table 1).

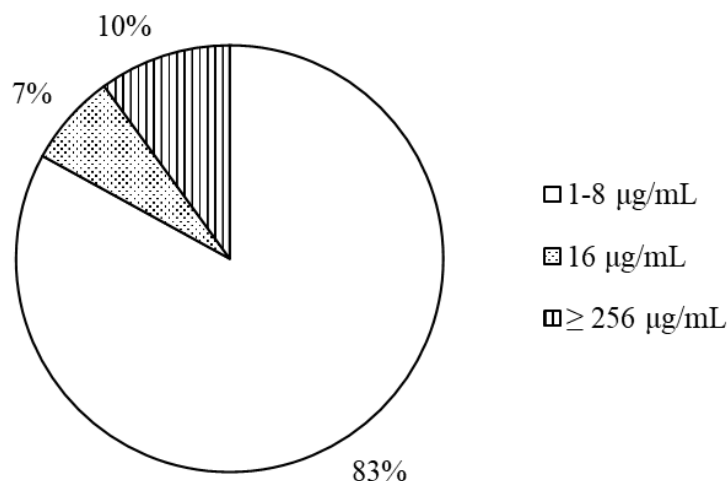


Figure 1. The repartition (%) of FF MIC ($\mu\text{g/mL}$) for 42 strains of *V. parahaemolyticus* collected in seven provinces of the Mekong Delta, Vietnam

3.2. Pharmacokinetics of florfenicol in plasma, hepatopancreas and muscle of whiteleg shrimp

After administering a single 10 mg/kg bw dose of FF, the concentration–time curves indicated that, at 30 min after FF ingestion, the highest FF concentrations were observed in the hepatopancreas of whiteleg shrimp, compared to lower levels in plasma and muscle (Figure 2).

The estimated PK parameters of FF in whiteleg shrimp plasma were determined by a one-compartment model. The results are shown in Table 2. The absorption rate constant (k_a) was 0.066 h^{-1} , the absorption half-life ($T_{1/2\text{abs}}$) was 10.44 h, the maximal plasma concentration (C_{max}) was $60.56\text{ }\mu\text{g/L}$, the time to reach the maximal plasma concentration (T_{max}) was 1.77 h, the apparent total body clearance after oral administration (Cl) was 9.75 L/kg/h , the apparent volume of distribution after oral administration (V_d) was 4.93 L/kg , the area under the plasma concentration–time curve from time 0 to infinity ($\text{AUC}_{0-\text{inf}}$) was $1026.07\text{ }\mu\text{g.h/L}$, the elimination rate constant (k_{el}) was 1.97 h^{-1} and the elimination half-life ($T_{1/2\text{el}}$) was 0.35 h.

Regarding the estimated PK parameters of FF in whiteleg shrimp tissues, the results indicated a markedly higher concentration in the hepatopancreas compared to muscle (Table 3). The maximum concentration of FF in the hepatopancreas reached $386.92\text{ }\mu\text{g/kg}$, while C_{max} in shrimp muscle was significantly lower at $11.76\text{ }\mu\text{g/kg}$. Similarly, based on $\text{AUC}_{0-\text{inf}}$ values, the distribution of FF in the hepatopancreas ($1660\text{ }\mu\text{g.h/kg}$) was much higher than that in shrimp muscle ($88.0\text{ }\mu\text{g.h/kg}$). In addition, T_{max} values for these two organs were similar, approximately 0.2 h. However, the T_{max} value of the hepatopancreas could not be accurately estimated because of the high standard deviation of FF concentrations in this organ (Figure 2).

Florfenicol amine, a metabolite of FF, was not detected in the plasma, muscle or hepatopancreas of shrimp.

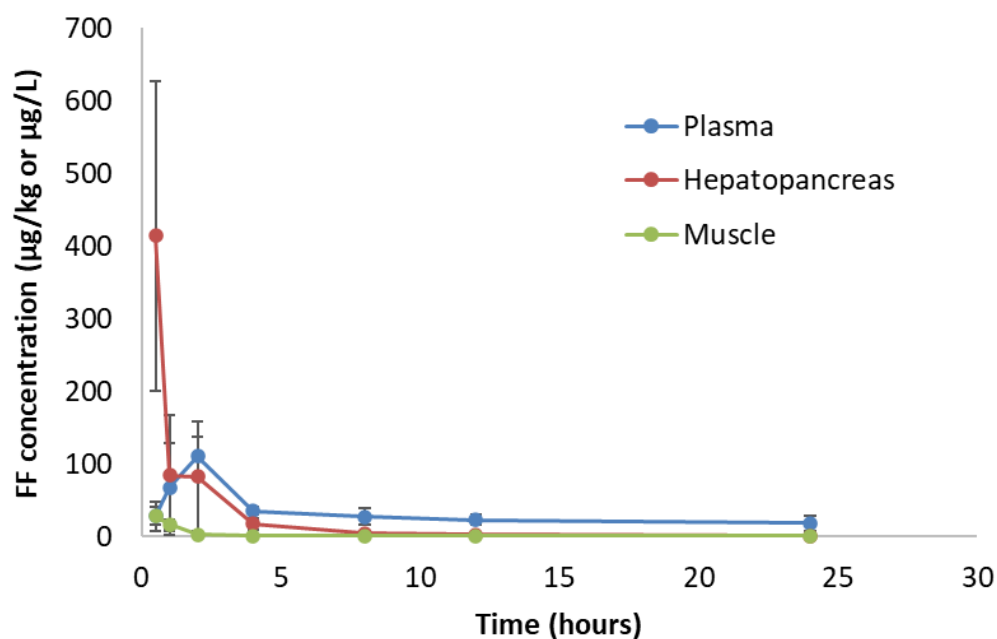


Figure 2. Florfenicol concentrations (mean $\pm$ SD, $n = 6$, $\mu\text{g/L}$ or $\mu\text{g/kg}$, wet basis) in the plasma, hepatopancreas and muscle of whiteleg shrimp after a single oral administration of 10 mg/kg bw

Table 2. Values for the main PK parameters of FF in whiteleg shrimp plasma after oral administration of a single dose of 10 mg FF/kg bw

PK Parameter	Unit	Plasma Estimate	CV%
k_a	h^{-1}	0.066	13.88
$T_{1/2abs}$	h	10.44	13.88
C_{max}	$\mu g/L$	60.56	7.45
T_{max}	h	1.77	10.41
Cl	L/kg/h	9.75	13.85
V_d	L/kg	4.93	30.51
AUC_{0-inf}	$\mu g.h/L$	1026.07	13.85
k_{el}	h^{-1}	1.97	17.96
$T_{1/2el}$	h	0.35	17.96

Values are presented as the mean of the population estimate, $n = 6$. Absorption rate constant (k_a), absorption half-life ($T_{1/2abs}$), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), area under the plasma concentration–time curve from time 0 to infinity (AUC_{0-inf}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (Cl) and apparent volume of distribution after oral administration (V_d).

Table 3. Values for the main PK parameters of FF in whiteleg shrimp hepatopancreas and muscle tissues after oral administration of a single dose of 10 mg FF/kg bw.

PK Parameter	Unit	Hepatopancreas Estimate	Muscle Estimate
$C_{\max}$	$\mu\text{g/kg}$	386.92	11.76
$T_{\max}$	h	0.19	0.20
$AUC_{0-\infty}$	$\mu\text{g.h/kg}$	1660	88.0

Values are presented as the mean of the population estimate, $n = 6$. Maximal tissue concentration ($C_{\max}$), time to maximal tissue concentration ($T_{\max}$) and area under the tissue concentration–time curve from time 0 to ∞ ($AUC_{0-\infty}$).

3.3. Depletion and withdrawal time of florfenicol in whiteleg shrimp muscle

The residual levels of FF in whiteleg shrimp muscle of both once-a-day and twice-a-day treatments were very low. The mean FF concentrations in shrimp muscle were $3.65 \mu\text{g/kg}$ for once-a-day treatment and $20.53 \mu\text{g/kg}$ for twice-a-day treatment on the first day of medication. After that, the FF levels of shrimp muscle increased and reached the highest mean concentration on day 3 of medication, at 9.35 and $49.29 \mu\text{g/kg}$ in the once-a-day and twice-a-day treatments, respectively. Florfenicol concentrations in shrimp muscle were eliminated very quickly; the FF residual level fell below the LOQ ($5 \mu\text{g/kg}$) at 24 h post-medication and was lower than the LOD ($2.5 \mu\text{g/kg}$) from day 3 after stopping medication in both treatments of feeding shrimp with medicated feed once-a-day and twice-a-day (Table 4). Florfenicol amine was not detected in all samples (LOD and LOQ of $5 \mu\text{g/kg}$ and $10 \mu\text{g/kg}$ for FFA, respectively).

Table 4. Residual levels of FF in whiteleg shrimp muscle during and after medication at a dose of 10 mg FF/kg bw fed once-a-day and twice-a-day during 3 consecutive days. Values are presented as mean $\pm$ SD, $n = 3$

Period	Sampling Time (Days)	FF Level (Once-a-Day Treatment) ($\mu\text{g/kg}$)	FF Level (Twice-a-Day Treatment) ($\mu\text{g/kg}$)
Medication	1	3.65 ± 0.16	20.53 ± 4.52
	3	9.35 ± 3.10	49.29 ± 10.1
After stopping medication	1	3.03 ± 1.61	2.95 ± 0.95
	3	<LOD	<LOD
	7	<LOD	<LOD
	14	<LOD	<LOD
	21	<LOD	<LOD

After three consecutive days of medication at a dose of 10 mg FF/kg bw once-a-day, and an ambient temperature of 26.5°C, the FF residues in shrimp muscle were lower than the MRL of 100 $\mu\text{g/kg}$ set by EU and Korea. The withdrawal time estimated for FF only administered twice-a-day, was 27.9 degree-days (2 days at 26.5°C) (Figure 3).

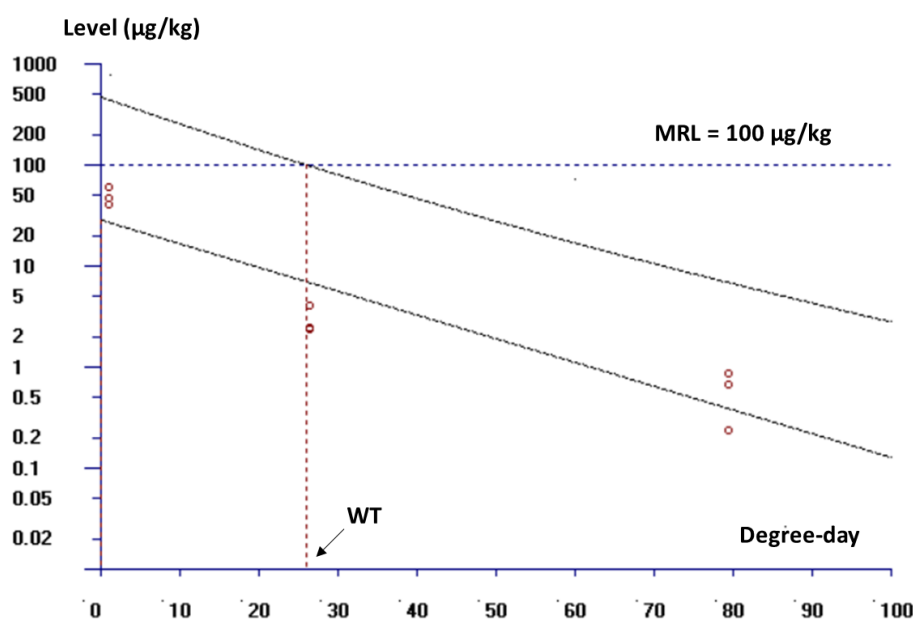


Figure 3. Plot of residual levels of FF in whiteleg shrimp muscle recorded at 1 h, 24 h and 72 h, corresponding to 1.1, 26.5 and 79.5 degree-days, respectively, following the cessation of treatment as a function of degree-days. Shrimp had been administered FF orally at a dose of 10 mg/kg bw, twice-a-day for three consecutive days, under ambient conditions (26.5°C). The withdrawal time was calculated as the time when the upper limit of the one-sided 95% confidence interval was below the MRL of 100 µg/kg.

3.4. Hepatopancreas histology of whiteleg shrimp

Prior to medication, histological examination of the hepatopancreas revealed a normal tissue structure with a full complement of B- and F-cells (Figure 4A). No abnormal clinical signs, such as mortality, color changed or reduced feed intake, were observed during medication. After oral administration of medicated feed twice-a-day for three consecutive days, a reduction in B-cell numbers was observed on day 3 of medication (Figure 4B). In Figure 4C, the numbers of B-cells in shrimp hepatopancreas were recovered on seven days after stopping medication (see also Figure 5).

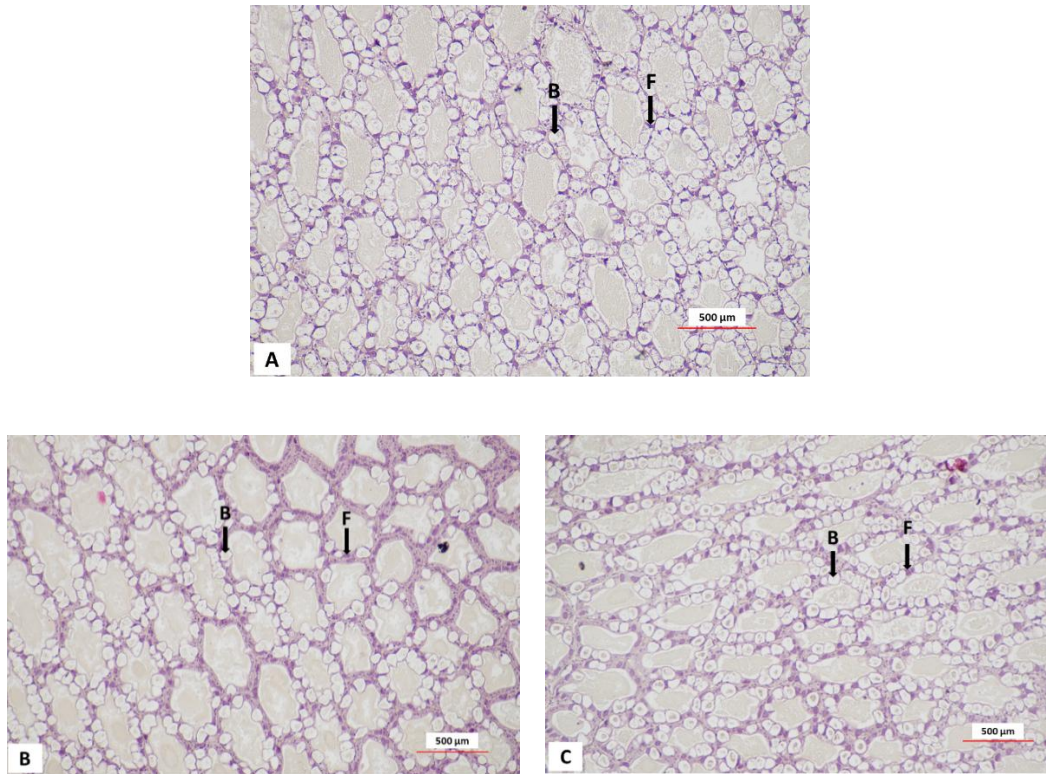


Figure 4. Representative histological section of shrimp hepatopancreas before (A) and after feeding with FF-mediated feed twice-a-day treatment on day 3 of medication (B) and 7 days after stopping medication (C) (100×). B—blasenzellen cells, F—fibrillenzellen cells

Results of the number of hepatopancreas B-cells after feeding shrimps with FF medicated feed once-a-day or twice-a-day for 3 consecutive days at a dose of 10 mg FF/kg bw are shown in Figure 5. The statistical analysis showed no significant difference in the B-cell counts between shrimps having received FF once-a-day or twice-a-day ($p > 0.05$). When considering the sampling time, it appeared that the hepatopancreas B-cells were significantly lower in samples collected on the third-day of medication than in those collected on day 7 after stopping medication or before medication ($p < 0.05$). Additionally, no interaction effect was observed between the doses applied and the sampling times on the numbers of B-cells ($p > 0.05$).

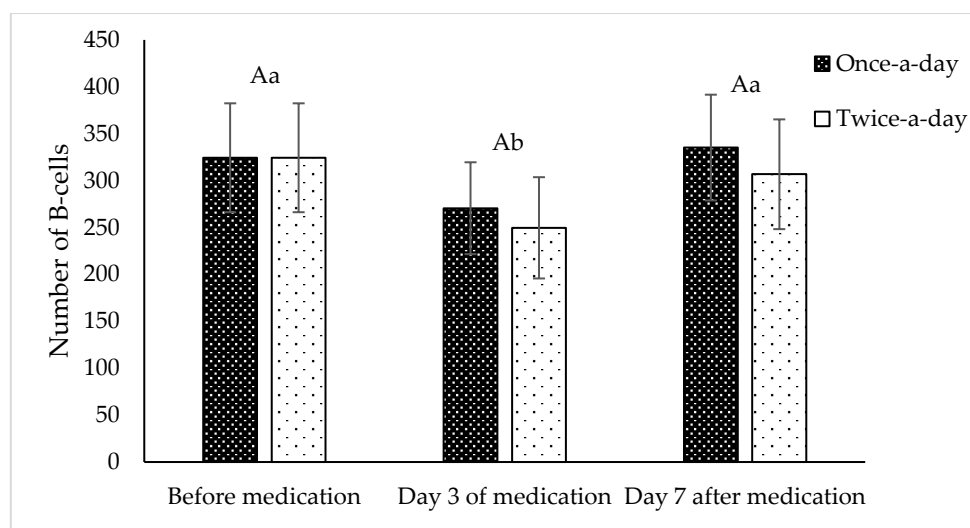


Figure 5. Number of B-cells of shrimp hepatopancreas before FF medication, on day 3 of medication and on day 7 after stopping medication in once-a-day (dark column) and twice-a-day (light column) treatments with 10 mg FF/kg bw. Values with similar capitalized letter showed no significant different between doses ($p > 0.05$). Values with similar small letter showed no significant different between sampling time ($p > 0.05$).

There was no significant difference in the number of F-cells between the applied doses (Table 5), i.e., feeding shrimp with FF-medicated feed once-a-day and twice-a-day ($p > 0.05$). Similarly, no significant different effect ($p > 0.05$) was found in the F-cell counts of shrimp across the different sampling times, i.e., there was no significant difference on day 3 of medication compared to before medication, and a similar trend was observed when comparing F-cell numbers of shrimp before medication and on day 7 after medication was stopped (Table 5). Similarly to B-cells, there was no interaction between the applied doses and the sampling times in the numbers of F-cells ($p > 0.05$). In addition, no specific histological changes, such as necrosis or vacuole formation, were observed in the hepatopancreas of shrimp during and after the medication period (Figure 4).

Table 5. The number of F-cells of the shrimp hepatopancreas before medication, on day 3 of medication and on day 7 after stopping medication in once-a-day and twice-a-day treatments with 10 mg FF/kg bw

	Number of F-Cells			p-value
	Before medication	Day 3 of medication	Day 7 after medication	
Once-a-day	164 ± 7	150 ± 20	160 ± 27	0.439
Twice-a-day	164 ± 7	153 ± 17	169 ± 21	
p-value	0.056			

4. Discussion

4.1. Antimicrobial activity of florfenicol on various *V. parahaemolyticus* strains

When using an agar disk diffusion method, the susceptibility of a bacterial strain to an antibiotic can be classified as “susceptible”, “intermediate” and “resistant” according to the observed inhibition zone diameter, i.e., ≥ 18 mm, 13–17 mm and ≤ 12 mm, respectively (CLSI, 2017; Jiang et al., 2020). In this study, all tested *V. parahaemolyticus* strains were susceptible (≥ 18 mm) to FF, except for 1 strain (belonging to Ca Mau province) with an inhibition zone diameter of 6 mm, and this strain also had an unusually high MIC (>256 $\mu\text{g/mL}$). Regarding the MIC test, the results were also varied among strains. These results illustrated that the strains of *V. parahaemolyticus* collected from different locations possess different levels of antibiotic susceptibility.

The low FF MIC values in this study were in accordance with FF MIC values against *Vibrio* spp. isolated from shrimp diseases in other studies. For example, FF MIC values were reported of between 0.5 and 4 $\mu\text{g/mL}$ in Ecuador, USA, Japan and Thailand (Mohny et al., 1992; Tipmongkolsilp et al., 2006), from 0.25 to 8 $\mu\text{g/mL}$ in Mexico (Roque et al., 2001), and of 8 $\mu\text{g/mL}$ in Ecuador by other authors (Sotomayor et al., 2019). In addition, the MIC values of FF in this study were low when compared to other antibiotics MIC values towards *V. parahaemolyticus* strains isolated from shrimp, with MICs for oxytetracycline, chloramphenicol, ampicillin and kanamycin of 54.5 to 88.6 $\mu\text{g/mL}$, 29.8 to 43.4 $\mu\text{g/mL}$, 89.5 to 126.6 $\mu\text{g/mL}$ and 21.5 to 28.9 $\mu\text{g/mL}$, respectively (Vaseeharan et al., 2005). Rebouças et al. (2011) reported an MIC value of 400 $\mu\text{g/mL}$ for oxytetracycline against *Vibrio* spp. strains. Florfenicol seems to be more effective than other antibiotics with similar bioavailability. Indeed, Rebouças et al. (2011) showed using a disk diffusion method that 31 strains of *Vibrio* spp. isolated from shrimp hepatopancreas and

hatchery water exhibited intermediate resistance to ampicillin, aztreonam, gentamicin, imipenem, oxytetracycline and ceftiofur, whereas FF was effective against all *Vibrio* isolates

Several previous studies reported that *V. parahaemolyticus* isolated from samples and environment sources exhibit resistance to multiple antibiotics, including amoxicillin, ampicillin, carbenicillin, colistin, gentamicin, tobramycin and the cephalosporin group, such as cefazolin, ceftazidime and cephalothin (Al-Othubi et al., 2014; Melo et al., 2011; Yano et al., 2011; Zanetti et al., 2001). In the study conducted by Tran et al. (2018), pathogenic *V. parahaemolyticus* strains isolated from seafood and water samples demonstrated high levels of resistance to streptomycin (84.6% at 10 µg), sulfisoxazole (57.7% at 250 µg) and ampicillin (57.7% at 10 µg). In the study of Rocha et al. (2016) on shrimp farming in Northeastern Brazil, antibiotic sensitivity tests on 70 strains of *Vibrio* isolated from water and sediment exhibited intermediate resistance to ampicillin, aztreonam, cephalothin, ceftriaxone and CFT. In another study on the resistance of *V. parahaemolyticus* isolated from shrimp samples in Malaysia, *V. parahaemolyticus* isolates were resistant to some antibiotics, i.e., amikacin (26.7%), tetracycline (36.6%), ceftazidime (46.7%), cefepime (50%), CFT (60%), amoxicillin-clavulanic acid (63.3%), and ampicillin (90%) (Saifedden et al., 2016).

