ANTIBACTERIAL EFFECT OF POSTBIOTICS FROM GUT BACTERIA OF WHITELEG SHRIMP *Litopeaneus vannamei* AGAINST AQUACULTURE BACTERIAL PATHOGENS

ANG CHUN YAO

BORNEO MARINE RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2022

ANTIBACTERIAL EFFECT OF POSTBIOTICS FROM GUT BACTERIA OF WHITELEG SHRIMP *Litopeaneus vannamei* AGAINST AQUACULTURE BACTERIAL PATHOGENS

ANG CHUN YAO

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BORNEO MARINE RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2022

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL : **ANTIBACTERIAL EFFECT OF POSTBIOTICS FROM GUT BACTERIA OF WHITELEG SHRIMP *Litopeaneus vannamei* AGAINST AQUACULTURE BACTERIAL PATHOGENS**

IJAZAH : **SARJANA SAINS**

BIDANG : **BIOTEKNOLOGI MARIN**

Saya **ANG CHUN YAO**, Sesi **2019-2022**, mengaku membenarkan tesis Sarjana ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis ini adalah hak milik Universiti Malaysia Sabah
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/):

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
☑	TIDAK TERHAD	

Disahkan Oleh,

ANITA BINTI ARSAD
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH

ANG CHUN YAO
MY1821015T

(Tandatangan Pustakawan)

Tarikh : 31 Mac 2022

(Dr. Mohammad Tamrin bin Mohamad Lal)
Penyelia

DECLARATION

I hereby declare that this dissertation is based on my original work except for citations and quotations which have been duly acknowledged.

Yao

24 November 2021

ANG CHUN YAO
MY1821015T

CERTIFICATION

NAME : **ANG CHUN YAO**

MATRIX NO : **MY1821015T**

TITLE : **ANTIBACTERIAL EFFECT OF POSTBIOTICS FROM GUT BACTERIA OF WHITELEG SHRIMP *Litopeaneus vannamei* AGAINST AQUACULTURE BACTERIAL PATHOGENS**

DEGREE : **MASTER OF SCIENCE**

FIELD : **MARINE BIOTECHNOLOGY**

DATE OF VIVA : **24 NOVEMBER 2021**

CERTIFIED BY;

Signature

SUPERVISOR
Dr. Mohammad Tamrin bin Mohamad Lal

ACKNOWLEDGEMENT

Firstly, I would like to thank the Centre for Postgraduate Studies and Borneo Marine Research Institute (BMRI) of Universiti Malaysia Sabah (UMS) for providing me the opportunity and resources to carry out this research and to finish my master study. I would like to thank UMS for the teaching assistance scheme (SPP), under the postgraduate assistance scheme for supporting me throughout my master study.

Secondly, I would like to express my gratitude to my supervisor, Dr. Mohammad Tamrin bin Mohamad Lal, who gave me the opportunity to carry out this research with the title “Antibacterial Effect of Postbiotics from Gut Bacteria of Whiteleg Shrimp *Litopenaeus vannamei* Against Aquaculture Bacterial Pathogens”. Starting from the planning of the research project, to submission of the research project proposal for research grant application, next during the conduct of the experiments within the research, and until the writing of this dissertation, he gave his undivided attentions and supports for me to complete this research.

Moreover, I would like to sincerely thank lab assistants of BMRI, Mrs. Veronica, Mrs Lusia and Mr. Herman. Next, I would like to thank the postgraduate seniors of BMRI, Suraini, Anita, and Patricia Pang, who gave guidance to my research. In addition, I would like to thank my friends, Stephanie, Ily, Crystal and Fatin who helped and encouraged me during my research. Lastly, I would like to thank my family members for their financial and mental supports.

Finally, special thanks to the Ministry of Higher Education of Malaysia for funding this research. This research is funded through the Fundamental Research Grant Scheme, FRG0502–1/2019.