Generally, the results of this study showed that *V. parahaemolyticus* strains were susceptible towards FF. This finding may partly explain why FF continues to be widely used in shrimp aquaculture for the treatment of bacterial diseases, such as necrotizing hepatopancreatitis (Morales-Covarrubias et al., 2012). However, inappropriate or sustained antibiotic application in food-producing environments promotes selective pressure that favors the survival and proliferation of resistant bacteria. Inappropriate, excessive, or prolonged antibiotic use can exert strong selective pressure on bacterial populations (Cabello, 2006; Romero et al., 2012). Within a bacterial community, susceptible bacteria are inhibited or eliminated following antibiotic exposure, whereas bacteria carrying resistance determinants, either through spontaneous mutations or acquired resistance genes, are more likely to survive. The reduction of susceptible competitors provides resistant bacteria with a selective advantage, allowing them to proliferate and progressively become dominant within the microbial population. Furthermore, continuous exposure to subtherapeutic antibiotic concentrations may facilitate the maintenance and dissemination of resistance genes through horizontal gene transfer mechanisms, including conjugation, transformation, and transduction (Romero et al., 2012). In aquaculture systems, antibiotic residues released into water and sediments may maintain long-term selective pressure on environmental microbiota, thereby contributing to the establishment of reservoirs of antimicrobial-resistant bacteria and resistance genes (Cabello, 2006). Susceptibility profiles may therefore vary among farming areas depending on local antibiotic-use practices and environmental conditions. Therefore, regular monitoring of the antimicrobial effectiveness of FF and the establishment of regional antimicrobial susceptibility databases for shrimp pathogens would support more prudent antibiotic use and improve disease-control strategies in shrimp farming. In addition, strengthening biosecurity measures, water-

quality management, and preventive approaches such as probiotics and improved farm management may help reduce dependence on antibiotics in shrimp aquaculture systems.

4.2. Pharmacokinetics of florfenicol in shrimp

The concentration of FF in the hepatopancreas was much higher than that in plasma and muscle, and the order was hepatopancreas > plasma > muscle. This is similar to what was shown in the study of Fang et al. (2013), Ren et al. (2016) and Feng et al. (2019), who showed shrimp hepatopancreas had the highest FF concentration. The antibiotics were absorbed directly from the shrimp stomach to the hepatopancreas after oral administration (Tu et al., 2010 and Ren et al., 2016). According to Verri et al. (2001) and Faroongsarng et al. (2007), the hepatopancreas plays a role in the metabolism and excretion of antibiotics in crustaceans. $T_{\max}$ values of shrimp organs in the current study were shorter than those in the study of Fang et al. (2013) following a single dose of 10 mg FF/kg bw in whiteleg shrimp by gavage feeding. The same authors reported $C_{\max}$ values in the hepatopancreas, plasma and muscle of 164.22 $\mu\text{g/g}$, 5.53 $\mu\text{g/mL}$ and 1.91 $\mu\text{g/g}$, respectively, and $\text{AUC}_{0-\infty}$ values for corresponding organs of 871.73 mg.h/kg , 41.27 mg.h/L and 15.97 mg.h/kg , respectively. These $C_{\max}$ and $\text{AUC}_{0-\infty}$ values were much higher than those in the present study. Feng et al. (2019) compared shrimp FF treatment by intramuscular injection and oral routes and showed that FF concentrations in plasma and tissues after oral administration were several times lower compared to those observed after FF intramuscular injection. However, the method of using antibiotics through feed (oral administration) is a commonly applied method in aquaculture in Vietnam.

After absorption and distribution, antibiotics will undergo elimination from the shrimp body. Florfenicol levels were rapidly eliminated in whiteleg shrimp plasma with a $T_{1/2\text{el}}$ value of 0.35 h and an apparent total body clearance of 9.75 L/kg/h . The absorption rate of antibiotics depends heavily on water temperature—the higher the water temperature, the more rapidly $T_{\max}$ is reached (Yang et al., 2019; Feng et al., 2019). For instance, in the current study, the $T_{\max}$ value (1.77 h) in shrimp plasma at 26.5°C was longer than that at 28°C (0.5 h) (Feng et al., 2019). There was evidence that drug absorption also depends on physiological differences between species, specifically, the structure of the intestine and the species-specific response to environmental factors (Kverme et al., 2019). For example, at the same applied dose (10 mg FF/kg bw), the $T_{\max}$ value in plasma of striped catfish was 3.70 h (Pham et al., 2023), while in the present study, the $T_{\max}$ value in shrimp plasma was 1.77 h. In addition, the PK parameters vary between different antibiotics, and according to the mode of drug administration and organ measurement as well as the dosage used (Table 6).

Table 6. Pharmacokinetic parameters in whiteleg shrimp on other antibiotics according to different routes of administration

Antibiotic	Muscle				Hepatopancreas				Plasma				References
	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	
	(h)	(mg/kg)	(mg.h/kg)	(h)	(h)	(mg/kg)	(mg.h/kg)	(h)	(h)	(mg/L)	(mg.h/L)	(h)	
Single dose													
Oxytetracycline *													
100 mg/kg bw	6	0.73	32.63	23.53	1	149.5	1015	14.89	6	27.77	691.3	11.01	(Ma et al., 2019)
Oxytetracycline **													
10 mg/kg bw	6	7.94	166.5	20.90	-	-	-	-	0	32.22	266.1	20.74	(Chiayvareesajja et al., 2006)
Thiamphenicol *													
10 mg/kg bw	2	2.98	29.33	6.84	1	204.2	1381	31.29	2	7.96	43.96	10.66	(Fang et al., 2013)
Sulfamethoxazole *													
83.3 mg/kg bw	1	21.5	203.9	6.53	0.5	89.54	381.1	8.22	1	36.52	395.3	13.76	(Ma et al., 2017)
Trimethoprim *													
16.7 mg/kg bw	1	0.78	1.90	2.12	0.5	73.17	321.3	4.53	1	0.89	4.82	7.38	(Ma et al., 2017)
Enrofloxacin ***													
10 mg/kg bw	0.25	5.81	90.1	52.3	0.5	11.23	274.2	75.8	0.083	4.87	75.8	19.8	(Fang et al., 2018)

Antibiotic	Muscle				Hepatopancreas				Plasma				References
	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	T _{max}	C _{max}	AUC _{0-∞}	T _{1/2el}	
	(h)	(mg/kg)	(mg.h/kg)	(h)	(h)	(mg/kg)	(mg.h/kg)	(h)	(h)	(mg/L)	(mg.h/L)	(h)	
Multiple dose													
Enrofloxacin * 30 mg/kg bw	1	1.96	34.75	10.92	3	16.29	314.9	15.86	1	19.64	299.6	16.07	(Ma et al., 2018)
Oxytetracycline * 100 mg/kg bw	6	1.67	66.05	35.39	6	124.3	1858	18.89	6	68.03	2049	20.52	(Ma et al., 2019)
Sulfamethoxazole * 83.3 mg/kg bw	1	47.8	403.5	10.92	1	73.73	756.5	11.33	1	85.89	1493	11.03	(Ma et al., 2017)
Trimethoprim * 16.7 mg/kg bw	1	0.80	3.03	2.71	1	46.50	190.2	9.65	1	1.14	4.84	5.25	(Ma et al., 2017)

T_{max} mean time to maximal concentration (h), C_{max} mean maximal concentration (mg/kg or mg/L), $AUC_{0-\infty}$ mean area under the concentration-time curve from 0 h to ∞ (mg h/kg or mg h/L), $T_{1/2el}$ mean elimination half-life of drug (h), * oral administration, ** intra-sinus administration, *** intramuscular administration

Florfenicol is classified in the group of time-dependent antibiotic (Dowling, 2013), which follow the time-dependent killing pattern, and according to Reed (2000), these antibiotics' effectiveness is dependent on the duration of pathogen exposure to the antibiotic. The time during which the tissue concentration exceeds the MIC ($T > MIC$) against a bacteria should be considered as a predictor of efficacy against the corresponding target bacteria. The therapeutic dose should be calculated to maintain the concentrations higher than the MIC towards the targeted bacterial strain. According to AliAbadi and Lees (2000), for time-dependent antimicrobials, the $T > MIC$ criteria should be used, and the value must be in the range of 40% to 50% of the dosing interval to achieve efficiency of treatment. In this study, the C_{max} value in shrimp plasma was 60.56 $\mu\text{g/L}$ (Table 2) and much lower than the MIC values of FF towards *V. parahaemolyticus* (Table 1). In theory, this means that AHPND caused by *V. parahaemolyticus* in whiteleg shrimp may not be treatable with a single dose of 10 mg FF/kg bw. It means that to ensure the effectiveness of this medication, the dose should be higher than 10 mg FF/kg bw to achieve the desired FF concentration in plasma or hepatopancreas, but the toxicity of FF should then be assessed in whiteleg shrimp. In addition, increasing the FF dose should be carefully evaluated because higher antibiotic exposure may prolong residue depletion in shrimp tissues and increase withdrawal times. Excessive or inappropriate antibiotic use may also contribute to increased selective pressure for resistant bacteria in aquaculture environments. Therefore, dose optimization should balance therapeutic effectiveness with food safety and prudent antibiotic use in shrimp farming systems.

4.3. Depletion of florfenicol in whiteleg shrimp muscle

Florfenicol residues were rapidly eliminated in shrimp muscle and became undetectable within 24 h after stopping medication, in both treatment frequencies (once-a-day and twice-a-day). To determine the withdrawal time, the study followed the definition of this parameter as the period required for drug residues in edible tissues to fall below a safe threshold for human consumption (Avunje et al., 2021). This calculation was based on the maximum residue limit (MRL) of 100 $\mu\text{g/kg}$ for the sum concentration of FF and its metabolite (FFA), as established by the European Commission (EC, 2010) and the Korean Ministry of Food and Drug Safety (2019). In this study, withdrawal time was estimated only for the twice-a-day treatment and was 27.9 degree-days (less than 2 days at 26.5°C). Although research on FF depletion has been performed in many fish species, those studies are limited for crustaceans. When comparing with fish, the rapid depletion of FF in shrimp muscle seen in this study is similar to the observations in the study of Pham et al. (2023) performed on stripped catfish with the same dosage. This is not the same for Asian seabass, as the FF residual level and withdrawal time of shrimp muscle was much lower than those reported by Rairat et al. (2023) on Asian seabass, where the muscle FF concentration was 0.28 $\mu\text{g/g}$ on day 4 and 0.15 $\mu\text{g/g}$ on day 10 after the last FF oral dose, and the withdrawal time was estimated up to 8 days at 25°C (approximately 204 degree-days). Besides the species, the difference may be due to the administration dose and the temperature, 10 mg/kg and

26.5°C (in this study) compared with 15 mg/kg and 25°C in the study of Rairat et al. (2023). The effect of temperature on depletion was also found in a study performed on tilapia, where the withdrawal time of FF at 22°C was longer than that at 28°C (Lin and Chen, 2021).

Compared to other commonly used antibiotics in whiteleg shrimp, FF demonstrated a relatively shorter depletion period and withdrawal time in this study. For instance, oxytetracycline administered at 4.5 g/kg feed for 14 consecutive days showed a withdrawal time of 96 h at 29°C (Avunje et al., 2021), while chloramphenicol and sulfamethoxazole, both administered at 2 g/kg feed for 3 consecutive days, showed longer withdrawal times of 139.7 h and 30.6 h, respectively, at 24°C (Wang et al., 2004). These comparisons highlight the influence of the type and the dose of the applied antibiotic on residue depletion and withdrawal time in shrimp muscle. Rapid depletion of FF is consistent with the results of Huynh et al. (2024) on CFT administered to shrimp at 25 mg/kg bw for 3 consecutive days; the CFT concentration in whiteleg shrimp muscle was below LOD after stopping medication for 24 h.

Generally, quick depletion of FF in shrimp muscle resulted in a short withdrawal time, suggesting that the health risk for humans consuming whiteleg shrimp treated with 10 mg FF/kg bw can be considered to be negligible.

4.4. Impact on shrimp hepatopancreas histology

The hepatopancreas has been investigated by histologists and biologists for more than a century (Caceci et al., 1988). The hepatopancreas of shrimp is constituted of many tubules. The tubule walls are composed of simple epithelium, containing four main types of cells, namely, E-cells (embryonalzellen), F-cells (fibrillenzellen), R-cells (restzellen) and B-cells (blasenzellen) (Pathak et al., 2018). In this investigation, the histology of the hepatopancreas was observed; however, only B- and F-cells were subjected to cell number counting, because the growth rate of shrimp is decreased during antibiotic application (as stated by shrimp famers), and the growth rate is related to digestive function in the shrimp hepatopancreas. According to Hu and Leung (2007), B- and F-cells in the shrimp hepatopancreas play key roles in digestive functions. F-cells are responsible for synthesizing digestive enzymes, which are stored within a large vacuole. These F-cells then transform into B-cells, which subsequently release the enzymes into the hepatopancreatic lumen via a holocrine secretion process (Nunes et al., 2014). Another reason is that the proportion of R-cells in the hepatopancreas of whiteleg shrimp was similar in both normal and growth-retarded shrimp, while the number of B-cells was lower in growth-retarded shrimp (Nima et al., 2022). Under a microscope, F-cells are very large with a cytoplasm that is noticeably metachromatic; B-cells are very prominent under light microscope with one or two very large vacuoles which are filled with a flocculent material and are usually so large that the nucleus is located to the periphery of the cell (Caceci et al., 1988; Nunes et al., 2014).

In the current study, there were no abnormal signs in the shrimp hepatopancreas histology during medication, and the cells of the shrimp hepatopancreases had no negative impacts when using FF-medicated feed at a dose of 10 mg FF/kg bw in both once-a-day and twice-a-day treatments. These results are similar to that obtained in a previous study on whiteleg shrimp treated with CFT at 25 mg/kg bw, showing no negative impacts on shrimp hepatopancreas cells during medication (Huynh et al., 2024). Regarding other antibiotics, several studies found negative effects on shrimp hepatopancreas histology, e.g., enrofloxacin (Maftuch et al., 2017) or oxytetracycline (Bray et al., 2006; Avunje et al., 2021), which showed that the higher the dose of antibiotics used, the more serious the impact on the hepatopancreas of shrimp. Therefore, evaluating hepatopancreatic health following antibiotic administration is important for assessing the biological safety of antibiotic use in shrimp culture.

In the present study, the temporary reduction in B-cell numbers observed on day 3 in shrimp receiving FF-medicated feed twice daily, followed by recovery after 7 days without medication, may suggest a transient adaptive response of the hepatopancreas rather than a persistent pathological alteration. Since B-cells are associated with digestive enzyme secretion and intracellular transport processes in the shrimp hepatopancreas (Nunes et al., 2014), the temporary decrease may reflect physiological adjustments during intensive antibiotic exposure. The subsequent recovery of B-cell numbers after cessation of medication indicates that the hepatopancreatic tissues retained the capacity for structural and functional recovery under the investigated treatment conditions. Moreover, the absence of severe histopathological signs, such as necrosis, tubular degeneration, or extensive vacuolization, further supports the interpretation that FF administration at the investigated dose did not induce irreversible hepatopancreatic damage in whiteleg shrimp. Although no severe histopathological alterations were observed at the investigated FF dose, the potential effects of higher doses or repeated treatments on hepatopancreatic tissues still require further investigation.

5. Conclusions

This study showed quick absorption and elimination of FF in the plasma, hepatopancreas and muscle of whiteleg shrimp after FF once oral administration, with a short elimination half-life of 0.35 h. The FF withdrawal time in shrimp muscle was 27.9 degree-days (2 days) when FF was administered twice-a-day and using an MRL of 100 µg/kg, which will ensure the safety of shrimp products for consumers. Oral therapeutic use of FF in shrimp slightly affected the histology of shrimp hepatopancreas during medication, but the organ structure recovered after 7 days of stopping medication. Further investigation of optimized FF dosing strategies may help improve therapeutic efficacy against *V. parahaemolyticus* in shrimp culture. However, such approaches should also consider antimicrobial stewardship principles, including the potential impacts on withdrawal periods, production costs, farmer compliance, public health, and the risk of promoting antimicrobial resistance.

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3.2.3 PHARMACOKINETICS AND WITHDRAWAL TIMES OF CEFOTAXIME IN WHITELEG SHRIMP (*PENAEUS VANNAMEI*) AFTER ORAL ADMINISTRATION

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Abstract

A high-performance liquid chromatography method coupled to tandem mass spectrometry was validated in order to study the pharmacokinetics (PK) of cefotaxime (CFT) in shrimp hepatopancreas and plasma, as well as its withdrawal time related to a maximum residue limit (MRL) in shrimp muscle. Pharmacokinetics parameters were investigated through oral medication at a single dose of 25 mg/kg shrimp body weight and subsequent hepatopancreas and plasma CFT concentration measurements at 0.5, 1, 2, 4, 8, 12 and 24 h after shrimp were fed with medication. The maximum concentration of CFT was observed after one hour in the hepatopancreas ($C_{\max}$, 19.45 ± 2.10 mg/kg) and 4 h in plasma (0.184 ± 0.061 mg/L). Based on a MIC of CFT of 4.13 mg/L against *V. parahaemolyticus* (known to cause AHPND in whiteleg shrimp), it was observed that the time during which the hepatopancreas CFT concentration was above the MIC was 23 h. An every 24 h CFT treatment could thus be effective in fighting against this bacterium in shrimp. The withdrawal time of CFT was determined after shrimp were fed with medicated feed once a day and twice a day for three consecutive days. Shrimp muscle was collected on day 1 and day 3 during medication and 1, 3, 7, 14 and 21 days after medication was stopped. Considering an MRL of 50 µg/kg, the withdrawal times were 8.5 degree-days (corresponding to 6.9 h at 29.5°C) after shrimp were fed with medicated feed once a day for 3 days and 95.5 degree-days (77.7 h at 29.5°C) after shrimp were fed with medicated feed twice a day for 3 days. Moreover, histological analysis revealed that feeding shrimp with CFT at the given dose in once- or twice-a-day treatments did not negatively impact the shrimp hepatopancreas.

Keywords: Cefotaxime; hepatopancreas; pharmacokinetics; *Vibrio parahaemolyticus*; whiteleg shrimp; withdrawal time.

Key Contribution: This study has determined the PK parameters and withdrawal time of CFT in whiteleg shrimp, supporting the rational use of this antibiotic in terms of shrimp disease treatment and food safety.

1. Introduction

Aquaculture is an important sector of Vietnam's economy, with total annual production accounting for 5.4 million tons in 2023, with shrimp and striped catfish culture industries being responsible for most of this production (VASEP, 2024b). Penaeid shrimp is a high-value export product produced mainly in the Mekong Delta in Vietnam and accounts for over 80% of shrimp production. Annually, the shrimp industry contributes about 40–45% to the total seafood export value, which is equivalent to USD 4.3 billion (VASEP, 2022). Like for other farmed aquatic animals, diseases, especially those caused by bacteria, frequently occur in the whiteleg shrimp culture industry with the development of intensive aquaculture. According to surveys performed previously in the Mekong Delta, Vietnam, common diseases causing heavy damage to shrimp reared in intensive systems are white feces disease (WFD), acute hepatopancreatic necrosis disease (AHPND), and white spot syndrome virus (WSSV), red body syndrome and black spot disease (Phu et al., 2019; Thinh et al., 2020). In order to control diseases in aquaculture, most farmers use antibiotics to prevent and treat bacterial infections (Chi et al., 2017; Phu et al., 2016; Rico et al., 2014). The improper use of antibiotics has led to residues found in exported shrimps. The Rapid Alert System for Food and Feed (RASFF) from the EU reported, from 2020 to 2022, residues of ciprofloxacin, oxytetracycline, sulfamethoxazole and nitrofurans metabolites in shrimp frozen products (RASFF, 2022).

Pharmacokinetics and residues in shrimp were investigated in previous studies, for several antibiotic classes, such as tetracyclines, e.g., oxytetracycline (Bermúdez-Almada et al., 1999; Ma et al., 2019; Nogueira-Lima et al., 2006; Reed et al., 2004; Uno et al., 2006; Wang et al., 2004); phenicols, e.g., FF (Fang et al., 2013; Ren et al., 2016), chloramphenicol, thiamphenicol (Fang et al., 2013); sulfonamides, e.g., sulfamethoxazole (Wang et al., 2004); quinolones, e.g., enrofloxacin (Maftuch et al., 2017; Fang et al., 2018; Ma et al., 2018); nitrofurans (Douny et al., 2013); and a combination of sulfamethoxazole and trimethoprim (Ma et al., 2017). Cefotaxime is a commonly used antibiotic in shrimp culture and plays an important role in disease treatment (Plosker et al., 1998; Thinh and Phu, 2021). CFT is a third-generation cephalosporin, which belongs to the class of beta-lactams; it has a broad spectrum of activity against aerobic Gram-negative as well as Gram-positive bacteria, while it shows variable activity against anaerobic bacteria (Carminc et al., 1983). This antibiotic is used in aquaculture, and its residue can be found in countries where aquaculture is developed, i.e., in China, the detection frequency of CFT was 40% in fish ponds around Tai Lake, with a level exceeding 10,000 ng/L (Song et al., 2016) and it was found in surface water of Xiangjiang River with a concentration of 830 ng/L (Lin et al., 2018). In Egypt, CFT is used in bacterial pathogen management in tilapia (Ali et al., 2021). In aquatic animals, studies related to side effects or PK of CFT are not common, and CFT was just investigated in tilapia (*Oreochromis niloticus*) in bacterial infectious treatment (Ali et al., 2021), but CFT PK has been studied in several terrestrial animals like goats (*Capra hircus*), buffalo calves (*Bubalus bubalis*), rats and camels (*Camelus dromedarius*) (Altayban et al., 2020; Atef et al., 1990; Golocorbin-Kon et al., 2009; Sharma

et al., 2005). Cefotaxime is used to control susceptible bacterial infections in aquatic and terrestrial animals, such as tilapia, horses, birds, dogs, cats and reptiles at the dose of 10–80 mg/kg body weight via intraperitoneal, intramuscular, intravenous or subcutaneous injection (Ali et al., 2021).

Although antibiotics are very effective in fighting against bacterial infectious diseases, they result in negative effects on the internal organs of terrestrial animals (Attia et al., 2019; Nale et al., 2012) as well as aquatic animals (Augusto et al., 1996; Bojarski et al., 2020; Islam et al., 2022; Maftuch et al., 2017; Manna et al., 2021; Rodrigues et al., 2019; Zhang et al., 2019). Exposure to CFT in *Artemia sinica*, an aquatic invertebrate, showed oxidation stress and changes in intestinal cell morphology and gut microbiota (Pang et al., 2024). Therefore, using CFT in disease management may result in a negative effect on cultured shrimp, especially in the hepatopancreas, which is responsible for chemical metabolism and the normal growth and development of shrimps and other crustaceans. The hepatopancreas (also called the midgut gland) is the most impacted organ during drug application or after chemical exposure. This organ, which is equivalent in vertebrates to the liver, pancreas and intestine, has been investigated by histologists and biologists for more than a century (Caceci et al., 1988). Because of its importance, any damage or change in the cell function of this organ could lead to negative effects, e.g., death, growth slowdown or immunodeficiency.

The outcomes of this research will, on the one hand, provide knowledge about CFT PK and withdrawal times in shrimp which will facilitate the establishment of guidelines for optimal use of this antibiotic in shrimp and, on the other hand, provide an improved understanding on the impact of CFT on hepatopancreas histology in whiteleg shrimp aquaculture during and after CFT medication.

2. Materials and methods

2.1. Chemicals

Standards of CFT and penicillin V, with a purity greater than 98% (Dr. Ehrenstorfer, 86199 Augsburg, Germany), were purchased and stored at -20°C . Stock solutions of CFT and penicillin V (1000 $\mu\text{g/mL}$) were prepared in a methanol and water mixture (1:1, v/v) and stored at -20°C . These stock solutions were then diluted to a concentration of 1 $\mu\text{g/mL}$ for use in sample fortification and standard curve preparation. Cefotaxime (99.9%) for medicated feed preparation was sourced from Phil Inter Pharma Co. Ltd. (75000 Binh Duong, Vietnam). C18 Bondesil powder, with a particle size of 40 μm , was obtained from Agilent (Santa Clara, CA, USA). Ultra-high-performance liquid chromatography water, methanol, acetonitrile, dipotassium hydrogen sulfate and 98% formic acid were acquired from Merck (64293 Darmstadt, Germany).

2.2. Experimental shrimp

Whiteleg shrimp were reared from post-larvae in the Faculty of Marine Science and Technology, College of Aquaculture and Fisheries, Can Tho University; the post-larvae were reared for

approximately 45 days before experimental processing. Shrimp were checked for CFT residues before the experiment. For each pond, 200 shrimp (13.8 ± 1.1 g/shrimp) were stocked in 2 m³ tanks to reach the stocking density of 100 shrimp/m³ and acclimated for 1 week prior experiment. Experimental tanks were fully equipped with aeration to maintain dissolved oxygen above 5 mg/L. During the experiment, water salinity was at 10‰, and alkalinity was adjusted at 140–160 mg CaCO₃/L; other parameters such as water temperature, pH and nitrite (NO₂⁻) were measured every two days. During acclimation, shrimp were fed commercial pellet feed (40% crude protein) at 3% shrimp biomass, four times a day, 6 am, 10 am, 2 pm and 6 pm. At each feeding time, after 45 min of feed provision, the excess feed (less than 5% of the total feed amount) was removed by siphoning.

2.3. Pharmacokinetics experiment

To determine the PK of CFT, shrimp were fed a single dose of 25 mg CFT/kg body weight. The experiment was performed using 6 tanks stocked with shrimp as described above (corresponding to 6 replicates). The concentration of CFT in feed was calculated based on the amount of feed fed to 1 kg of shrimp biomass. Before every medication, medicated feed was prepared by spraying a solution of CFT over the feed. Cefotaxime (1 g) was dissolved in 30 mL of distilled water and mixed with 200 g feed, which corresponded to 5 mg CFT/g feed or 0.5% of CFT in feed. The medicated feed was then covered with 2% squid oil and left for 15 min before shrimp feeding at 6 am. Shrimp received then antibiotic-free feed at 10 am, 2 pm and 6 pm. Analytical results in two samples showed that the content of CFT in feed was 0.48% and 0.54%, close to the theoretical concentration. Shrimp blood and hepatopancreas samples were collected at 0.5, 1, 2, 4, 8, 12 and 24 h after medication. At each sampling time, three shrimp were randomly collected from each tank to constitute one replicate. The replicate number used in this experiment ($n = 6$) was determined with reference to previously published pharmacokinetic studies in shrimp and other aquatic species employing comparable experimental approaches (Ma et al., 2017; Yang et al., 2014; Xu et al., 2022). Blood samples were collected from the pericardial cavity of shrimp using a 1 mL syringe soaked with anti-coagulation solution (EDTA 0.01 M, NaCl 0.338 M, glucose 0.115 M, trisodium citrate dihydrate 0.03 M). Blood samples from three shrimp in one tank were pooled, placed into 1.5 mL centrifuge tubes and centrifuged at 10,000 rpm for 5 min to collect plasma. All samples were stored at -80°C until analysis. Meanwhile, hepatopancreases from 3 individual shrimp in each tank were collected into marked plastic bags and stored at -80°C until analysis. During sampling, collected samples were preserved on ice to avoid CFT concentration changes in collected tissues.

2.4. Determination of the withdrawal times of cefotaxime and hepatopancreas histological observation

The withdrawal times of CFT in shrimp were determined after oral administration. Shrimp were fed medicated pellet feed (prepared as described above) at the dose of 25 mg CFT/kg body weight once a

day at 6 am (three tanks) and twice a day (three tanks) at 6 am and 6 pm for three consecutive days. After three days of medication, shrimp were fed CFT-free feed for 21 days, four times a day, at 6 am, 10 am, 2 pm and 6 pm. On days 1 and 3 during medication, samples were collected 1 h after medication for the “once-a-day treatment” group and 1 h after the second dose for the “twice-a-day treatment” group. Sampling was continued on days 1, 3, 7, 14 and 21 after the last medication. At each sampling time, 10 shrimp were randomly collected from one tank, and the shrimp muscle without shells was pooled, minced and kept in a -20°C freezer until analysis.

The samples for hepatopancreatic histological observations were also collected in this experiment one day before medication, one hour after the last dose of medication (day 3 of medication) and 7 days after the medication was stopped to evaluate the impact on shrimp hepatopancreases. At each sampling time, three shrimp were collected from each tank. Histological sampling and preparation were performed as described by Lightner (1996).

2.5. Cefotaxime quantification

The analytical method for CFT residue analysis was adapted from the LC-MS/MS method for the determination of beta-lactam antimicrobials described by the United States Department of Agriculture (USDA, 2011).

2.5.1. Sample extraction and chromatographic and mass spectrometry conditions

For the extraction of CFT, 3.0 ± 0.05 g of homogenized shrimp muscle, hepatopancreas or feed was introduced into a 50 mL Falcon tube and spiked with 60 μL of 1 $\mu\text{g/mL}$ penicillin V solution as an internal standard. Shrimp plasma (0.5 mL) was also fortified with 10 μL of 1 $\mu\text{g/mL}$ penicillin V solution. The extraction, chromatographic and mass spectrometry conditions were as described by Phu et al. (2019). A Waters ACQUITY ultra-high-performance LC system (Waters, Milford, MA, USA) and a Hypersil GOLD C18 3×100 mm, 1.9 μm C18 column (Thermo Scientific, Waltham, MA, USA) were used. The mobile phases included two solvents: solvent A containing methanol/acetonitrile (1/1, v/v) and 0.1% formic acid, and solvent B containing water and 0.1% formic acid (the gradient elution is detailed in Table 1). Under such conditions, the retention times of CFT and penicillin V were 3.1 and 4.04 min, respectively. The following transitions (precursor > product ion, m/z) were used for quantitation and confirmation (collision energy indicated in brackets): CFT 456.1 > 396.1 (10 eV) and 456.1 > 324.1 (14 eV); penicillin V (IS) 351.1 > 160.1 (16 eV) and 351.1 > 114.1 (35 eV).

Table 1. The HPLC mobile phase conditions for cefotaxime analysis.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0	0.3	20	80
1	0.3	60	40
1.5	0.3	80	20
3.0	0.3	80	20
3.01	0.3	20	80
6.0	0.3	20	80

2.5.2. Quantification of cefotaxime residues

The concentrations of CFT residues were calculated using matrix-matched calibration curves prepared with CFT solutions at different concentrations and penicillin V, used as the internal standard. For the matrix-matched calibration curves, 3.0 ± 0.05 g of homogenized blank (antimicrobial free) shrimp muscle, hepatopancreas and feed samples and 0.5 mL of plasma were fortified with standard solutions of CFT at 6 concentrations (0, 10, 20, 40, 80 and 160 $\mu\text{g/kg}$ for hepatopancreas and feed and $\mu\text{g/L}$ for plasma) and 60 μL of a 1 $\mu\text{g/mL}$ penicillin V solution, except for plasma where 10 μL of a 1 $\mu\text{g/mL}$ penicillin V solution was added. The fortified samples were prepared on the same day as the analysis of the samples.

2.5.3. Method validation

Since no certified reference material was available, the validation was conducted according to Commission Decision No. 2021/808/EC (European Commission, 2021) by recovering known amounts of CFT added to a blank matrix. Validation for recovery, repeatability and within-laboratory reproducibility was carried out using 21 blank shrimp muscle samples fortified at three concentrations: 100 $\mu\text{g/kg}$ (MRL) and 0.1 and 1.5 times the MRL, i.e., 10 and 150 $\mu\text{g/kg}$. Repeatability was assessed from seven repeated analyses performed on the same day, expressed as the coefficient of variation (CV) of the mean CFT concentration ($\mu\text{g/kg}$). Within-laboratory reproducibility was determined by calculating the mean and CV from results obtained over three independent days. Recovery was calculated by comparing the measured concentrations to the fortified concentrations for each sample. Mean recovery and CV, both expressed as %, were derived from all results obtained over the three days for each fortification level. The decision limit CC_α ($\alpha = 5\%$ for substances with an MRL) was determined as the average concentration of the blank samples fortified at the MRL concentration of CFT (100 $\mu\text{g/kg}$) under within-laboratory reproducibility conditions, plus 1.64 times the corresponding

standard deviation. In addition, selectivity/specificity, matrix effect and ruggedness were determined according to Commission Decision No. 2021/808/EC (European Commission, 2021). The limit of detection (LOD) was calculated as 3.3 times and the limit of quantification (LOQ) was calculated as 10 times the standard deviation of the response divided by the slope of the matrix-matched calibration curve. The standard deviation of the response was determined following the ICH guidelines (ICH, 2005) via the responses of 21 blank samples, integrating the area of the chromatographic peak at the retention time corresponding to the expected compound.

2.6. Pharmacokinetics data analysis

Cefotaxime plasma and hepatopancreas concentration data were modeled using a naive pooled population approach based on a non-compartmental model with first-order absorption and elimination. The following PK parameters were calculated: maximal concentration ($C_{\max}$), time to maximal concentration ($T_{\max}$), area under the concentration–time curve from time 0 to 24 h ($AUC_{0-24\text{ h}}$), elimination rate constant (k_{el}), elimination half-life ($T_{1/2\text{el}}$).

All PK data from shrimp plasma and hepatopancreases were computed using the linear-up log-down trapezoidal method using Phoenix 8 (Certara, Princeton, NJ, USA).

2.7. Withdrawal times calculation

European Medicine Agency guidelines (EMA, 2022) were used to assess the withdrawal time. The concentration of CFT in muscle was measured at various time points post-treatment and analyzed using linear regression against time (degree-days) with the statistical software WT 1.4, developed by Hekman (2004). Degree-days were calculated by multiplying the average daily water temperature (in Celsius) by the total number of days the temperature was measured. The withdrawal period was defined as the time when the upper one-sided 95% tolerance limit for the CFT residue concentration fell below the MRL.

2.8. Histological analysis

Shrimp hepatopancreases were collected and injected with Davidson's AFA (ethanol–formalin–acetic acid) fixative for 24 h and then transferred and preserved in 70% ethanol solution. After fixation, the hepatopancreases were trimmed. Before we proceeded with casting, the samples were dehydrated by soaking in 70%, 80%, 95% and 100% ethanol and xylene before being put in paraffin. Then, the samples were cut into 6 μm thick sections and put in warm water at 45–50°C to make the paraffin stretch and stick on the sections, and then the slides were stained with hematoxylin and eosin (H&E) dyes (Yang et al., 2007). Histological sections of the hepatopancreases were examined by microscope (Novex, the Netherlands).

3. Results

3.1. LC-MS/MS method validation

The validation evaluated linearity, specificity, selectivity, signal per noise, stability, precision (repeatability and within-laboratory reproducibility), recovery and decision limit (CC_α) according to Commission Decision No. 2021/808/EC (European Commission, 2021) (Table 2).

Table 2. Validation parameters of the LC-MS/MS method for quantification of CFT in shrimp muscle tissue

Parameters		Results
LOD ($\mu\text{g/kg}$)		1.5
LOQ ($\mu\text{g/kg}$)		4
CC_α ($\mu\text{g/kg}$)		108
Linearity (R^2)		0.999
Selectivity/specificity	Identification point	5
	Relative standard deviation of the ion ratio (%)	± 15
	Minimum acceptable retention time (%)	0.3
Matrix effect (%)		11.63
Ruggedness (%)		93
Repeatability at 3 concentrations (measured concentration in $\mu\text{g/kg}$, $n = 7$) (%)	0.1 MRL (10 $\mu\text{g/kg}$)	5.95
	1 MRL (100 $\mu\text{g/kg}$)	4.97
	1.5 MRL (150 $\mu\text{g/kg}$)	3.64
	0.1 MRL (10 $\mu\text{g/kg}$)	6.00
	1 MRL (100 $\mu\text{g/kg}$)	5.14

Parameters	Results	
Within-laboratory reproducibility at 3 concentrations (measured concentration in $\mu\text{g/kg}$, $n = 3$) (%)	1.5 MRL (150 $\mu\text{g/kg}$)	3.79
	0.1 MRL (10 $\mu\text{g/kg}$)	88–107
Recovery at 3 concentrations (min–max, $n = 3$) (%)	1 MRL (100 $\mu\text{g/kg}$)	89–106
	1.5 MRL (150 $\mu\text{g/kg}$)	90–102

LOD: limit of detection, LOQ: limit of quantification, CC_a: decision limit, MRL: maximum residue limit.

LOD and LOQ of CFT were 1.5 $\mu\text{g/kg}$ and 4 $\mu\text{g/kg}$ for shrimp muscle, respectively. The comparison of CFT calibration curves prepared from blank shrimp tissue and standard solutions showed that the slope of the calibration curve in the solution was significantly lower ($p = 0.002$) than the one in the matrix (Figure 1), while the coefficients of determination (R^2) were above 0.999 for both curves, in the matrix or in the solvent.

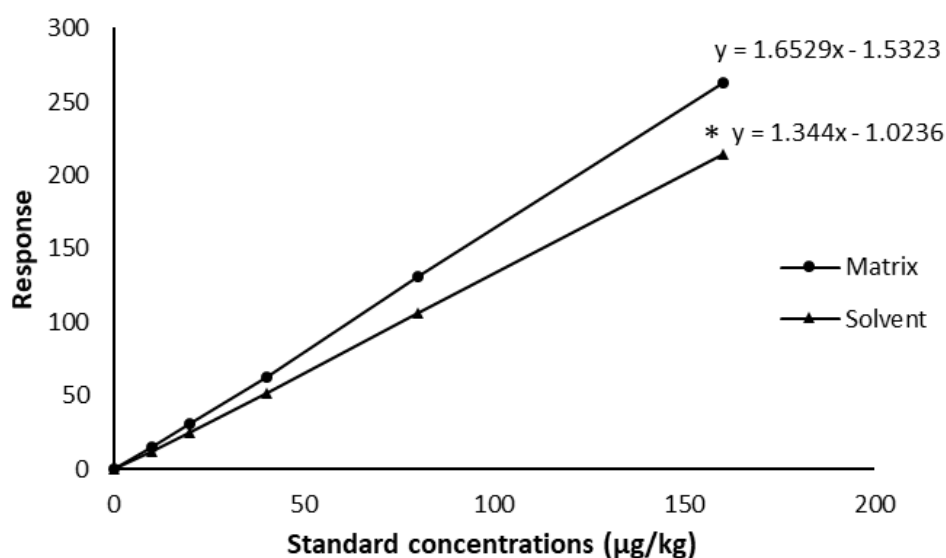


Figure 1. Cefotaxime matrix–matched (using blank shrimp muscle) and solvent calibration curves obtained using the LC–MS/MS method; asterisk (*) value presented significant difference in slope values between matrix–matched and solvent calibration ($p < 0.05$)

The specificity of the method was evaluated by comparison of the chromatograms obtained when analyzing blank shrimp muscle samples with those obtained when analyzing the CFT–fortified samples. No peak was observed in the blank samples at the retention time of CFT (Figure 2A). The chromatogram of CFT obtained from the analysis of blank shrimp muscle fortified with 10 µg/kg is shown in Figure 2B.

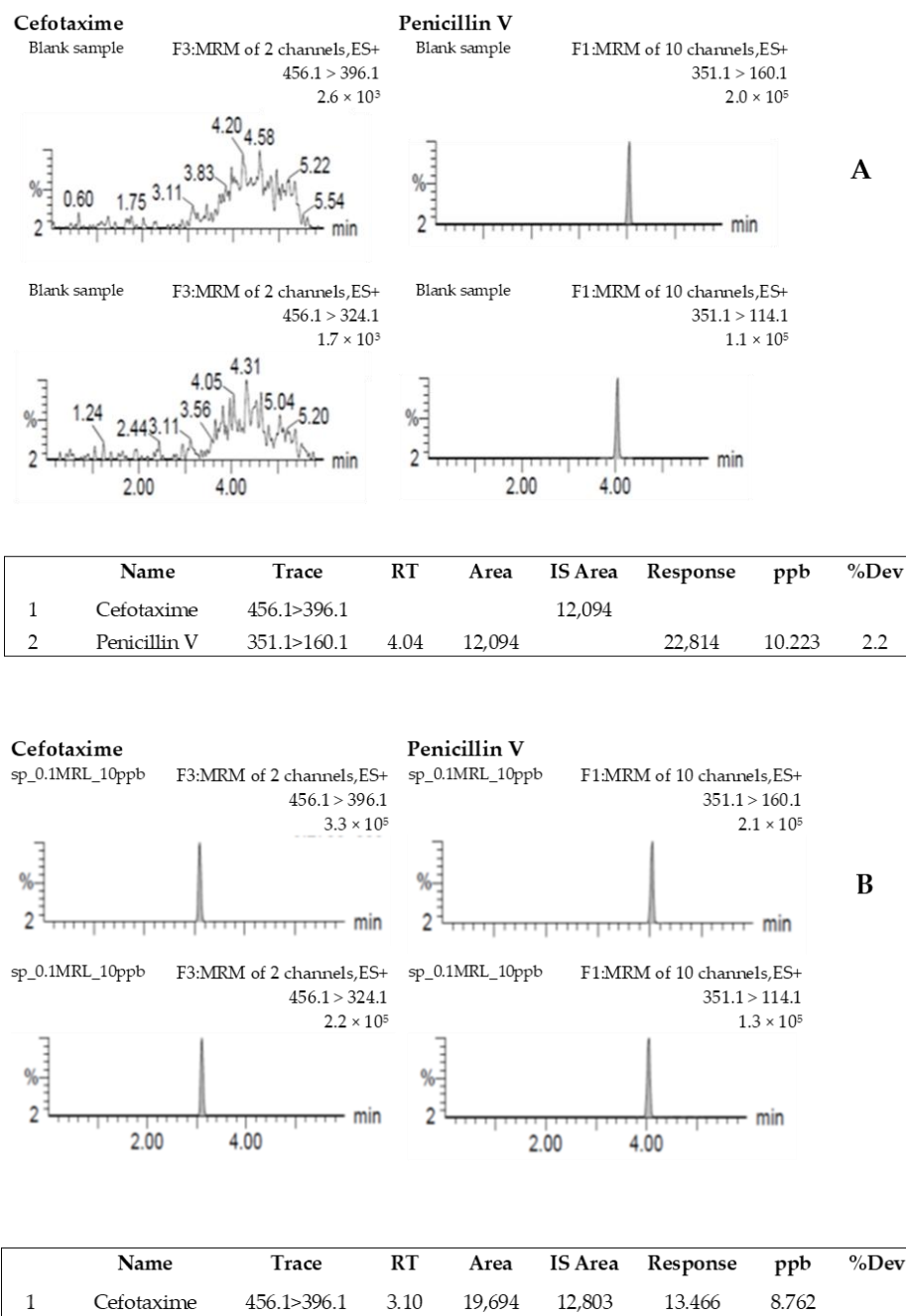


Figure 2. Chromatogram resulting from the analysis of a blank shrimp sample spiked with internal standard (penicillin V) (A) and the analysis of a blank shrimp sample spiked with 10 µg/kg of CFT and penicillin V (B)

Results for repeatability, within-laboratory reproducibility, recovery rate and decision limit (CC_α) are presented in Table 2. The mean concentrations obtained for the repeatability were $9.39 \pm 0.57 \mu\text{g/kg}$, $94.23 \pm 4.36 \mu\text{g/kg}$ and $141.89 \pm 5.71 \mu\text{g/kg}$ at the fortification levels 0.1 MRL, MRL and 1.5 MRL, respectively, with a coefficient of variation of 5.95%, 4.97% and 3.64%, respectively. The average concentrations obtained from the within-laboratory reproducibility study at the fortification levels 0.1 MRL, MRL and 1.5 MRL were 9.62, 95.4 and 144 $\mu\text{g/kg}$ for CFT with a coefficient of variation of 6.00%, 5.14% and 3.79%, respectively, with corresponding recoveries ranging between 88% and 107%. From the within-laboratory reproducibility study, the value of CC_α was 108 $\mu\text{g/kg}$ for CFT.