Ang Chun Yao

24 November 2021

ABSTRACT

Whiteleg shrimp (*Litopenaeus vannamei*) aquaculture is very important industry in Sabah, but disease outbreak commonly happens. Various disease control strategies such as specific pathogen free postlarvae, tighter disease and effluent management, phage therapy, antibiotic, vaccination, passive immunization, phytobiotic, probiotic, prebiotic, synbiotic and parabiotic have been introduced but each has its own shortcomings. Thus, postbiotics are explored as an alternative disease control strategy. Crude postbiotics were extracted through ethyl acetate from gut bacteria isolates of whiteleg shrimp, namely P17 (*Enterobacter* sp.), P18 (*Bacillus thuringiensis*), P19 and P20 (*Lactobacillus plantarum*), P21 (*Staphylococcus* sp.) and P22 (*Bacillus cereus*). Next, the best crude postbiotics with the most antibacterial effects against aquaculture bacterial pathogens (*Vibrio alginolyticus* ATCC 17749, *Vibrio anguillarum* ATCC 19264, *Vibrio parahaemolyticus* SS07 and *Photobacterium damselae* SS16) were selected for subsequent tests. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected crude postbiotics against the pathogens were determined. Growth curves of *V. parahaemolyticus* SS07 in 1x and 2x MIC of selected crude postbiotics were determined. The effects of temperatures (40, 60, 80, 100 and 121°C) and types of buffers (acetate and phosphate-buffered saline (PBS)) on the antibacterial activities of postbiotics were determined. The antibacterial component of selected crude postbiotics was identified. Safety of the selected crude postbiotics was examined through toxicity testing on brine shrimp (*Artemia*). Crude postbiotics of P17, P18 and P20 were selected, and the MIC and MBC range for the selected crude postbiotics were 10-25% and 10-30%, respectively. The MIC and MBC determined enable optimum dose of postbiotics for maximum disease protection. The exponential growth of *V. parahaemolyticus* SS07 was suppressed at 1x and 2x MIC of the selected crude postbiotics. Antibacterial activities of the selected crude postbiotics maintained at 40-121°C, and in acetate and PBS buffers. Selected crude postbiotics were identified as organic acids. The selected crude postbiotics were toxic to *Artemia* sp.. However, oral administration of postbiotics through feeds might be safe. Therefore, *in vivo* feeding trials of postbiotics is recommended. Postbiotics have the potential as disease control strategy for whiteleg shrimp. However, optimization of the environmental conditions for postbiotics production, organic solvent extraction and purification methods are recommended for accurate future *in vitro* trials.

ABSTRAK

KESAN ANTIBAKTERIA POSTBIOTIK DARI BAKTERIA USUS UDANG PUTIH Litopenaeus vannamei TERHADAP PATOGENIK BAKTERIA AKUAKULTUR

Akuakultur udang putih (Litopenaeus vannamei) adalah industri penting di Sabah, namun penyakit berjangkit kerap berlaku. Pelbagai strategi pengawalan penyakit seperti postlarva bebas patogen, pengurusan penyakit dan efluen yang lebih ketat, terapi phage, antibiotik, vaksinasi, immunisasi pasif, fitobiotik, probiotik, prebiotik, sinbiotik dan parabiotic telah diperkenalkan, namun masing-masing ada kelemahan. Jadi, postbiotik diterokai sebagai strategi pengawalan penyakit alternatif. Posbiotik disari dari pencilan bakteria usus udang putih iaitu P17 (Enterobacter sp.), P18 (Bacillus thuringiensis), P19 dan P20 (Lactobacillus plantarum), P21 (Staphylococcus sp.) dan P22 (Bacillus cereus) melalui etil asetat. Seterusnya, postbiotik terbaik dengan kesan antibakteria terbanyak terhadap patogen bakteria akuakultur (Vibrio alginolyticus ATCC 17749, Vibrio anguillarum ATCC 19264, Vibrio parahaemolyticus SS07 dan Photobacterium damselae SS16) akan dipilih untuk ujian seterusnya. Kepekatan merencat minimum (MIC) dan kepekatan bakteriasid minimum (MBC) postbiotik terpilih terhadap patogen akan ditentukan. Keluk pertumbuhan V. parahaemolyticus SS07 dalam 1x dan 2x MIC postbiotik terpilih akan ditentukan. Kesan suhu (40, 60, 80, 100 dan 121 °C) dan jenis penimbal (asetat dan larutan garam fosfat-bertimbal (PBS)) terhadap aktiviti antibakteria postbiotik akan ditentukan. Komponen antibakteria postbiotik terpilih akan dikenalpasti. Keselamatan postbiotik terpilih akan diperiksa melalui ujian ketoksikan pada udang brin (Artemia). Postbiotik dari P17, P18 dan P20 telah dipilih, dan julat MIC dan MBC untuk postbiotik terpilih masing-masing ialah 10-25% dan 10-30%. MIC dan MBC membolehkan penggunaan dos optimum postbiotik untuk perlindungan maksimum daripada penyakit. 1x dan 2x MIC postbiotik terpilih dapat menindas pertumbuhan eksponen V. parahaemolyticus SS07. Aktiviti antibakteria postbiotik terpilih dikekalkan pada 40-121°C, dan dalam penimbal asetat dan PBS. Postbiotik dikenalpasti sebagai asid organik. Postbiotik terpilih adalah toksik kepada udang brin. Namun, pemberian postbiotik dalam makanan melalui mulut mungkin selamat. Jadi, ujian in vivo untuk postbiotik dalam makanan disyorkan. Postbiotik berpotensi menjadi strategi kawalan penyakit udang putih. Namun, pengoptimuman keadaan persekitaran untuk penghasilan postbiotik, kaedah pengekstrakan pelarut organik dan penulenan disyorkan supaya ujian in vitro lebih tepat pada masa hadapan.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AHL - N-acyl-homoserine lactone