The analytical method was fully validated for shrimp muscle. For plasma and hepatopancreas, the LOD and LOQ were in the same order as those for muscle, i.e., 1.5 $\mu\text{g/L}$ and 4 $\mu\text{g/L}$ for plasma and 1.5 $\mu\text{g/kg}$ and 4 $\mu\text{g/kg}$ for hepatopancreas, respectively.

3.2. Pharmacokinetic parameters of cefotaxime in plasma and hepatopancreas of whiteleg shrimp

Non-compartment modeling was used to describe the PK of CFT in whiteleg shrimp after single oral dose administration. The results (Figure 3A, B) show large fluctuations in hepatopancreas and plasma CFT concentrations probably due to individual variations in antibiotic absorption between shrimp.

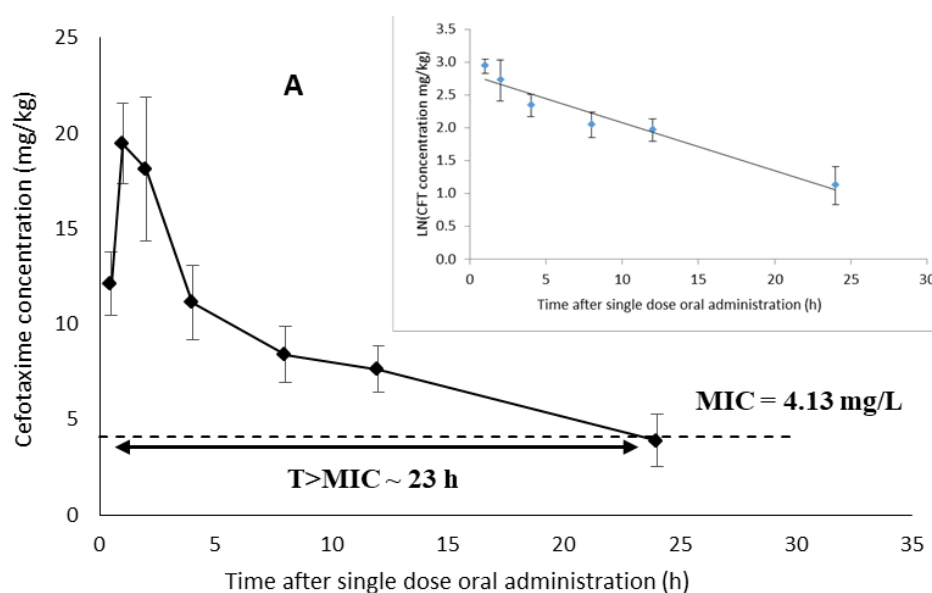
The results showed that the concentration of CFT in the hepatopancreas was much higher than that in plasma. The maximum hepatopancreas concentration (C_{max}) was 19.45 mg/kg and was obtained after 1 h, while C_{max} in plasma was 0.184 mg/L and was reached after 4 h of medication (Table 3). In addition, the $\text{AUC}_{0-24 \text{ h}}$ value in shrimp hepatopancreas was 199.64 mg.h/kg, while plasma had an $\text{AUC}_{0-24 \text{ h}}$ value of 2.15 mg.h/L, also showing that the distribution of CFT in shrimp hepatopancreas is much higher than in plasma. In addition, the elimination half-life ($T_{1/2\text{el}}$) of CFT in plasma (8.78 h) was shorter than that in the hepatopancreas (11.23 h).

Table 3. Main PK parameters of CFT in hepatopancreas and plasma after a single oral administration at a dose of 25 mg CFT/kg shrimp. Values of $C_{\max}$, $T_{\max}$ and $AUC_{0-24\text{ h}}$ are presented as mean $\pm$ standard error, $n = 6$

PK Parameter	Hepatopancreas	Plasma
$C_{\max}$ (mg/kg; mg/L)	19.45 $\pm$ 2.10	0.184 $\pm$ 0.061
$T_{\max}$ (h)	1 $\pm$ 0.20	4 $\pm$ 1.07
$AUC_{0-24\text{ h}}$ (mg.h/kg; mg.h/L)	199.64 $\pm$ 16.22	2.15 $\pm$ 0.35
k_{el} (1/h)	0.062	0.079
$T_{1/2\text{el}}$ (h)	11.23	8.78

Maximal tissue/plasma concentration ($C_{\max}$), time to maximal tissue/plasma concentration ($T_{\max}$), area under the tissue/plasma concentration–time curve from time 0 to 24 h ($AUC_{0-24\text{ h}}$), elimination rate constant (k_{el}), elimination half-life ($T_{1/2\text{el}}$).

Regarding PK/PD, based on a minimal inhibitory concentration (MIC) of CFT towards *V. parahaemolyticus* strains of 4.13 mg/L, the time during which the CFT concentration in the hepatopancreas was above the MIC was 23 h (Figure 3A).



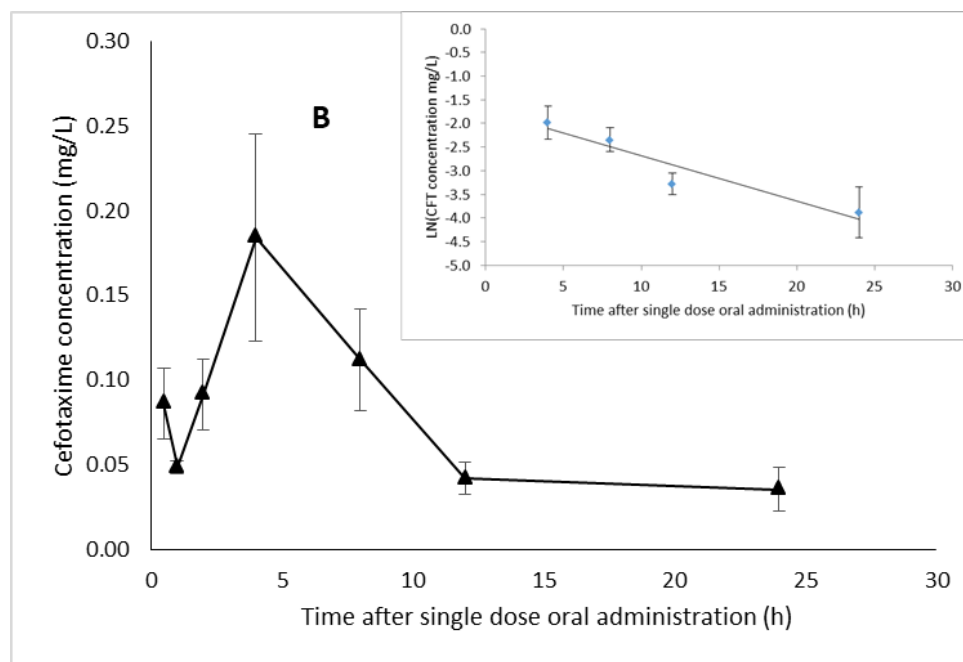


Figure 3. Concentration of CFT versus time and elimination of CFT (insert) in hepatopancreas (A) and plasma (B) of whiteleg shrimp ($n = 6$) after administration of a single oral dose (25 mg/kg shrimp); the data are expressed as mean $\pm$ standard error; MIC, minimal inhibitory concentration (dotted line); $T > \text{MIC} \sim 23$ h, time during which the concentration remains above the MIC (A)

3.3. Depletion of cefotaxime in muscle of whiteleg shrimp

The residual concentrations of CFT in whiteleg shrimp muscle measured in “once a day” and “twice a day” treatment groups are shown in Figure 4. On the first day, after 1 h of medication, the CFT concentrations in shrimp muscle were 9.75 ± 8.64 $\mu\text{g/kg}$ and 13.1 ± 4.09 $\mu\text{g/kg}$ in the “once a day” and “twice a day” treatment groups, respectively. At 3 days of medication, the CFT concentration in shrimp muscle increased and reached a peak of 23.2 ± 8.02 $\mu\text{g/kg}$ (“once a day” treatment group) and 93.52 ± 8.49 $\mu\text{g/kg}$ (“twice a day” treatment group). After the medication had been stopped for 24 h, the CFT concentration in shrimp muscle was below the LOD (1.5 $\mu\text{g/kg}$) in the treatment of feeding shrimp with medicated feed once a day and below the LOQ (4 $\mu\text{g/kg}$) in treatment of feeding shrimp with medicated feed twice a day (2.29 ± 0.80 $\mu\text{g/kg}$). Three days after medication, as well as after 7, 14 and 21 days after medication was stopped in both treatments, CFT concentration was not detected anymore in muscle.

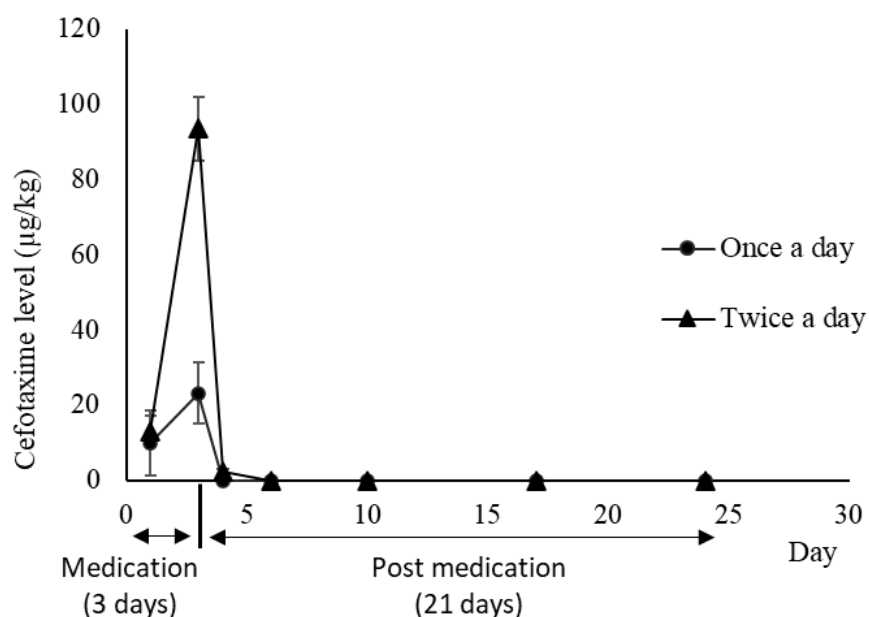


Figure 4. Depletion of CFT ($\mu\text{g/kg}$) in whiteleg shrimp muscle during and after medication at a dose of 25 mg/kg shrimp when fed with CFT once a day and twice a day, during 3 consecutive days (mean $\pm$ SD, $n = 3$)

After three consecutive medications at an ambient temperature of 29.5°C and at a dose of 25 mg/kg, the calculated withdrawal times (WTs) for an MRL of 50 $\mu\text{g/kg}$ were 8.5 degree-days (corresponding to 6.9 h) and 95.5 degree-days (77.7 h) for once- and twice-a-day medication treatments, respectively (Figure 5A and 5C). For an MRL of 100 $\mu\text{g/kg}$, they were 2.5 degree-days (2.0 h) and 74.1 degree-days (60.3 h), respectively (Figure 5B and 5D).

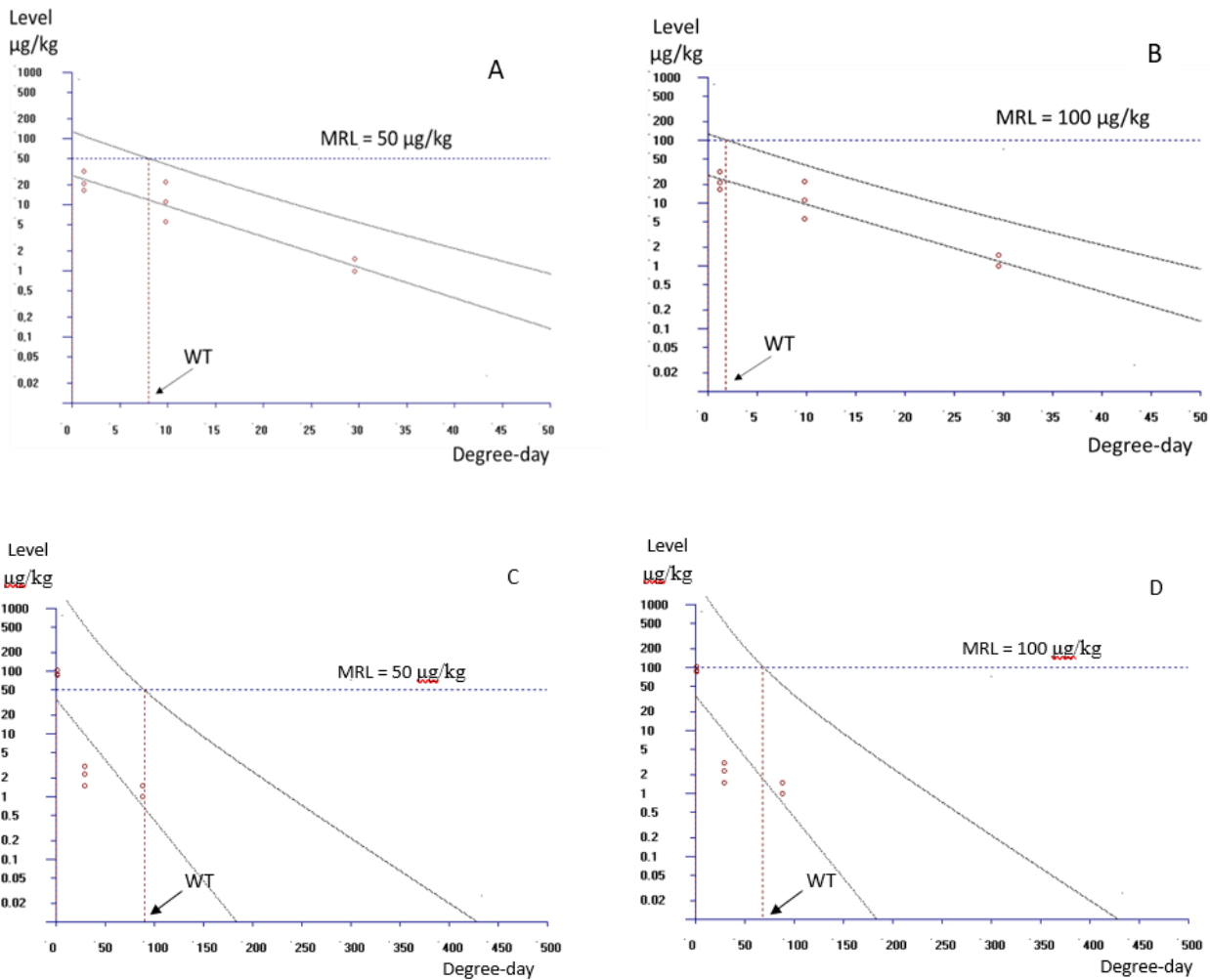


Figure 5. Plot of the CFT residual concentrations in shrimp muscle recorded at 1, 8 and 24 h for once-a-day treatment and 1, 24 and 72 h for twice-a-day treatment as a function of degree-days.

The withdrawal times (WTs) were calculated as the time when the one-sided 95% upper tolerance limit was below the MRL (50 µg/kg and 100 µg/kg) when shrimp were fed with CFT once a day (A,B) and twice a day (C,D) for 3 consecutive days at a dose of 25 mg/kg shrimp, at ambient temperature 29.5°C

3.4. Hepatopancreas histology

The hepatopancreas histology before CFT medication showed no abnormalities. The hepatopancreatic tissue structure displayed the full presence of B, R and F cells (Figure 6A).

After shrimp were fed with medicated feed once a day or twice a day for 3 consecutive days, the number of B, F and R cells remained unchanged on day 3 of medication (Figure 6B). Seven days after medication was stopped, the numbers of B, F and R cells in shrimp hepatopancreases were also normal (Figure 6C).

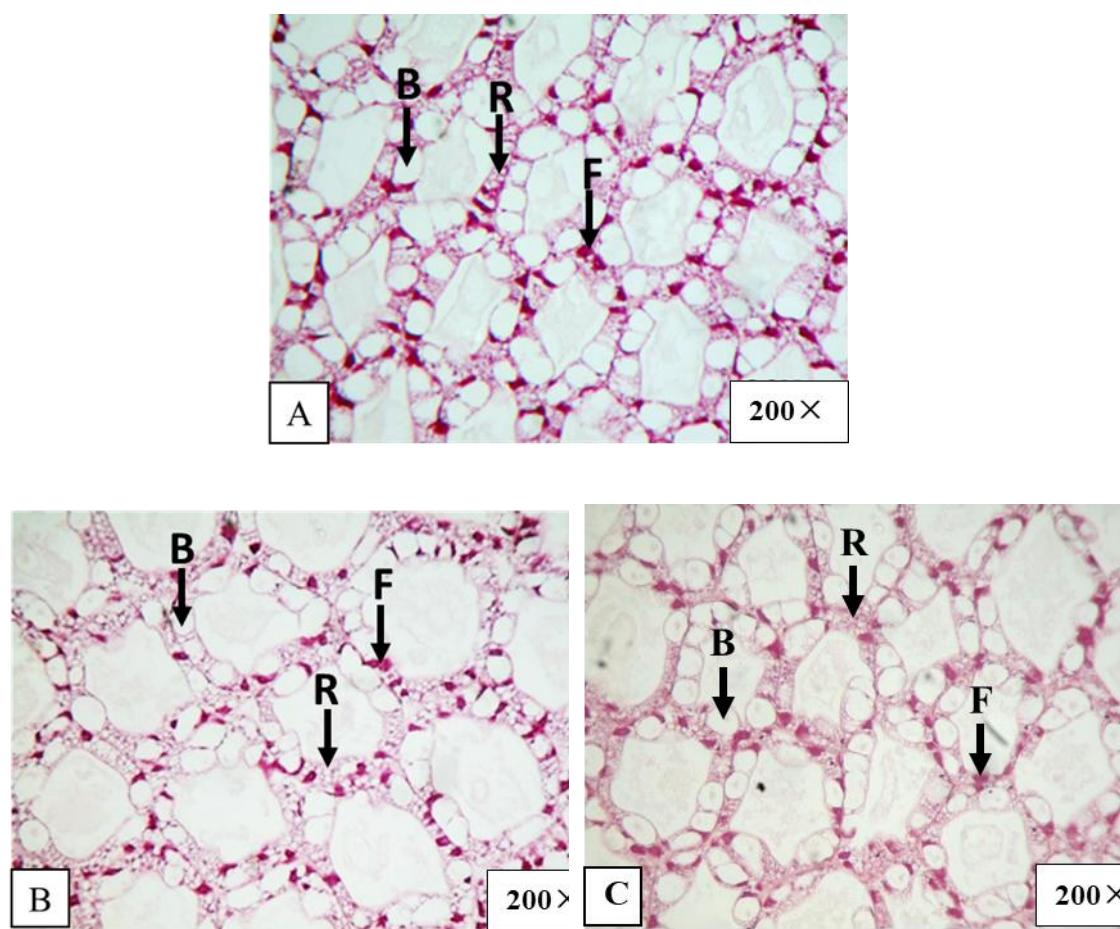


Figure 6. Histological section of shrimp hepatopancreas before (A) and after shrimp were fed with CFT -medicated feed of twice-a-day treatment on day 3 of medication (B) and 7 days after the medication was stopped (C) (200×)

4. Discussion

4.1. Method validation for cefotaxime analysis

The developed analytical method was validated for shrimp muscle. The coefficient of determination was high (R^2 above 0.99), for both matrix and solvent CFT calibration curves, indicating a linear relationship between the responses and CFT concentrations. Slope values between matrix-matched and solvent calibration were 1.65 ± 0.04 and 1.34 ± 0.01 , respectively, showing a significant matrix effect. Therefore, the method validation and the analysis of CFT in shrimp muscle were performed using calibration curves established in spiked blank shrimp muscle. This result is in agreement with the findings of previous studies on the validation of antibiotic drug analysis procedures in shrimp and aquaculture samples (Fedorova et al., 2014; Lopes et al., 2012; Saxena et al., 2018).

The repeatability and within-laboratory reproducibility (expressed as coefficients of variation on results obtained on the same day and on different days, respectively) at three fortified levels did not exceed 6% and were both much lower than the threshold set by Commission Decision No. 2021/808/EC (European Commission, 2021) of 22%, respectively. The trueness, assessed through recovery at three different levels, ranged between 88% and 107% at the fortification levels of 0.1 MRL, MRL and 1.5 MRL, which meets the tolerance range for trueness of 80% to 120% of Commission Decision No. 2021/808/EC (European Commission, 2021). The decision limit (CC_{α}) was 108 $\mu\text{g/kg}$, close to the corresponding MRL of 100 $\mu\text{g/kg}$. The parameters of selectivity/specificity and matrix effect were below the threshold set by Commission Decision No. 2021/808/EC (European Commission, 2021).

4.2. Cefotaxime pharmacokinetics in whiteleg shrimp

The results of the PK investigation showed that the concentrations of CFT in the hepatopancreas were very high, indicating a preferential distribution of CFT in this organ. The PK data of CFT in aquatic animals are very limited, but CFT PK has been studied extensively for some terrestrial animals. In this study, CFT concentration in shrimp plasma showed a longer elimination half-life ($T_{1/2\text{el}} = 8.78 \text{ h}$) compared with other animals, i.e., in buffalo calves ($T_{1/2\text{el}} = 0.94 \text{ h}$) (Sharma et al., 2005) or goats ($T_{1/2\text{el}} = 1.03 \text{ h}$) (Dutta et al., 2019). However, the elimination of CFT in this study was faster than that in Arabian camels (15.46 h) (Altayban et al., 2020). In addition, the C_{max} value of CFT in shrimp plasma (0.18 $\mu\text{g/mL}$) was much lower than that reported in some fish species when using antibiotics of the same group, i.e., cephalosporins. For example, the C_{max} values of cephalosporins in dogfish treated with a dose of 6.6 mg/kg body weight (Fayette et al., 2019), in tilapia treated with 5 mg/kg body weight (Khalil et al., 2016), in olive flounder treated with 10 mg/kg body weight and in olive flounder treated with 20 mg/kg body weight (Seo et al., 2021) have been reported to be 3.75, 12.32, 7.61 and 13.34 $\mu\text{g/mL}$, respectively. Regarding the T_{max} value, previous studies found that it strongly varies between species. For example, in whiteleg shrimp (in this study), the T_{max} was 4 h; this value is shorter than that of dogfish (96 h), but it was longer in comparison with tilapia (0.74 h) and olive flounder (0.42 h for 10 mg/kg dose and 0.67 h for 20 mg/kg dose). The difference may be due to the difference in the circulatory systems of these species, i.e., in animals with an open circulatory system, all the internal organs and structures are constantly bathed in hemolymph (Fang et al., 2018). In addition, the absorption phase and effective distribution are rapid, and both have a close relationship with an open circulation system (Wu et al., 2006). In fish that have a closed circulatory system, blood from the gills goes to most organs before reaching the liver (where the drug can be metabolized) (Owen et al., 2007).

The results of this study showed that the C_{max} and $\text{AUC}_{0-24 \text{ h}}$ of CFT in hepatopancreases are much higher than those in plasma. A similar result was found in the experiment of Fang et al. (2013), where the C_{max} values of thiamphenicol and FF in plasma (7.96 $\mu\text{g/mL}$ and 5.53 $\mu\text{g/mL}$) of whiteleg shrimp

(medicated at a dose of 10 mg/kg body weight) was much lower than the C_{max} values in the shrimp hepatopancreas (204 $\mu\text{g/kg}$ and 164 $\mu\text{g/kg}$, respectively). Fang et al. (2018), who treated whiteleg shrimp by intramuscular injection of enrofloxacin at a dose of 10 mg/kg body weight, showed the same pattern in AUC values. These results indicate that there is an accumulation of CFT in the whiteleg shrimp hepatopancreas. The $T_{1/2el}$ value is an important PK parameter that describes the rate and duration of antibiotic elimination from the body (Fang et al., 2013). Notably, the $T_{1/2el}$ value of CFT observed in this study was similar to that of sulfamethoxazole and trimethoprim in whiteleg shrimp after single-dose and multiple-dose oral administration (Ma et al., 2017).

Cefotaxime exhibits time-dependent bactericidal activity, and the time during which the tissue concentration is above the MIC ($T > \text{MIC}$) has been reported as an appropriate PK/PD index (Craig, 1995; Gustafsson et al., 2001). Craig (1995) also stated that cephalosporins including CFT show maximal effectiveness in several animal pathogens when the time of tissue concentration is above the MIC for 60 to 70% of the dosing interval. In addition, Gustafsson et al. (2001) reported that the average maximal antimicrobial effect was achieved when the concentration of CFT exceeded the MIC for 50% of the dosing interval. In the present study, based on an MIC for CFT towards *V. parahaemolyticus* strains of 4.13 mg/L, the PK/PD characteristics of CFT in the shrimp hepatopancreas toward this bacterium resulted in a $T > \text{MIC}$ of approximately 23 h (Figure 3A). Generally, in shrimp farming, farmers apply antibiotics once a day during medication, suggesting a dosing interval of 24 h corresponding to approximately 95% of $T > \text{MIC}$. Hence, this illustrates the therapeutic efficiency of CFT in treating AHPND caused by *V. parahaemolyticus* in whiteleg shrimp. Generally, CFT must be used prudently as CFT is a third-generation cephalosporin which is not a first-line antibiotic. It is considered a critically important antibiotic, so it should be used only when necessary and must be combined with PK information to optimize therapeutic effectiveness while minimizing unnecessary antibiotic exposure and the risk of antimicrobial resistance development. In addition, appropriate withdrawal periods should be respected to ensure residue compliance and food safety of shrimp products. The results of this study illustrate that the dose which is proposed here is expected to be efficient in *V. parahaemolyticus* treatment in shrimp disease.

4.3. Cefotaxime depletion in whiteleg shrimp

The withdrawal time is the time required to eliminate drug residues in edible tissues to a safe level for consumers (Avunje et al., 2021). MRLs for CFT have not been established in European legislation or elsewhere. In this study, both 50 $\mu\text{g/kg}$ and 100 $\mu\text{g/kg}$ were assumed as the MRL of CFT in edible shrimp muscle. On one hand, the MRL of 50 $\mu\text{g/kg}$ of amoxicillin was chosen because both antibiotics are similar in terms of rapid elimination from muscle. On another hand, the MRL of 100 $\mu\text{g/kg}$ of

oxytetracycline was selected because CFT and oxytetracycline have relatively equal acute toxicity in mice (approximately 7000 mg/kg as LD₅₀ value) (Berezhinskaia et al., 1990; US EPA, 2012).

In the current research, after 3 days of CFT medication at the dose of 25 mg/kg, the CFT WTs were lower than those of other groups of antibiotics. For example, the withdrawal time of oxytetracycline in whiteleg shrimp after oral administration at the dose of 4.5 g/kg feed for 14 consecutive days was 96 h at 29°C (corresponding to 116 degree-days), for the MRL of 100 µg/kg set by the EU, Canada and India (Avunje et al., 2021); in another study of Wang et al. (2004), the WTs in shrimp muscle of chloramphenicol, sulfamethoxazole and oxytetracycline at the dose of 2 g/kg feed after twice-a-day oral administration for 3 consecutive days were 139.7 h (139.7 degree-days), 30.6 h (30.6 degree-days) and 90.3 h (90.3 degree-days) at 24°C, respectively.

4.4. The effect of cefotaxime on whiteleg shrimp hepatopancreas histology

Shrimp hepatopancreases are composed of numerous hepatopancreas tubules. The tubule walls are constituted of a simple epithelium, which contains four cell types including blasenzellen cells (B cells), embryonic cells (E cells), fibrillenzellen cells (F cells) and restzellen cells (R cells). The B, F and R cells are differentiated from E cells which are located at the narrow distal end of the tubule. Young R cells and F cells are located at a short distance away from the distal region, and B cells are at the middle and proximal regions of the tubules (Pathak et al., 2018). In this study, there was no negative impact of CFT on the B, F and R cells of shrimp hepatopancreases, contrary to what was observed for other antibiotics. Maftuch et al. (2017) reported that hepatopancreatic cells were destroyed through shrinkage of microvilli, atrophy, inflammatory cell infiltration and plasma leakage from blood vessels when whiteleg shrimp were fed with enrofloxacin at a dose of 200 mg/kg body weight. Likewise, the hepatopancreatic cells of whiteleg shrimp exhibited degeneration and necrosis when oxytetracycline was used at doses of 9 and 18 g/kg feed (Avunje et al., 2021). Similarly, when feeding whiteleg shrimp at 13.5 and 22.5 g oxytetracycline/kg feed, Bray et al. (2006) reported reduced levels of lipid droplet storage in the hepatopancreas R cells, some necrosis and sloughing of the hepatopancreas tubule epithelium, the general absence of hepatopancreas lipids, and moderate atrophy of the hepatopancreas tubules in the proximal portion, and at the higher dose, tubular atrophy in the hepatopancreatic cells was observed until 42 days; this research showed that the higher the dose of antibiotics used, the more severe the impact on the shrimp hepatopancreas. Besides the adverse effect of antibiotics, a decrease in the number of B cells in shrimp hepatopancreas was also found when feeding shrimp with other chemicals like an organic acid blend that consisted of formic, lactic, malic and citric acids (Romano et al., 2015). These findings suggest that CFT administration at the investigated dose did not induce detectable hepatopancreatic damage in whiteleg shrimp under the experimental conditions applied in

this study. Nevertheless, further investigations on repeated treatments or higher doses would be useful to better evaluate the long-term biological safety of CFT use in shrimp aquaculture.

5. Conclusions

A single-dose oral administration of 25 mg/kg in whiteleg shrimp revealed quick absorption and distribution. The concentrations of CFT were much higher in the shrimp hepatopancreas than in plasma, indicating that the antibiotic can be sufficient in *V. parahaemolyticus* infection control during shrimp culture. After oral administration of CFT at a dose of 25 mg/kg, the withdrawal time ranged from approximately 6.9 h to 78 h (at 29.5°C water temperature), depending on the dosing regimen (once or twice daily administration), based on a MRL of 50 µg/kg, ensuring consumer food safety. No negative effect was found in hepatopancreas tissue at the given dose. However, as a limitation, this study did not test the effectiveness of feeding shrimp with CFT in shrimp with *Vibrio* spp. infection challenge. Moreover, the withdrawal time of CFT should be determined in field practice.

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3.3 RESIDUES AND RISK ASSESSMENT OF ANTIBIOTICS IN COMMERCIAL WHITELEG SHRIMP (*PENAEUS VANNAMEI*) IN CAN THO CITY, VIETNAM

The previous studies in this work showed that antibiotics are widely used in whiteleg shrimp farming in the Mekong Delta, and several commonly used compounds were identified through the survey. The experimental studies also provided information on the PK and residue depletion of selected antibiotics in shrimp. Although withdrawal times were established under experimental conditions, the actual occurrence of antibiotic residues in commercial shrimp products has not been fully evaluated. In addition, import markets still report cases of non-compliance with international regulations. Taking these aspects into account, the presence of antibiotic residues in shrimp products available on the local market remains a concern, especially in relation to food safety and consumer exposure. Therefore, it is important to evaluate the occurrence of antibiotic residues in commercial shrimp at local market and to assess the potential risk for consumers. In the present study, antibiotic residues were measured in whiteleg shrimp samples collected from supermarkets and local markets in Can Tho city, Vietnam. The dietary exposure of consumers to the detected antibiotic residues was also estimated based on shrimp consumption data.

RESIDUES AND RISK ASSESSMENT OF ANTIBIOTICS IN COMMERCIAL WHITELEG SHRIMP (*PENAEUS VANNAMEI*) IN CAN THO CITY, VIETNAM

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Abstract

The aim of this study was to evaluate the risk, for consumer health of the presence of antibiotic residues in commercial whiteleg shrimp in Can Tho City, Vietnam. Thirty whiteleg shrimp samples were collected from local markets and supermarkets for screening by Premi®Test and Beta-star®, while quantification was performed by Enzyme-Linked Immunosorbent Assay (ELISA) and Liquid Chromatography–tandem Mass Spectrometry (LC-MS/MS). Five antibiotics were detected by LC-MS/MS, with the levels in the order DOX (52.87 µg/kg), followed by oxytetracycline (OTC, 31.5 µg/kg), enrofloxacin (ENR, 8.02 µg/kg), ciprofloxacin (CIP, 1.47 µg/kg), and sulfamethoxazole (SMZ, 1.04 µg/kg). The estimated daily intakes (EDI; µg/kg body weight/day) were then calculated based on local shrimp consumption data. The calculated EDI values were compared with the acceptable daily intake values to assess the risk. Based on median and P95 shrimp consumption data (125 and 250 g per person per day, respectively, n=191), the maximum antibiotic residue concentrations detected in shrimp and the mean body weight of the interviewed participants, the EDI values ranged from 0.001 to 0.120 µg/kg body weight/day for median consumption and from 0.002 to 0.241 µg/kg body weight/day for P95 consumption. All EDI values were substantially lower than their respective acceptable daily intake values, indicating negligible health risks associated with the consumption of whiteleg shrimp among the surveyed population.

Keywords: antibiotic residues, consumption, EDI, exposure, whiteleg shrimp.

1. Introduction

Aquaculture is an essential industrial sector that provides jobs, livelihoods, food and nutrition for millions of people around the world. Vietnam's shrimp industry has seen positive growth in both production and export between 2018 and 2024. The shrimp farming area expanded at an average annual rate of 5%, while the production gradually increased by 8.4% per year, largely due to advancements in whiteleg shrimp culture productivity. Whiteleg shrimp production consistently increased, showing an 82% growth over 6 years, with an average annual rise of 13%. Conversely, black tiger shrimp production experienced a slight decline, averaging a 1.5% decrease per year, resulting in an 8% drop after 6 years, with growth remaining relatively stagnant compared to whiteleg shrimp (VASEP, 2025).

Vietnam has many advantages in developing the shrimp industry, which enabled shrimp production to play an important role in Vietnam's seafood exports during the past two decades. Unfortunately, the Vietnamese shrimp sector has faced major problems since the introduction of the intensive and super intensive farming systems, due to increased stocking density and weather- and water-related environmental problems, disease outbreaks have frequently occurred (Nguyen et al., 2020). The shrimp aquaculture industry has faced many serious diseases due to pathogens such as viruses, bacteria, parasites, water quality parameters, or some unidentified pathogens around the world. Among these causes, viral and bacterial diseases are dominant, e.g., acute hepatopancreatic necrosis disease (AHPND) and white spot disease (WSD) are common diseases (Tran et al., 2013; Flegel & Sritunyaluucksana, 2018; Thinh et al., 2020). To control diseases and to mitigate these issues, antibiotics have been widely used for disease prevention and treatment in shrimp culture (Rico et al., 2014; Phu et al., 2016; Chi et al., 2017). Tai (2012) reported that more than 30 types of antibiotics were used in shrimp culture in 2004. In recent years, the trend of antibiotic use has been decreasing, shrimp farmers in Northern Vietnam mainly used oxytetracycline for shrimp disease management (Chi et al., 2017). While antibiotics such as amoxicillin, cotrim (a combination product of sulfamethoxazole and trimethoprim), and oxytetracycline have been commonly used by shrimp farmers in the South of Vietnam (Phu et al., 2019; Thinh et al., 2020). These studies collectively suggest that antibiotic use patterns in Vietnamese shrimp aquaculture vary among farming regions and production systems, although tetracycline-group antibiotics remain among the most commonly applied compounds. The differences observed between Northern and Southern Vietnam may reflect variations in disease occurrence, farming intensity, product availability, and local management practices. In addition, the continued use of multiple antibiotic classes indicates that disease prevention and treatment in shrimp aquaculture still rely considerably on antimicrobial intervention.

Although this practice may offer short-term benefits, the misuse or overuse of antibiotics poses significant risk to consumer health and environment. Antibiotic residues in shrimp products can persist until harvest if appropriate withdrawal periods are not followed. These residues, if present in commercial shrimp, may lead to adverse health effects for consumers, and contribute to antimicrobial

resistance concerns (Le et al., 2005; Lulijwa et al., 2019). Regulatory authorities in many countries have established maximum residue limits (MRLs) for antibiotics in seafood to ensure consumer safety. Specifically, the Rapid Alert System for Food and Feed (RASFF) from the EU reported that shrimp products from several regions in Vietnam occasionally contained antibiotic residues exceeding permitted levels, e.g., from 2020 to 2024, the presence of residues of oxytetracycline, DOX, tetracycline and sulfamethoxazole in shrimp frozen products was reported (RASFF, 2025). Given the importance of shrimp in the Vietnamese diet and the potential health impacts of antibiotic residues, monitoring antibiotic residues in commercial shrimp products is essential to assess compliance with food safety standards and the risk of potential exposure of consumers. The findings will provide scientific evidence to support more rational antibiotic use practices in shrimp culture and to enhance food safety control for shrimp products in Vietnam to protect consumer health.

Risk assessment or food safety assessment is a scientific approach used to estimate and evaluate the potential harm that chemicals in food may pose to a particular organism, population, or ecosystem (Benford, 2013). Risk assessment is one component of risk analysis, together with risk management and risk communication (Teaf et al., 2004; Haimes, 2011). The process of risk assessment involves four main steps, i.e., hazard identification, hazard characterization, exposure assessment, and risk characterization. In the final step, risk characterization is conducted by comparing the estimated exposure level with toxicological reference values, most commonly the ADI. In contrast, risk management involves the development and implementation of regulatory or practical measures based on risk-assessment outcomes while also considering policy, economic, and societal aspects. For example, once the ADI is established through scientific assessment, risk management may use this information to establish MRLs for veterinary drugs in food products (Haimes, 2011).

2. Material and method

2.1 Reagents

For screening analysis, the Premi-Test Starter Kit (Art. No. ZPT-2000, R-Biopharm AG, Darmstadt, Germany) and the Beta-Star® test kit (Beijing, China) were utilized.

For ELISA test, the Fluoroquinolones Elisa (Generic test, 5101FLUQG) purchased from Arnhem Netherlands, and RIDASCREEN® Tetracyclin, Art. No. R3505 (Darmstadt, Germany).

For LC-MS/MS analysis, high purity chemicals (> 98%) including DOX, oxytetracycline (OTC), enrofloxacin (ENR), ciprofloxacin (CIP), sulfamethoxazole (SMX) standards were purchased from Dr. Ehrenstorfer® (Augsburg, Germany). The instrument used for antibiotic quantification was a Waters ACQUITY Ultra-High-Performance Liquid Chromatograph (UHPLC) (Waters, Milford, MA, USA) with an Acquity UPLC CSH C18 column, 2.1 mm × 50 mm, 1.7 µm (Waters, Milford, MA, USA). C18 Bondesil powder with a particle size of 40 µm was supplied by Agilent (Santa Clara, CA, USA). Other

chemicals like acetonitrile, methanol and distilled water were obtained from Merck (Darmstadt, Germany).

2.2 Sample collection

Whiteleg shrimp were selected for the screening of antibiotic residues from five local markets and five supermarkets in Can Tho City in 2023. Three different periods of sampling were chosen with a one-month interval. For each type of market/supermarket, three samples were collected (corresponding to three different times). The sampling strategy was designed to reflect potential consumer exposure to antibiotic residues through shrimp consumption in Can Tho City. Samples were collected from five local markets and five major supermarkets, which represent the principal retail sources of shrimp products available to consumers. Three sampling campaigns were conducted at one-month intervals, and one shrimp sample was collected from each market and supermarket during each sampling period, resulting in a total of 30 samples. This approach was intended to account for both spatial variation among retail outlets and temporal variation in marketed shrimp products. Shrimp muscle without shells was minced and kept in a -20°C freezer until analysis. Firstly, all shrimp muscle samples were screened to detect the residues of antibiotics by Premi-test (for all antibiotic group) and Beta-star (for beta lactam group). After screening, samples with positive results were subjected to ELISA and LC-MS/MS analysis for residue quantification.

2.3 Antibiotic Residue Screening

2.3.1 Sample extraction

To extract shrimp juice for qualitative antibiotic residue screening, approximately 10–20 grams of shrimp muscle were placed into a zip-lock bag, which was then sealed and placed into a second zip-lock bag. The double-bagged samples were incubated in a water bath at 64°C for 15 minutes. After incubation, the shrimp juice released during the heating process was collected for further analysis.

2.3.2 Premi®Test procedure

For the Premi®Test (Premi-Test Starter Kit, Art. No. ZPT-2000), 100 μL of the collected shrimp juice was transferred onto the agar surface inside the test ampoule. The ampoules were incubated at room temperature for 20 minutes. Residual juice was then removed, and the ampoules were rinsed twice using deionized distilled water. After washing, the ampoules were sealed with the supplied perforated foil. Incubation was conducted at $64 \pm 0.5^{\circ}\text{C}$ using the Premi-Test incubator. The results were interpreted once the negative control showed a clear color transition to yellow, typically observed between 3 hours and 3.5 hours. Ampoules that remained purple were considered positive. Only the lower two-thirds of the agar medium in the ampoule were considered for result interpretation.

2.3.3 Beta-Star® kit procedure

For the Beta-Star® test kit, 100 µL of shrimp juice was mixed with 25 µL of reconstituted receptor solution in a sterile Eppendorf tube. The mixture was incubated for 3 minutes at 47.5°C. A Beta Star stick was then carefully inserted into the tube, followed by a further 2-minute incubation at 47.5°C. The result was visually interpreted: two red lines indicated a negative result, whereas one red line indicated a positive result for the presence of antibiotic residues.

2.4 Quantification of antibiotic residues

The quantification of antibiotic residues in whiteleg shrimp muscle was assessed using two analytical methods: Enzyme-Linked Immunosorbent Assay (ELISA) on 30 samples for group-specific screening and Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) on 10 representative samples for specific compound identification and quantification. The objectives were to assess the presence and levels of commonly used antibiotic groups and individual antibiotics, and to compare the efficacy and accuracy of the two methods. ELISA analysis was used to screen for two major antibiotic groups, i.e., tetracyclines and fluoroquinolones, across all 30 shrimp muscle samples. Meanwhile, ten samples that tested positive in the screening analysis were analyzed by LC-MS/MS. The antibiotics were selected for analysis based on the result from screening analysis, common antibiotics used in shrimp culture and antibiotic excretion capacity. Five types of antibiotics were selected for analysis, i.e., DOX, oxytetracycline (OTC), enrofloxacin (ENR), ciprofloxacin (CIP) and sulfamethoxazole (SMZ).

2.4.1 Fluoroquinolone group analysis by ELISA

Sample extraction. Approximately 10 g of sample was homogenized, and 1 g of the homogenate was transferred into a test tube. Three milliliters of 80% methanol in sample dilution buffer were added, followed by mixing for 15 minutes. The mixture was then centrifuged at $2000 \times g$ for 10 minutes, and 2 mL of the supernatant was transferred to a glass tube. The supernatant was evaporated under a mild stream of nitrogen at 50°C, and the residue was reconstituted with 1 mL of 8% methanol in sample dilution buffer. After vortexing for 1 minute, the solution was centrifuged at $2000 \times g$ for 15 minutes, and 50 µL of the lower layer was used for the ELISA test.

Analysis procedure. The ELISA kit (Fluoroquinolones Elisa (Generic test) 5101FLUQG) was used for fluoroquinolone group quantification. Standard solutions of norfloxacin (0.157–5 µg/L) or sample extracts (50 µL) were pipetted in duplicate into the 96-well plate. Subsequently, 25 µL of conjugate (norfloxacin-HRP) and antibody solution (25 µL) were added, and the plate was incubated for 1 h in the dark at 4°C. After washing three times with rinsing buffer, substrate/chromogen solution (100 µL) was added and the plate was incubated at room temperature (20–25°C) for 30 minutes. The reaction was stopped with 100 µL of stop solution, and absorbance was measured immediately at 450 nm.

2.4.2 Tetracycline group analysis by ELISA

Sample extraction. Shrimp samples were first homogenized, and 1 g of the homogenate was transferred into a centrifugal screw-cap vial. Each sample was mixed with 9 mL of 20 mM PBS buffer (0.55 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ + 2.85 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ + 9 g NaCl, pH 7.4) and vortexed thoroughly. The mixture was then shaken for 10 minutes to facilitate extraction, followed by centrifugation at $4000 \times g$ for 10 minutes at room temperature (20–25°C). Finally, 50 μL of the upper aqueous phase was collected for use in the assay.

Analysis procedure. An indirect competitive ELISA (RIDASCREEN® Tetracyclin, Art. No. R3505) was carried out for tetracycline group quantification. Briefly, standard solutions of tetracycline (0–18 $\mu\text{g/L}$) or sample extracts (50 μL) were pipetted into the 96-well plate, and then 50 μL of anti-tetracycline antibody was added to each well and incubated for 1 h at room temperature (20–25°C). After discarding and washing three times with 250 μL of diluted wash buffer, 100 μL of conjugate was added and incubated for 15 minutes. Wells were again washed three times before adding 100 μL of substrate/chromogen and incubating for 15 minutes in the dark. Finally, 100 μL of stop solution was added, and absorbance was measured at 450 nm within 30 minutes.

2.4.3 Quantitative analysis by LC-MS/MS

The extraction and quantification of DOX and OTC from whiteleg shrimp muscle were performed using a modified method of Vinh et al. (2024). In brief, a 3.0 ± 0.1 g portion of the homogenized sample was transferred into a 50 mL tube, fortified with 60 μL of internal standard (penicillin V, 1000 ng/mL), and left at room temperature for 15 minutes. The extraction process involved adding 1 mL of 0.1 M phosphate buffer at a pH of 8.0, vortexing for 1 minute, and then 20 mL of acetonitrile was introduced, followed by vortexing for 30 sec and sonication for 15 minutes in an Elma Ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany). To collect the supernatant, the tubes were then centrifuged at $3501 \times g$ for 5 minutes. The extract volume was adjusted with acetonitrile to a final volume of 24 mL, and 8 mL was transferred into a 15 mL glass test tube containing 40 mg of Bondesil C18. After centrifugation again at $3501 \times g$ for 4 minutes, the supernatant was collected in a 10 mL glass test tube and evaporated at $40 \pm 1^\circ\text{C}$ in a water bath under nitrogen flow. The dried residues were reconstituted in 1 mL of mobile phase before inject into the LC–MS/MS system.

Quinolone (ENR and CIP) and SMZ were quantified by LC-MS/MS method which was described by Sun et al. (2012). Briefly, 2.00 ± 0.05 g of each sample homogenized was placed into 50 mL centrifuge tubes. Samples were then fortified with 200 μL of antibiotic standard to obtain a concentration of 10 ng/g (sulfanilamides) and 1 ng/g (quinolones). Four mL of water were added, and samples were vortexed for 1 minute. A 10 mL solution of 1% acetic acid in acetonitrile were added to each tube. Tubes were capped and vortexed for 1 minute. The extraction salts (4 g Na_2SO_4 , 1 g NaCl) were added to each tube. Sample tubes were capped tightly and vigorously shaken for 1 minute. Tubes were centrifuged at

5,000 rpm for 5 minutes at 4°C, then allowed to stand for 30 minutes. Dispersive-SPE: A 6 mL aliquot of the upper acetonitrile layer was transferred into a 15 mL tube, which contained 50 mg of PSA, 150 mg of C18EC and 900 mg of anhydrous Na₂SO₄. The tubes were tightly capped and vortexed for 1 minute and then centrifuged at 5,000 rpm for 5 minutes. A 4 mL aliquot of the upper acetonitrile layer was transferred into another tube and dried by N₂ flow at 40°C. Samples were reconstituted into 1 mL of 2:8 acetonitrile/H₂O, and then centrifuged at 10,000 rpm for 10 minutes. The upper layer was transferred to an autosampler vial, and inject into LC-MS/MS system.

2.5 Consumption study

A total of 200 adult consumers from Can Tho City were interviewed to collect information about dietary habits, particularly focusing on the consumption of aquatic products (including whiteleg shrimp) and other common sources of protein. The survey targeted shrimp consumers in Can Tho City. Participants were recruited from local markets and supermarkets, which represent the principal retail locations where shrimp products are commonly purchased by consumers. Based on the sample-size estimation formula of Yamane (1967), previously described in the Methodology section of the manuscript “The use of drugs and chemicals in super intensive whiteleg shrimp aquaculture in the Mekong Delta, Vietnam”, the estimated sample size ranged from approximately 100 to 400 participants according to the population size and accepted margins of error. Therefore, 200 participants were considered an appropriate sample size to capture general shrimp-consumption patterns among consumers in the surveyed area. Adult respondents were selected because they were responsible for food purchasing and household meal preparation and were therefore able to provide reliable information regarding household shrimp consumption patterns. Household shrimp consumption was converted to an estimated shrimp consumption per person by dividing the total amount of shrimp consumed by the number of household members aged 5–70 years who consumed shrimp, excluding children under 5 years of age, elderly individuals over 70 years of age, and persons with shrimp allergy.

Estimated daily antibiotic intake (EDI, µg antibiotic per kg body weight per day) was calculated by multiplying the residue concentration of a compound by the daily estimated amount of food intake and dividing the result by the average body weight (Juan et al., 2010). According to Kumari et al. (2020) and Bishnoi et al. (2025), the risk was assessed by calculating the Hazard Quotient (HQ). An HQ value higher than or equal to 1 suggests that the particular compound has the potential to cause adverse effects on human health, while a value lower than 1 indicates negligible effects and therefore does not pose a significant risk. The formula is as follows:

$$HQ = \frac{\text{Estimated daily intake (EDI)}}{\text{Acceptable daily intake (ADI)}}$$

where, EDI was calculated using the formula:

$$EDI = \frac{(\text{mg of antibiotic per kg of food}) \times (\text{Daily intake of food})}{\text{Body weight (kg)}}$$

This method allowed for an estimation of potential exposure to antibiotic residues through regular dietary practices.

3. Results

3.1 Screening analysis of antibiotic residues

The screening analysis of antibiotic residues in shrimp muscle samples were performed using Premi®Test and Beta-star® kits. The results for each test are presented below, along with observations from the control samples, as detailed in Table 1. The Premi®Test detected positive signals in eight samples (26.7%, n=30), while twenty-two samples (73.3%, n=30) exhibited negative results with this test. Remarkably, the Beta star test returned negative results for all thirty (100%) shrimp muscle samples screened, suggesting that β -lactam residues were absent or below the detection limit in all tested samples.

Table 1. Positive ratio according to market (%) after screening analysis of antibiotic residues in shrimp muscle

Market	Premi test	Beta star
Supermarket (n=15)	20.0	0
Local Market (n=15)	33.3	0
Overall (n=30)	26.7	0

3.2 Quantification of antibiotic residues in whiteleg shrimp muscle by ELISA and LC-MS/MS

For the tetracycline group, there were 13 positive samples, with the highest concentration of 1.84 $\mu\text{g/kg}$ observed in a supermarket sample (4.1). In the fluoroquinolone group, 16 samples were detected with concentrations ranging from 0.30 to 7.33 $\mu\text{g/kg}$. The highest concentration was determined in supermarket sample 5.2. However, many samples showed concentrations below the quantification limit (<LOQ) in both antibiotic groups.

The result presented that antibiotic residues in 5/10 samples were lower than LOD for all five antibiotics (Table 2). Doxycycline was the most prevalent compound, reaching 52.87 $\mu\text{g/kg}$, followed by OTC, ENR, CIP, and SMZ. OTC was notably detected at a high concentration of 31.50 $\mu\text{g/kg}$ in a sample

collected from a supermarket, another supermarket sample contained multiple residues, including DOX, ENR, CIP and SMZ (Table 2).

Table 2. Antibiotic residues ($\mu\text{g/kg}$) of target antibiotics in shrimp sample collected from supermarket and market through ELISA and LC-MS/MS

Sample code	ELISA ($\mu\text{g/kg}$)		LC-MS/MS ($\mu\text{g/kg}$)				
	Tetracycline group	Fluoroquinolone group	DOX	OTC	ENR	CIP	SMZ
1.1	1.58	<LOQ	<LOD	31.50	<LOD	<LOD	<LOD
1.2	1.19	0.59	<LOD	<LOD	<LOD	<LOD	<LOD
1.3	0.79	0.62					
2.1	1.76	0.61					
2.2	0.55	<LOQ					
2.3	0.98	<LOQ	23.36	2.28	<LOD	<LOD	<LOD
3.1	<LOQ	<LOQ					
3.2	<LOQ	<LOQ					
3.3	0.62	2.14					
4.1	1.84	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
4.2	1.24	<LOQ					
4.3	0.57	0.88					
5.1	<LOQ	2.27					
5.2	1.59	7.33	52.87	<LOD	8.02	1.47	1.04
5.3	<LOQ	<LOQ					

Sample code	ELISA (µg/kg)		LC-MS/MS (µg/kg)				
	Tetracycline group	Fluoroquinolone group	DOX	OTC	ENR	CIP	SMZ
6.1	1.06	0.80					
6.2	<LOQ	0.30	<LOD	<LOD	0.81	0.42	<LOD
6.3	<LOQ	0.30					
7.1	<LOQ	0.38					
7.2	<LOQ	0.32					
7.3	0.71	<LOQ	<LOD	13.70	<LOD	<LOD	<LOD
8.1	<LOQ	0.30					
8.2	<LOQ	<LOQ					
8.3	<LOQ	<LOQ					
9.1	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
9.2	<LOQ	2.36	<LOD	<LOD	<LOD	<LOD	<LOD
9.3	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
10.1	<LOQ	<LOQ					
10.2	<LOQ	0.42					
10.3	<LOQ	0.45					

Sample codes from 1.1 to 5.3 represent samples collected from supermarkets, while sample codes from 6.1 to 10.3 represent samples collected from local markets. DOX- doxycycline, OTC-oxytetracycline, ENR-enrofloxacin, CIP-ciprofloxacin, SMZ- sulfamethoxazole, LOQ-limit of quantification, LOD-limit of detection.

3.3 Exposure assessment of the consumer to chemical residues through whiteleg shrimp product consumption

3.3.1 General information, knowledge and attitude of surveyed peoples about the risk

The result provided detail an exposure assessment of consumers in Can Tho City, Vietnam to chemical residues, particularly antibiotics, found in whiteleg shrimp products. This assessment first gathered general information and dietary habits from a population of 200 surveyed consumers, noting that most participants were female (92.5%), whereas males accounted for only 7.5% (Table 3). This high proportion of female participants may be explained by the fact that women are more commonly responsible for purchasing food products at local markets and supermarkets in Vietnam. In addition, the consumption assessment was based on household shrimp consumption and the total number of household members, with consumption values expressed per person per day.

The surveyed population had a mean age of 39.7 ± 9.9 years and a mean body weight of 54.9 ± 8.0 kg. Regarding knowledge and attitude towards potential risk, 68% of individuals recognized the risks associated with antibiotic residues in aquaculture products, while 32% reported no awareness. In terms of dietary habits, whiteleg shrimp product was highly favored, with 88% of respondents indicating that they liked to consume shrimp (Table 3). The survey information demonstrated that shrimp is an important protein source for consumers. This may result in health problems if shrimp or shrimp products are not safe in term of contaminated with antibiotics or chemicals.

Table 3. General information and shrimp consumption habits of the surveyed consumers

Item	Value	
	1	2
Age (1, mean $\pm$ SD)	39.7 ± 9.9	
Body weight (kg) (1, mean $\pm$ SD)	54.9 ± 8.0	
Gender (1, % male, 2, % female)	7.5	92.5
Aware about the risk of antibiotic residues (1, % known about risk; 2, not known about risk)	68	32
Perspective on shrimp eating (1, like to eat (%); 2, do not like to eat (%))	88	12

3.3.2 Exposure assessment to antibiotic through whiteleg shrimp consumption

About the amount of shrimp consumed by people living in Can Tho, the consumption survey indicated that 95.5% of respondents regularly consumed whiteleg shrimp. Therefore, the consumption assessment was based on 191 respondents who reported consuming whiteleg shrimp. The median daily consumption of whiteleg shrimp was 125 g/person/day. The maximum and minimum reported consumptions were 375 g and 12.5 g/person/day, respectively, while the P95 consumption level reached 250 g/person/day (Table 4).

Table 4. Percentage of customers consuming whiteleg shrimp in their meals (n=191), and amount of whiteleg shrimp consumed daily

	Values
Percentage of consumer %	95.5
Median (g/person/day)	125
Max (g/person/day)	375
Min (g/person/day)	12.5
P95 (g/person/day)	250

Based on these consumption levels, the estimated daily intake (EDI) of each antibiotic was calculated using the maximum residue concentrations detected in the supermarket and market groups (Table 2), the average body weight of 54.9 kg (Table 3), and the median (125 g/person/day) and P95 (250 g/person/day) shrimp consumption levels (Table 4). The calculated EDIs were subsequently compared with their corresponding ADI values (Table 5). The ADI values applied in this study were 3 µg/kg bw/day for DOX and oxytetracycline (EMA, 2015), and 2 µg/kg bw/day for both enrofloxacin and ciprofloxacin (JECFA, 1998, cited in Ji et al., 2010). The ADI for sulfamethoxazole was 50 µg/kg bw/day (JECFA, 1995). For most analyzed antibiotics, the EDI values were extremely low, whether calculated using median or P95 consumption levels. Among the detected antibiotics, the highest calculated EDI values, based on “high consumption” consumers (P95, 250 g/person/day), were observed for DOX (0.241 µg/kg bw/day) from the supermarket source, corresponding to 8% of the ADI. Oxytetracycline ranked second, reaching 0.143 µg/kg bw/day in supermarket samples, accounting for 4.8% of its ADI, whereas residues from local market samples contributed to lower exposure levels (2.1% of ADI). Regarding the remaining antibiotics, including enrofloxacin, ciprofloxacin, and

sulfamethoxazole, the corresponding EDI values were also substantially lower than their respective ADI values (Table 5).

Overall, the exposure assessment revealed that although antibiotic residues were detected in shrimp muscle, all calculated HQ values remained below 1 under both median and P95 consumption scenarios, indicating that consumer health risks associated with shrimp consumption were within acceptable limits.

Table 5. Estimated dietary exposure and hazard quotient (HQ) of the surveyed population to the investigated antibiotics through whiteleg shrimp consumption

Antibiotic	Market	EDI ($\mu\text{g/kg bw/day}$)		ADI ($\mu\text{g/kg bw/day}$)	HQ in case of P95 consumption
		Median consumption	P95 consumption		
Doxycycline	Supermarket	0.120	0.241	3	8.0×10^{-2}
	Local market	ND	ND		-
Oxytetracycline	Supermarket	0.072	0.143	3	4.8×10^{-2}
	Local market	0.031	0.062		2.1×10^{-2}
Enrofloxacin	Supermarket	0.018	0.036	2	1.8×10^{-2}
	Local market	0.002	0.004		2.1×10^{-3}
Ciprofloxacin	Supermarket	0.003	0.007	2	3.5×10^{-3}
	Local market	0.001	0.002		1×10^{-3}
Sulfamethoxazole	Supermarket	0.002	0.005	50	1×10^{-4}
	Local market	ND	ND		-

EDI: estimated daily intake; ADI: acceptable daily intake; HQ: hazard quotient; P95: 95th percentile consumption; ND: not detected.

4. Discussion

The analysis clearly demonstrated the distinct capabilities of ELISA and LC-MS/MS for antibiotic residue quantification. LC-MS/MS is generally considered more accurate and precise for antibiotic residue analysis due to its specificity for individual compounds, whereas, in our study, ELISA provided group-level screening result. There were some different results found between the applied methods, e.g., in sample 2.3 collected from a supermarket, ELISA detected the tetracycline group at 0.98 µg/kg, while LC-MS/MS identified DOX at 23.36 µg/kg and OTC at 2.28 µg/kg. The sum of these two tetracyclines quantified by LC-MS/MS (25.64 µg/kg) is significantly higher than the ELISA result (Table 2), suggesting that the ELISA result appears to underestimate the total tetracycline concentration compared to the sum of individual compounds quantified by LC-MS/MS. Similarly, this phenomenon was also observed in other samples (1.1, 5.2 and 7.3). For the fluoroquinolone group, LC-MS/MS detected ENR concentrations in shrimp muscle of 8.02 and 0.81 µg/kg in samples collected from supermarket and local market, respectively; whereas, CIP levels were 1.47 and 0.42 µg/kg (Table 2), and the total ENR and CIP levels quantified by LC-MS/MS were higher than those of the results quantified by ELISA. This highlights ELISA's potential to miss specific antibiotic residues or its limitations in detecting individual compounds within a group, even when present at high concentrations. Moreover, some samples tested positive by ELISA but were below the detection limit of LC-MS/MS. Therefore, the agreement between the methods should be investigated in further studies or the screening test should be revalidated in shrimp muscle samples for all groups of antibiotics.

These findings underscore the critical differences between the two methods. ELISA serves as a useful and rapid screening tool for the presence of antibiotic groups, offering a preliminary indication of contamination. However, LC-MS/MS provides superior accuracy and specificity by identifying and quantifying individual antibiotic compounds, making it the preferred method for definitive confirmation and precise quantification of antibiotic residues in animal muscle (Stolker & Brinkman, 2005). In fact, the validated LC-MS/MS workflows have been used to quantify a wide range of veterinary drugs in shrimp and fish with high sensitivity and robust performance for regulatory monitoring (Ljubojević Pelić et al., 2024). The results in this study align with prior research in Vietnam as well as elsewhere, where LC-MS/MS has been shown to provide more accurate quantification of antibiotic residues in shrimp and other aquatic products. For instance, the study on monitoring of antibiotic residues in aquatic products in urban and rural areas of Vietnam by Uchida et al. (2016) which used LC-MS/MS on hundreds of samples, detecting residues of enrofloxacin and other antibiotics with more reliable sensitivity than ELISA methods. Similarly, Pham et al. (2015) also analyzed antibiotic residues in freshwater aquaculture using ELISA method, and then confirmation and more precise quantification were performed via LC-MS/MS. In addition, several comparative studies have reported cases of ELISA producing false-positive results requiring confirmation by LC-MS/MS, or conversely where LC-MS/MS revealed residues (or different analytes) that immunoassays could not distinguish (Jayasinghe

et al., 2023). Therefore, LC-MS/MS is preferable for confirmation and quantification, while ELISA remains useful as a rapid screening tool.

The consumption data confirm that whiteleg shrimp is a major component of the diet for nearly the entire surveyed population, as indicated by the 95.5% consumption rate and the strong preference (88%). This high consumption rate underscores the critical importance of monitoring antibiotic residues in this product. The findings demonstrate that for all five target antibiotics, the exposure risk remains very low, even when considering the “high consumption” consumers (P95). This suggests that the current level of antibiotic residues in whiteleg shrimp consumed by the population in Can Tho City, Vietnam, as represented by this sample, does not pose a public health risk. The exposure assessment outcomes reported here are consistent with previous regional studies. Pham et al. (2015) surveyed antibiotic residues in Vietnamese fish and shrimp aquaculture products, i.e., reported EDIs were generally well below established ADIs, supporting our results that even with relatively high shrimp consumption (P95), the risk to consumers remains low (Table 5). Similarly, Tsai et al. (2019) found that residue levels of chloramphenicol, enrofloxacin, flumequine in shrimp from Taiwan were lower than the MRLs, and the risk assessment of quinolone showed that the EDI was below 1% of the relevant ADIs, supporting the conclusion that shrimp consumption posed negligible risk, which a finding consistent with the result in the present study. Moreover, the research of Andriyono et al. (2022) in Banyuwangi, Indonesia reported negative or very low levels for several antibiotics, i.e., oxytetracycline, tetracycline, chloramphenicol with correspondingly very low EDI and HQ values, remained within safe limits, which aligns with our finding of low exposure levels. In addition, Yu et al. (2018) reported that although the quite high levels of sulfamethoxazole were found in bream fish samples, consumption of cultured fish was not significantly associated with human health risks in China, according to the calculated EDI values. However, a potential risk to human safety still exists if these compounds accumulate over time. Therefore, routine monitoring of antibiotic residues in shrimp is critical to ensure the safety of shrimp products and protect consumers.

It should be noted that the present risk assessment considered only antibiotic exposure through shrimp consumption and did not account for potential exposure from other food commodities. Therefore, the calculated HQ values reflect shrimp-specific exposure rather than total dietary exposure and may underestimate cumulative antibiotic intake. Nevertheless, the findings provide important baseline information on consumer exposure to antibiotic residues from white leg shrimp, one of the most frequently consumed aquatic food products in the study area. As the first study assessing white leg shrimp consumption patterns and associated antibiotic exposure in Can Tho city, Vietnam, the results contribute valuable data for food safety monitoring, risk management, and future cumulative dietary exposure assessments.

5. Conclusion

The calculated EDI and HQ values for antibiotics (DOX, OTC, ENR, CIP, and SMX) in whiteleg shrimp were far below the corresponding ADI values, and all HQ values were below 1, indicating negligible health risks for consumers. Nevertheless, the detection of antibiotic residues in commercial shrimp products suggests the need for continued monitoring and stricter management of antibiotic use in aquaculture. In addition, strict compliance with withdrawal periods and adherence to good aquaculture practices are essential to ensure food safety and protect public health.

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Competing Interests

The authors declare that they have no competing interests to declare that are relevant to the content of this article.

Animal welfare and Ethics statement

The experiments and animals used in this work were followed and treated according to Decision No. 3965/QD-DHCT Date October 15 2021 “Can Tho University Regulation on Ethics in animal experimentation” ULR: <https://dra.ctu.edu.vn/images/upload/news/246.pdf>.

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4. Discussion, Conclusions and Perspectives

4.1 Discussion

4.1.1 Integrated interpretation of the main findings

When considered together, the findings of this thesis provide a comprehensive overview of antibiotic use and its implications in shrimp farming, combining information from field practices, controlled experimental studies, and consumer exposure to antibiotics at the market level. Although each study had a distinct objective, the results need to be interpreted together to understand the broader implications for shrimp production and food safety. By integrating data from these studies, this thesis provides a comprehensive evidence base to support the rational antibiotic use and the mitigation of contamination risks in the shrimp production system in Vietnam.

The initial survey was conducted in super-intensive whiteleg shrimp farms in the Mekong Delta, Vietnam. The identification of CFT, FF, and DOX as commonly applied antibiotics provided the practical basis for subsequent studies. This step ensured that subsequent experimental work was based on actual production practices rather than theoretical assumptions.

Controlled experimental studies of selected antibiotics were conducted to generate quantitative data on PK and withdrawal times after oral administration. Differences in absorption, exposure, and elimination patterns were observed among the compounds, leading to different PK parameters and PK/PD indices relevant to antimicrobial efficacy. These findings demonstrate that antibiotics used in shrimp farming exhibit compound-specific behavior, which directly influences treatment efficacy and withdrawal time determination.

In addition to determining PK parameters and withdrawal times, the evaluation of hepatopancreas histology provided further insight into the biological responses associated with antibiotic exposure. Although residue concentrations decreased over time, tissue changes were still observed during and after antibiotic exposure, suggesting that the biological impact of antibiotics may not be fully reflected by residue data alone.

Furthermore, the analysis of antibiotic residues in commercial whiteleg shrimp products and the associated consumer risk assessment provided an additional practical perspective to the experimental studies. The consumer risk assessment helped to clarify the residue findings within the context of food safety.

4.1.2 Comparative insights from the evaluation of selected antibiotics in shrimp aquaculture

4.1.2.1 Comparative pharmacokinetics and pharmacodynamics of selected antibiotics

Pharmacokinetics

When examined under comparable experimental conditions, DOX, FF, and CFT showed different PK profiles in whiteleg shrimp following oral administration. In shrimp aquaculture, oral administration via medicated feed represents the most practical and widely applied method of antibiotic delivery,

compared to injection or immersion methods. The effectiveness of oral therapy depends not only on the pharmacological properties of the compound but also on feed-related factors, including palatability, stability of the active ingredient within the feed, and protection of the drug during feeding and ingestion. These characteristics may influence drug intake and subsequent absorption kinetics in aquatic species (Daniel, 2009). Therefore, interpretation of PK parameters in shrimp should consider the particular context of medicated feed administration. Although all three antibiotics were administered orally, their absorption, systemic exposure, elimination, and tissue distribution were not similar (Table 1).

Table 1. Comparative pharmacokinetic parameters of DOX, FF, and CFT in plasma, hepatopancreas, and muscle of whiteleg shrimp following oral administration

Organs	Antibiotic	Dose (mg/kg)	C _{max} (µg/mL or µg/g)	T _{max} (h)	t _{1/2} (h)	AUC (µg.h/mL or µg.h/g)
Plasma	DOX	20	1.09	1.60	4.78	9.45
	FF	10	0.0606	1.77	0.35	1.026
	CFT	25	0.184	4.00	8.78	2.15
Hepatopancreas	DOX	20	2.37	0.23	NR	31.72
	FF	10	0.387	0.19	NR	1.66
	CFT	25	19.45	1.00	11.23	199.64
Muscle	DOX	20	0.46	2.00	NR	5.55
	FF	10	0.0118	0.20	NR	0.088

NR: not reported

In shrimp plasma, DOX showed the highest distribution among the three antibiotics, as evidenced by its AUC value and relatively prolonged elimination half-life (4.78 h). In contrast, FF exhibited significantly lower AUC values and a shorter elimination half-life (0.35 h). The higher clearance value observed for FF indicates faster removal from systemic circulation compared to DOX. Although the T_{max} values of DOX and FF were relatively similar, their elimination half-life differed considerably, leading to distinct overall exposure profiles. Cefotaxime showed a different PK profile. Although C_{max}

of CFT was lower than that of DOX at the administered dose, its elimination half-life (8.78 h) was almost twice as long as that of DOX (4.78 h). Furthermore, the $T_{\max}$ of CFT was 4 h, indicating slower absorption compared to DOX and FF.

In tissues, DOX and FF concentrations in the hepatopancreas were much higher than those measured in muscle. Based on the values of $C_{\max}$ and AUC, the concentrations of both antibiotics followed the general order hepatopancreas > plasma > muscle, suggesting that the hepatopancreas retained higher levels of the administered antibiotics over time. Notably, the $C_{\max}$ values of CFT in the hepatopancreas were 8 times higher than DOX and 49 times higher than FF, indicating a marked accumulation of CFT in this organ under the experimental conditions.

It should be noted that the administered doses differed among the three antibiotics (20 mg/kg for DOX, 10 mg/kg for FF, and 25 mg/kg for CFT). Therefore, caution should be exercised when interpreting direct quantitative comparisons of $C_{\max}$ and AUC values, as these antibiotics belong to different pharmacological classes. The purpose of this comparison was not to quantitatively rank the level of antibiotic distribution but rather to identify differences in PK patterns between antibiotics within the same species.

Pharmacodynamics

Based on PK/PD calculations using the $T > \text{MIC}$ criterion for time-dependent antimicrobials, the three antibiotics demonstrated different levels of predicted therapeutic performance against *V. parahaemolyticus* (known to cause AHPND in whiteleg shrimp). For FF, the $C_{\max}$ values in shrimp organs were below the MIC value against *V. parahaemolyticus*. In theory, this means that AHPND caused by *V. parahaemolyticus* in whiteleg shrimp may not be treatable with a single dose of 10 mg FF/kg bw. For DOX, using an MIC value of 2 µg/mL against *V. parahaemolyticus*, the time during which hepatopancreas concentrations exceeded the MIC was approximately 4 h. For time-dependent antimicrobials, antibacterial efficacy is generally associated with $T > \text{MIC}$ covering approximately 40–50% of the dosing interval (AliAbadi & Lees, 2000). Based on this criterion, a $T > \text{MIC}$ of approximately 4 h would therefore require a dosing interval of approximately 8 h to achieve sufficient therapeutic efficacy. Regarding CFT based on a MIC value of 4.13 µg/mL for CFT against *V. parahaemolyticus*, it was observed that the time during which the hepatopancreas CFT concentration was above the MIC was 23 h. In shrimp farming, farmers apply antibiotics once a day during medication, suggesting a dosing interval of 24 h corresponding to approximately 95% of $T > \text{MIC}$. Hence, this suggests the therapeutic efficacy of CFT in treating AHPND caused by *V. parahaemolyticus* in whiteleg shrimp.

In this thesis, the use of hepatopancreas concentrations for PK/PD interpretation was considered biologically relevant, as this organ is the main target of AHPND and the primary site of pathological damage caused by *V. parahaemolyticus* in shrimp. Based on the PK/PD index, CFT achieved the highest

proportion of the dosing interval where hepatopancreas concentrations remained above the MIC, followed by DOX, while FF showed the lowest predicted therapeutic efficacy at the administered dose. In these studies, the administered doses were selected to reflect those commonly used by shrimp farmers in their shrimp farming practices. Nevertheless, these findings suggest that empirical use of these doses does not necessarily guarantee sufficient therapeutic efficacy against *V. parahaemolyticus*. Particularly, insufficient coverage of the target site may lead to suboptimal treatment outcomes or prolonged disease persistence. Therefore, applying antibiotics using fixed dosing practices without considering PK/PD principles may result in inconsistent treatment effectiveness and therapeutic failure, while also increasing the risk of antimicrobial resistance development in shrimp production systems.

Nevertheless, it should also be recognized that the PK/PD data generated in this thesis were obtained under controlled experimental conditions in which environmental parameters were adjusted to approximate commercial shrimp farming practices in the Mekong Delta. Nevertheless, environmental and management-related factors such as salinity fluctuations, water quality variation, stocking density, feeding practices, disease pressure, and seasonal changes may differ substantially among commercial shrimp farms and may influence antimicrobial susceptibility patterns, antibiotic pharmacokinetics, and therapeutic efficacy in shrimp. Hence, periodic evaluation of antimicrobial susceptibility and treatment efficacy under practical farming conditions would be beneficial to support more effective and sustainable antibiotic use in shrimp aquaculture over time.

4.1.2.2. Differences in depletion and withdrawal times of selected antibiotics

The depletion patterns of DOX, FF, and CFT in whiteleg shrimp muscle showed differences during and after medication. Residues of all three antibiotics reached the highest concentration at day 3 of medication, and gradually decreased after stopping medication. Among these antibiotics, DOX reached the highest levels in muscle during medication, indicating a relatively higher persistence in shrimp muscle tissue compared to the other two antibiotics. In particular, residual levels increased from day 1 to day 3 of medication, specifically under twice-daily administration, indicating accumulation during repeated dosing, and then the DOX levels were below the LOD after 14 days onwards in both feeding regimes. In contrast, FF and CFT exhibited lower muscle levels and a more rapid decline following withdrawal, with concentrations falling below the LOD from day 3 after stopping medication under both treatment regimens (once-daily and twice-daily administration of medicated feed). This rapid decline indicates limited retention in shrimp muscle tissue. Although high concentrations of CFT were observed in the hepatopancreas during PK study, the drug did not show significant accumulation in muscle tissue. Overall, data on decline showed that muscle sustainability followed the general trend of higher DOX than CFT and FF under the tested conditions.

Regarding withdrawal times, withdrawal time refers to the period needed for drug residues in edible tissues to decrease to a safe level for consumer consumption (Avunje et al., 2021). Withdrawal times varied among the three antibiotics and were influenced by dosing frequency of administration, the

applied MRL values, and ambient temperature. Based on a MRL of 100 µg/kg, DOX exhibited a markedly longer withdrawal time in whiteleg shrimp muscle compared with the other two antibiotics under the tested conditions. Particularly, DOX showed the longest withdrawal period, ranging from approximately 6 to 8 days depending on dosing frequency at 26.5°C. In contrast, FF and CFT showed significantly shorter WTs, maintaining concentrations below the MRL value within 2 to 3 days even under twice-daily administration at 26.5°C and 29.5°C, respectively.

The longer withdrawal time observed for DOX may be due to its greater lipophilicity compared with beta-lactam antibiotics such as CFT, which facilitates broader tissue distribution and may contribute to its higher apparent volume of distribution. Drugs with higher tissue penetration and protein binding are generally associated with prolonged persistence in edible tissues (Øie, 1986; Toutain & Bousquet-Mélou, 2004). In contrast, CFT, a hydrophilic beta-lactam, typically remains confined to extracellular compartments and is eliminated more rapidly, which may explain its shorter withdrawal period. Furthermore, ambient temperature may affect the elimination process in aquatic species, as metabolic and excretory processes accelerate at higher temperatures (Rigos & Troisi, 2005).

Currently, official withdrawal times for the studied antibiotics in shrimp have not been specifically established in Vietnam. Although some commercial antibiotic products used in shrimp aquaculture provide withdrawal recommendations on product labels, these values are often based on limited available information and may vary among manufacturers. Therefore, the withdrawal times determined in this study provide scientifically based data that may support more reliable recommendations for shrimp farming practices. These findings may also support veterinary pharmaceutical companies in providing withdrawal recommendations on product labels for shrimp aquaculture products, while also assisting regulatory authorities in developing antibiotic use guidelines and disseminating practical withdrawal recommendations to shrimp farmers.

4.1.2.3 Effects of antibiotic exposure on shrimp hepatopancreas health

The hepatopancreas plays a central role in digestion, metabolism, and xenobiotic processing in crustaceans, and is therefore considered a sensitive indicator of shrimp health status (Caceci et al., 1988; Manan et al., 2015). The hepatopancreas is known as a primary site of xenobiotic metabolism, where the drug and its residual compounds are catabolized and eliminated from the shrimp body (Rewitz et al., 2006). Consequently, temporary cellular alterations in this organ may occur during exposure to antimicrobial compounds.

Histological evaluation of the hepatopancreas following exposure to DOX, FF, and CFT, based on B- and F-cell counts, revealed transient alterations following antibiotic administration. Regarding B cells, a significant reduction in B cell numbers was observed on day 3 of medication in the three antibiotic groups, suggesting a general hepatopancreatic response to antibiotic exposure. Afterward, the number of B-cells in the shrimp hepatopancreas recovered within seven days after stopping medication. B cells

are associated with enzyme storage and secretion into the tubule lumen through holocrine processes (Hu & Leung, 2007). A temporary decrease in their abundance during active medication may indicate modified secretory dynamics or increased cellular turnover linked to drug metabolism. For F-cells, a statistically significant reduction was detected only in the DOX-treated group, particularly under the twice-daily regimen, whereas FF and CFT did not induce comparable alterations. According to Hu and Leung (2007), F-cells synthesize and store digestive enzymes in large vesicles before differentiating into B-cells. These enzymes are then released into the hepatopancreatic lumen via a holocrine secretion process facilitated by B-cells (Nunes et al., 2014). Although CFT showed high peak concentrations in the hepatopancreas in the single-dose PK study, DOX exhibited longer persistence at the organism level, as reflected by extended withdrawal times and slower depletion in muscle. Prolonged exposure during repeated dosing may therefore contribute to transient modulation of enzyme-producing F cells. Similar alterations in hepatopancreatic cellular structure and digestive tissues have been reported in other crustacean species exposed to xenobiotics or veterinary compounds, including crayfish and crabs, where the hepatopancreas was identified as a highly sensitive organ involved in detoxification and metabolic adaptation processes (Gao et al., 2021; Kadam & Raut, 2018).

Nevertheless, full recovery of both B and F cell numbers within seven days after stopping medication indicates that the observed changes were reversible. Therefore, the mild and reversible histological changes observed in these studies likely reflect the role of the hepatopancreas in drug processing rather than toxic injury, further supporting the PK findings of low tissue exposure and rapid drug clearance at the applied dosages. In shrimp, previous studies evaluating other antibiotics or veterinary drugs, including enrofloxacin, oxytetracycline, and maduramicin, reported more severe histopathological alterations in the hepatopancreas, such as cellular degeneration, tubular deformation, and vacuolization, particularly when higher doses or prolonged exposure periods were applied (Avunje et al., 2021; Maftuch et al., 2017; Bray et al., 2006). Additionally, these studies also showed that the higher the dose of antibiotics used, the more severe the impact on the hepatopancreas of shrimp. These findings underscore the potential risks associated with inappropriate or excessive antibiotic use in aquaculture systems. Prolonged or high-dose exposure to antibiotics may adversely affect shrimp health, particularly in sensitive organs such as the hepatopancreas.

4.1.3 Residue occurrence in commercial shrimp and implications for consumer risk assessment

For the study related to consumer risk assessment, antibiotics were selected for LC-MS/MS analysis based on the preliminary screening results, antibiotic excretion capacity and common antibiotics used in shrimp culture (Phu et al., 2019; Thinh et al., 2020). Hence, five types of antibiotics, i.e., DOX, oxytetracycline, enrofloxacin, ciprofloxacin and sulfamethoxazole were chosen for quantitative analysis. Although antibiotics such as amoxicillin, CFT and FF are frequently used in shrimp culture,

they were not prioritized for confirmatory LC–MS/MS quantification, because these antibiotics are generally characterized by relatively rapid elimination, resulting in muscle tissue concentrations that decline below detectable levels within short withdrawal periods (Pham et al., 2023; Vinh et al., 2024). Therefore, these antibiotics were considered less likely to persist in shrimp muscle at measurable concentrations in commercial products on the markets. In addition, qualitative screening for beta-lactam groups yielded negative results in all analysed samples. The LC-MS/MS results suggest that CFT, despite being widely reported to be used in shrimp farming, was not detected in edible tissues at market stage, possibly due to its comparatively rapid depletion. This interpretation is consistent with the results of the depletion experiment, where CFT residues declined rapidly following the cessation of medication. The residue analysis in commercial shrimp products and the depletion studies provide complementary evidence regarding the limited persistence of β -lactam residues in market-ready shrimp. Besides, among the detected antibiotics in this study, DOX and oxytetracycline showed the highest concentrations in analysed samples. This finding is consistent with the depletion studies presented in previous chapters of this thesis, where DOX exhibited slow muscle tissue depletion and prolonged persistence in shrimp compared to antibiotics such as CFT and FF, which may explain their higher concentrations observed in market samples. According to the food intake survey conducted in this study, shrimp consumption was reported by 95.5% of respondents, with 88% indicating a strong preference. The estimated average consumption was 116 g/person/day. Such a high consumption level underscores the importance of continuous monitoring of antibiotic residues in this product. Regarding the consumer exposure assessment, based on the detected residue concentrations and the average body weight of the interviewed participants, the estimated EDI values ranged from 0.0004 to 0.08 $\mu\text{g/kg bw/day}$. All calculated EDI values were significantly lower than their corresponding ADI values established by the EMA (2015) for DOX and oxytetracycline (3 $\mu\text{g/kg bw/day}$), by JECFA (1998) for enrofloxacin and ciprofloxacin (2 $\mu\text{g/kg bw/day}$), and by JECFA (1995) for sulfamethoxazole (50 $\mu\text{g/kg bw/day}$), resulting in HQ values well below 1, even under high-consumption scenarios (P95). These results indicate that the health risk to consumers from antibiotic residues in commercial shrimp is negligible under current exposure levels. The exposure assessment results are also in agreement with previous regional studies (Pham et al., 2015; Tsai et al., 2019; Andriyono et al., 2022; Yu et al., 2018), which similarly reported low risk of adverse health effects associated with antibiotic residues in shrimp.

Although current data do not indicate a toxicological risk, they underscore the importance of ongoing monitoring and prudent antibiotic use to prevent increasing residue levels. Because Vietnamese consumers have a relatively high shrimp consumption rate, averaging nearly 125 g/day and up to 250 g/day for high consumers, maintaining low residue levels of antibiotics is crucial to ensuring food safety and consumer confidence. However, even if repeated use of substances at subtherapeutic concentrations may not pose a direct health concern, it could contribute to the development of antibiotic resistance

(Marshall & Levy, 2011; Cabello et al., 2016). Furthermore, the consistent detection of several antibiotics in the same sample suggests that antibiotic use in shrimp culture remains widespread.

Although the estimated dietary exposure risk was negligible under the investigated consumption scenarios, the continued detection of antibiotic residues in commercial shrimp products remains an important concern from regulatory and international trade perspectives. Many export markets apply strict residue monitoring programs and maintain zero-tolerance policies for certain antibiotics in aquaculture products. Consequently, even when residue concentrations do not pose an immediate toxicological risk to consumers, such findings may still contribute to increased border inspections, trade restrictions, or reduced consumer confidence in shrimp products. These findings therefore highlight the importance of prudent antibiotic use, withdrawal time compliance, and continuous residue surveillance to support sustainable shrimp production and maintain the competitiveness of Vietnamese shrimp in international markets.

4.1.4 Implications for antimicrobial use and antibiotic resistance in shrimp aquaculture

The use of antibiotics in animal production systems has long been recognized as a factor contributing to the emergence and dissemination of antimicrobial resistance (Teale, 2002). Several authors (Bogaard and Stobberingh, 2000; Russell, 2002; Teale, 2002) are concerned about the negative effects due to the use of antibiotics in agriculture and aquaculture, particularly in intensive production systems where bacterial diseases are common and antimicrobial therapy is frequently applied.

Antibiotic use patterns and selection pressure at farm level

The survey conducted in this study demonstrated that nearly half of the interviewed shrimp farms reported antibiotic use during the production cycle (48.1%). Importantly, treatment decisions were predominantly based on farmers' experience rather than laboratory diagnosis or susceptibility testing. In addition, treatments were applied for 3–5 days per episode, with up to four treatment episodes per crop in some cases. A proportion of farms reported the use of more than one antibiotic within the same production cycle. These findings indicate that antimicrobial exposure in intensive shrimp farming is not a rare or isolated event but may occur repeatedly within a single production cycle. Sequential or multi-class antibiotic use has been discussed as a factor that can increase the complexity of selection pressures imposed on bacterial populations (Marshall & Levy, 2011). The observed usage patterns suggest that bacterial communities in shrimp production systems may experience intermittent and repeated antimicrobial exposure. The doses selected for the experimental PK studies (20 mg/kg for DOX, 10 mg/kg for FF, and 25 mg/kg for CFT) were based on those commonly applied in practice. Therefore, the PK and PK/PD results obtained under experimental conditions in the thesis are directly relevant to field scenarios.

Evaluating treatment efficacy based on PK/PD approach

The PK/PD analysis using the time above the minimum inhibitory concentration ($T > MIC$), defined as the duration during which drug concentrations remain above the MIC, for time-dependent antimicrobials revealed substantial differences among the investigated compounds. Cefotaxime achieved approximately 95% $T > MIC$ under once-daily dosing, indicating sustained exposure above the MIC for most of the dosing interval. In contrast, DOX achieved shorter $T > MIC$ durations and required consideration of the lowest observed MIC value to meet theoretical efficacy targets. FF did not reach concentrations above the MIC in hepatopancreas or hemolymph at the administered field-representative dose (as discussed in the section 8.2). For time-dependent antimicrobials, maintaining drug concentrations above the MIC for a sufficient proportion of the dosing interval is considered critical for bacterial suppression (AliAbadi & Lees, 2000). The PK/PD approach to dosage design was established to relate antimicrobial exposure to antibacterial response and to support rational dose optimization (Toutain et al., 2002). Hence, the integration of PK/PD approach into dosage design has been proposed as an important strategy not only to improve therapeutic outcomes but also to reduce the probability of resistance selection. According to Drlica and Zhao (2007) and Blondeau (2009), the mutant selection window suggests that when antimicrobial concentrations are close to or below the MIC value, bacteria that are less sensitive to the drug may survive and gradually increase in number. The present findings indicate that empirical dosing regimens commonly applied in shrimp farming do not necessarily achieve optimal PK/PD targets. Particularly, exposure below the MIC, as observed for FF, represents a pharmacological situation in which bacteria may encounter sub-inhibitory concentrations without effective suppression. Even in the case of DOX, the observed $T > MIC$ target was achieved only when the lowest MIC value among the evaluated strains was considered. Therefore, prolonged or repeated exposure to suboptimal antimicrobial concentrations may increase the risk of antimicrobial resistance development (Blondeau, 2009; Toutain et al., 2021).

Mechanisms and emergence of antibiotic resistance in shrimp aquaculture

The exposure profiles identified in the present study should also be considered in light of established molecular mechanisms of antimicrobial resistance. Bacterial resistance is commonly mediated through three principal strategies: active efflux of antimicrobial compounds, enzymatic inactivation of the drug, and alteration or protection of the antimicrobial target (Davies and Davies, 2010; Blair et al., 2015; Munita and Arias, 2016). Efflux systems consist of membrane-associated transport proteins that actively export antimicrobial molecules out of the bacterial cell, so that intracellular concentrations no longer reach inhibitory levels (Poole, 2007). Resistance depends on enzymes that chemically modify or degrade the drug before it can interact with its target, typical examples include β -lactamases and other modifying enzymes (Wright, 2005). In addition, bacteria may also become less susceptible through changes in the structure of the drug target or through proteins that interfere with drug binding, allowing cellular processes to continue despite the presence of the antimicrobial agent (Blair et al., 2015). Davies

& Davies (2010) indicate that the antibiotic classes investigated in this thesis, namely tetracyclines, phenicols and β -lactams, can each be associated with the three principal resistance mechanisms outlined above. These mechanisms may occur individually or in combination within bacterial populations.

In addition to these molecular mechanisms, it is important to consider how resistance emerges and spreads within bacterial populations. Resistance can arise through spontaneous genetic mutations or through the acquisition of resistance genes via horizontal gene transfer mechanisms, including conjugation, transformation, and transduction (Munita & Arias, 2016; von Wintersdorff et al., 2016). Once established, resistant bacteria or resistance genes can disseminate within aquaculture environments through water, sediments, and microbial communities, facilitating their persistence and wider distribution (Done et al., 2015; Cabello et al., 2016). The widespread use of antibiotics in aquaculture systems can further promote these processes by selecting for resistant bacterial populations. Under antibiotic exposure, susceptible bacteria may be inhibited or eliminated, whereas bacteria carrying resistance determinants, either through spontaneous mutations or acquired resistance genes, are more likely to survive. The reduction of susceptible competitors provides resistant bacteria with a selective advantage, allowing them to proliferate and progressively become dominant within the microbial population (Cabello, 2006; Romero et al., 2012).

Although the present thesis primarily focused on therapeutic efficacy, residue depletion, withdrawal times, and food-safety aspects of antibiotic use in shrimp aquaculture, the environmental fate of these compounds should also be considered when evaluating treatment strategies. In aquaculture systems, a proportion of administered antibiotics may be released into surrounding water and sediments through uneaten feed, excretion, and farming effluents (Cabello, 2006). The persistence of antibiotic residues in aquatic environments may vary among compounds and could influence their environmental impact. For example, tetracycline-group antibiotics have been reported to exhibit strong adsorption to sediments and persistence in aquatic environments (Samuelsen et al., 1992; Chen et al., 2017; Harrower et al., 2021; Miliasevic et al., 2024). Therefore, the selection of antibiotics for shrimp disease treatment should consider not only therapeutic efficacy and food safety, but also the environmental persistence of antibiotic residues in aquaculture systems.

In the Mekong Delta, antibiotic-resistant *Vibrio* species associated with shrimp diseases have been reported in recent studies. For instance, antibiotic susceptibility testing of *V. parahaemolyticus* strains isolated from diseased shrimp in Vietnam showed resistance to multiple commonly used antibiotics, particularly β -lactams such as amoxicillin and cephalexin, as well as other groups including sulfonamides and macrolides (Oanh et al., 2022). These findings indicate that resistant bacterial populations are already present in aquaculture systems. Notably, a considerable proportion of isolates exhibited multidrug resistance, being resistant to three or more antibiotics, suggesting the impact of repeated antibiotic use in intensive aquaculture practices (Thi et al., 2024). *Vibrio* spp., including *V. parahaemolyticus*, are recognized as major causative agents of shrimp diseases such as AHPND (Tran

et al., 2013; De Schryver et al., 2014). Therefore, the presence of resistant strains may complicate treatment strategies and reduce therapeutic effectiveness, suggesting that antibiotic resistance may be impacting disease management in shrimp farming systems in the Mekong Delta.

From a practical perspective, the results of this thesis suggest that antimicrobial use in shrimp farming should be considered from two distinct perspectives: antibiotic residue control related to food safety and consumer exposure, and antimicrobial resistance associated with bacterial selection pressure and reduced treatment effectiveness. The combined evaluation of farm practices and PK and PK/PD data illustrates that dosing regimens applied under field conditions may influence drug exposure at the target site and, consequently, treatment efficacy. Although the resistance genes were not investigated in the present thesis, repeated treatments and insufficient drug exposure may allow less susceptible bacteria to survive under certain conditions. In this context, strengthening the management of antibiotic use in shrimp production can support more effective treatments and contribute to minimizing the emergence and spread of antibiotic resistance.

In a broader context, the findings of the present thesis also highlight the importance of responsible antimicrobial use in shrimp aquaculture from One Health and sustainability perspectives. Inappropriate antibiotic use and non-compliance with residue regulations may affect not only shrimp health management, but also environmental quality, public health, and international market access for shrimp-exporting countries such as Vietnam. Therefore, reducing antimicrobial dependency through improved biosecurity, water quality management, optimized farming practices, and preventive strategies such as probiotics may contribute to more sustainable aquaculture systems and help limit the selection pressure associated with antimicrobial resistance (Milijasevic et al., 2024).

4.2 Conclusions

The survey conducted in super-intensive shrimp farming systems in the Mekong Delta showed that farmers used many types of chemicals and antibiotics. The CFT, DOX and oxytetracycline were frequently used to control disease outbreaks such as AHPND and WFD. Most farmers used probiotic products to improve water quality and feed digestibility, reflecting farmers' efforts to enhance shrimp health, though product quality sometimes varied. Nevertheless, farmers decided on the types of chemicals used mainly based on their experience. Many farmers knew about the banned antibiotics; however, a lack of antimicrobial susceptibility testing data led to improper use of antibiotics.

The PK studies demonstrated that CFT, FF and DOX were rapidly absorbed and eliminated in whiteleg shrimp after oral administration. In the present study, CFT (25 mg/kg bw), FF (10 mg/kg bw), and DOX (20 mg/kg bw) were administered through medicated feed. The concentrations of the investigated antibiotics were consistently higher in the shrimp hepatopancreas than in hemolymph, indicating the important role of this organ in antibiotic distribution and elimination. The integration of PK data with the antimicrobial susceptibility data of *V. parahaemolyticus* (MIC of 4.13 mg/L) suggested that the CFT dosing regimen could provide sufficient drug exposure in shrimp hepatopancreas ($C_{\max}$ of 19.45 ± 2.10 mg/kg at $T_{\max}$ of 1.0 ± 0.20 h) to inhibit the pathogen, whereas the investigated doses of FF and DOX were likely insufficient to achieve effective therapeutic exposure against most tested isolates.

The estimated withdrawal times varied depending on the dosing regimen and the applicable MRL, ranging from approximately 2.0 to 77.7 hours for CFT, around 2 days for FF, and 6 to 10 days for DOX. The very short withdrawal times (2.0 hours) estimated for CFT regimen mainly reflect the rapid elimination of the compound in shrimp. However, in practical farming conditions, withdrawal management is generally implemented on a daily rather than hourly basis. Conversely, the relatively longer withdrawal times estimated for DOX may require additional consideration in relation to harvest planning under commercial shrimp farming conditions. These results provide useful reference data for establishing appropriate withdrawal times and improving antibiotic management in shrimp aquaculture.

Histological examination of the shrimp hepatopancreas showed that oral administration of FF and DOX caused slight but reversible changes that resolved after stopping medication, while CFT did not show negative effects at the tested dose.

Regarding the occurrence of antibiotic residues in commercial whiteleg shrimp collected from markets and supermarkets in Can Tho City, DOX was detected at the highest concentration (52.87 $\mu\text{g/kg}$), followed by oxytetracycline (31.5 $\mu\text{g/kg}$), while enrofloxacin, ciprofloxacin, and sulfamethoxazole were found at lower levels (ranging from 1.04 to 8.02 $\mu\text{g/kg}$). The EDI and HQ values calculated for the detected antibiotics (DOX, OTC, ENR, CIP, SMX) from whiteleg shrimp were far below their recommended ADI values and <1 , indicating negligible health risks for consumers.

Beyond optimizing antibiotic usage and withdrawal management, future shrimp aquaculture should progressively move toward more sustainable and resilient farming systems. Improved biosecurity, better water-quality management, appropriate stocking densities, and the use of probiotics, prebiotics, and immunostimulants may help reduce the dependence on antibiotics while improving shrimp health and disease prevention. In addition, the adoption of advanced production systems such as biofloc technology, recirculating aquaculture systems, and integrated farming models may contribute to more sustainable shrimp production with reduced environmental impacts and improved product quality.

4.3 Perspectives

This thesis provides useful information to support a more appropriate use of antibiotics in whiteleg shrimp aquaculture, as well as to improve food safety for consumers. However, several areas require further investigation to strengthen and expand these findings.

Further studies should investigate a broader range of antibiotics used in shrimp farming, as well as their PK, residue depletion and effectiveness under different culture conditions. Evaluating these parameters under field farming conditions would be important, as environmental factors such as temperature, salinity, and feeding practices may influence antibiotic behavior in shrimp. In addition, the integration of molecular and genomic approaches to better understand antimicrobial resistance in shrimp pathogens, particularly *V. parahaemolyticus*, would provide deeper insights into treatment effectiveness and resistance development.

The results of this study also highlighted differences in the effectiveness of the investigated antibiotics. Cefotaxime showed the potential to reach effective exposure against *V. parahaemolyticus*, while the investigated doses of FF and DOX appeared insufficient for most tested isolates. Therefore, future studies should evaluate higher dosing regimens of FF and DOX to determine whether effective therapeutic exposure can be achieved while maintaining shrimp health and ensuring product safety.

From a practical perspective, the findings of this thesis could support the development of more appropriate guidelines for antibiotic use in shrimp farming, including evidence-based dosing regimens and withdrawal times. Strengthening shrimp farmer training would also be important to reduce reliance on experience-based practices.

The detection of antibiotic residues in commercial shrimp samples indicates that regular monitoring programs should be maintained to better understand the occurrence of residues in aquaculture products. In addition, antibiotic exposure assessments should be conducted for consumers, particularly for people living in the Mekong Delta, where aquaculture production is intensive and antibiotic use is common. Risk assessment of antibiotic residues in aquaculture products is still limited in Vietnam and should be further considered by Vietnamese authorities in order to reduce potential risks to human health and to improve the safety and quality of aquaculture products.

In the longer term, the development of more sustainable and potentially antibiotic-free shrimp farming systems should be encouraged through alternative approaches such as improved farm management practices, probiotics, prebiotics, immunostimulants, herbal products, and better biosecurity measures to reduce the dependence on antibiotics in shrimp aquaculture. Improved water-quality control, appropriate stocking density, pond hygiene, and early disease prevention may also help reduce disease outbreaks and the need for antibiotic treatments. In addition, the adoption of advanced aquaculture systems such as recirculating aquaculture systems, biofloc technology, and integrated farming models including shrimp–seaweed, shrimp–rice, and shrimp–mangrove systems may contribute to reducing

antibiotic dependence in shrimp culture. Furthermore, strengthening farmer education and improving access to technical guidance on responsible antibiotic use remain essential for sustainable disease management in shrimp farming. Such approaches will contribute to sustainable aquaculture development, improved product quality, and enhanced consumer confidence in shrimp products from Vietnam.

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Appendices

APPENDIX 1: QUESTIONNAIRE FOR SHRIMP FARMS

The questionnaire presented in Appendix 1 was developed by the research team in consultation with specialists in shrimp aquaculture and included both quantitative and qualitative components. Prior to the main survey, the questionnaires were piloted with seven shrimp farming households and subsequently refined to improve clarity, consistency, and relevance to local farming practices. Interviews were conducted by trained interviewers under technical guidance from scientists specialized in shrimp aquaculture.

Farms were randomly selected from official farm lists provided by the provincial Departments of Agriculture and Rural Development. In cases where selected farmers declined participation or were unavailable, replacement farms were randomly selected from the same province to maintain the planned sample size and geographical distribution.

During the interviews, the objectives of the study were clearly explained to participants, and verbal informed consent was obtained prior to data collection. Written consent forms were not used because farmers were generally reluctant to sign formal documents during field interviews. Instead, interviewers introduced themselves as researchers affiliated with Can Tho University and verbally explained the purpose of the study, the voluntary nature of participation, and the confidentiality of collected information. Personal information, including names, addresses, and telephone numbers, was collected only for communication and survey-management purposes and was not disclosed to third parties. Data were anonymized during coding and analysis to ensure participant confidentiality.

At the time of the study, no formal institutional ethical-review procedure was available at Can Tho University for questionnaire-based surveys of this type. Nevertheless, the survey was conducted following general ethical principles for voluntary participation, confidentiality, and responsible handling of personal information.

I. General information of farm

1. Name: _____ Gender: _____ Age: _____
2. Address: _____
3. Phone number: _____
4. Number of year of farming: _____ years; Lined ponds: _____ years
5. Training: Yes / No, Times/year: _____ Trainer: _____
Content: _____
6. Number of grow-out ponds: _____ ponds; Total area: _____ m²; Depth: _____ m

7. Number of nursery ponds: _____ ponds; Area: _____ m²
8. Number of sedimentation ponds: _____ ponds; Area: _____ m²
9. Number of ready ponds: _____ ponds; Area: _____ m²
10. Wastewater ponds: _____ ponds; Area: _____ m²
11. Stocking density: _____ shrimp per m²; Survival rate: _____ %
12. Nursery stage before grow-out: Yes / No
13. Nursery duration: _____ days → transfer to _____ pond
14. Salinity: _____ ppt
15. Crops/year: _____ Culture duration/crop: _____ months
16. Stocking period: Year-round / Months: _____
17. Post-larvae source: _____
18. Feed source: _____
19. FCR: _____
20. Harvest yield: _____ tons (kg)/pond
21. Harvest size: _____ shrimp/kg
22. Checking antibiotic before selling? Yes / No
23. The farm is following any kind of standards/practices or certification scheme? Yes / No (BMP, GlobalGAP, BAP, ASC, Others: _____)

II. Shrimp diseases

1. Disease occurrence during culture

- Acute hepatopancreatic necrosis disease: Month _____ Times/crop _____
 - White feces disease: Month _____ Times/crop _____
 - White spot disease: Month _____ Times/crop _____
 - Yellow head disease: Month _____ Times/crop _____
 - EHP (Enterocytozoon hepatopenaei): Month _____ Times/crop _____
 - Other diseases: _____
-

2. Identification of diseases and their characteristic clinical signs:

III. Use of chemicals

1. Pond preparation

Lime (CaO / CaCO₃): _____ kg/pond

Chlorine: _____ kg/pond

KMnO₄: _____ kg/pond

2. Water treatment in ponds

Water circulation: Yes / No

Brief description of the water treatment process:

Chemicals used:

Lime: CaO _____ or CaCO₃ _____ kg/pond/m³

Chlorine: _____ kg/pond

Iodine: _____ L/pond

BKC: _____ L/pond

TCCA: _____ kg/pond

Potassium permanganate: _____ kg/pond

Formalin: _____ L/pond

Probiotics: _____ kg/pond

Others: _____

3. During the culture period

Lime (CaO / CaCO₃): _____ kg/pond; frequency: once every _____ days

Chlorine: _____ kg/pond; frequency: once every _____ days

Iodine: _____ L/pond; frequency: once every _____ days

BKC: _____ L/pond; frequency: once every _____ days

TCCA: _____ kg/pond; frequency: once every _____ days

Potassium permanganate: _____ kg/pond; frequency: once every _____ days

Formalin: _____ L/pond; frequency: once every _____ days

Virkon: _____ L/pond; frequency: once every _____ days

Hydrogen peroxide (H₂O₂): _____ L/pond; frequency: once every _____ days

Others: _____; frequency: once every _____ days

Antiparasitic drugs: Ivermectin, Praziquantel, Mebendazole, Hadaclean, Others _____

Dosage: _____ g/mL/kg feed or kg shrimp; frequency: once every _____ days

Probiotics: mixed with feed / applied to water (daily or weekly)

Feed application: product name, company, main composition:

Water application: product name, company, main composition:

Supplementation:

Minerals: _____; feeding frequency: once every _____ days

Vitamins: _____; feeding frequency: once every _____ days

Others: _____

Antibiotics

Enrofloxacin _____ Doses and application times/crop

Ciprofloxacin _____ Doses and application times/crop
 Chloramphenicol _____ Doses and application times/crop
 Amoxicillin _____ Doses and application times/crop
 FF _____ Doses and application times/crop
 Sulfonamides +trimethoprim _____ Doses and application times/crop
 Doxycycline _____ Doses and application times/crop
 Oxytetracycline _____ Doses and application times/crop
 Others: _____

Do you know about the banned antibiotic? Who showed you? Say some types if you know?

IV. Health aspect: circle the answer

1. Do the farmer keep records of amounts of chemicals used during operation? **Yes / No**
 2. Are drugs/chemicals administered according to:
 - a. safety instructions described on the package.
 - b. by veterinarian/technicians.
 - c. extensionist.
 - d. experiences.
 3. Do you buy chemical and use it directly or buy it and store it to use later?

 4. Is there a designated storage cabinet for drugs, or are they stored in the house? **Yes / No**
 5. Are gloves used when mixing drugs? **Yes / No**
 6. Are masks or protective equipment used when applying chemicals? **Yes / No**
 7. Is hygiene performed after handling drugs? **Yes / No**
 8. Are there any symptoms such as itching, burns, or eye irritation during drug use? **Yes / No**
- If yes, specify the causative agent: _____

Trends in chemical and drug use:

Increased antibiotic use compared to before: **Yes / No**

Increased disinfectant use compared to before: **Yes / No**

Increased probiotic use compared to before: **Yes / No**

APPENDIX 2: RISK ASSESSMENT QUESTIONNAIRE

This questionnaire was used to collect information on shrimp-consumption habits in Can Tho City, Vietnam for the assessment of consumer exposure to antibiotic residues through whiteleg shrimp consumption. Before the interview, the objectives of the study were explained to participants and verbal informed consent was obtained. Participation was voluntary, and respondents could decline to answer any question. Personal information, including names, addresses, and telephone numbers, was collected only for communication and survey-management purposes and was not disclosed to third parties. Data were anonymized during coding and analysis to ensure participant confidentiality.

At the time of the study, no formal institutional ethical-review procedure was available at Can Tho University for questionnaire-based surveys of this type. Nevertheless, the survey was conducted following general ethical principles for voluntary participation, confidentiality, and responsible handling of personal information.

I. General information

1. Name:
2. Phone number:
3. Address:
4. Job:
5. Health status:
- Body weight:
- Age:
- Gender:
6. Do you follow any diet according to physician or vegetarian?
 - According to physician ☐
 - Not according to physician ☐
 - Vegetarian ☐
 - If according to physician, please specific:.....
7. Number of vegetarians in your family?.....
8. Total number of members in your family?.....
9. Number of children (< 5 years old), and elder (>70 years old)
10. Number of members do not eat or are allergic to shrimp?
11. Market refer

Do not like

Like

Very like

Super Market

Local Market

12. Do you have any knowledge about the risk of antibiotic residues in food?

Yes ☐

No ☐

If yes, could you specific the source of information?

.....

13. Do you think antibiotic/chemical residue is risk to health?

Yes ☐

No ☐

14. Do you apply any method to reduce the risk?

Yes ☐

No ☐

If yes, please specific:

15. What kind of food is the most risk of contamination of antibiotics/chemicals?

Meat ☐

Fish ☐

Shrimp ☐

Vegetable ☐

Other _____

II. Food uptake information for your family?

16. Do you like to eat shrimp?

Do not like ☐

Like ☐

Very like ☐

17. What is the main protein source? How many kg/day and number of eating day/week (per family, latest day and week of eating)? How interesting?

	kg/day	Number of eating day/week	Do not like	Like	Very like
Whiteleg shrimp					
Black tiger shrimp					
Other shrimps					
Freshwater fish					
Marine fish					
Pork					
Beef					

Chicken					
Others					

18. Where is the origin of consuming shrimp?

Supermarket	Local market	Wild shrimp	Others