AHPND - Acute Hepatopancreatic Necrosis Disease

ASEAN - Association of Southeast Asian Nations

BLIS - Bacteriocin-like Inhibitory Substances

BLS - Bacteriocin-like Substances

CFS - Cell-free Supernatants

EMS - Early Mortality Syndrome

EPS - Exopolysaccharides

GIFT - Genetically Improved Farmed Tilapia

IL - Interleukin

LAB - Lactic Acid Bacteria

LPS - Lipopolysaccharides

LTA - Lipoteichoic Acid

MBC - Minimum Bactericidal Concentration

MIC - Minimum Inhibitory Concentration

NaCl - Sodium Chloride

NaOH - Sodium Hydroxide

OMP - Outer Membrane Proteins

PBS - Phosphate-buffered Saline

PG - Peptidoglycan

PHB - Poly β-hydroxybutyrate

PL - Postlarvae

P17-O - Crude Postbiotic Extract of Isolate P17 from the Organic Layer

P17-I - Crude Postbiotic Extract of Isolate P17 from the Interphase Layer

P17-A - Crude Postbiotic Extract of Isolate P17 from the Aqueous Layer

P18-O - Crude Postbiotic Extract of Isolate P18 from the Organic Layer

P18-I - Crude Postbiotic Extract of Isolate P18 from the Interphase Layer

P18-A - Crude Postbiotic Extract of Isolate P18 from the Aqueous Layer

P20-O - Crude Postbiotic Extract of Isolate P20 from the Organic Layer

P20-I - Crude Postbiotic Extract of Isolate P20 from the Interphase Layer

P20-A - Crude Postbiotic Extract of Isolate P20 from the Aqueous Layer

RM - Ringgit Malaysia

SCFAs - Short Chain Fatty Acids

SPF - Specific Pathogen Free

TLR - Toll-like receptor

TSA - Tryptone Soy Agar

TSB - Tryptone Soy Broth

UV - Ultraviolet

WPS - Wall polysaccharides

WSSV - White Spot Syndrome Virus

LIST OF SYMBOLS

& - And

~ - Around

°C - Degree Celcius

g - Gram

h - Hour

kDa - Kilodalton

< - Less than

≤ - Less than or equal to

L - Litre

μL - Microliter

μm - Micrometer

mg/mL - Milligram per millilitre

mL - Millimeter

min - Minute

M - Molarity

≥ - More than or equal to

x - Multiply

nm - Nanometer

OD - Optical Density

% - Percentage

± - Plus minus sign

rpm - Revolutions per minute

U/mg - Units per milligram

v/v - Volume concentration

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) aquaculture is very important to Sabah. Production of whiteleg shrimp has contributed RM321.08 million in 2014 (Borneo Post, 2015). Investment in whiteleg shrimp aquaculture in Sabah had been done by four companies, Sunlight Inno Sdn Bhd, KB Aquaculture, Pegagau Aquaculture Sdn Bhd and QL Aquamarine Sdn Bhd in Pitas, Kota Belud, Tawau and Kudat, respectively (Borneo Post, 2015). As of 2020, production of whiteleg shrimp in Sabah has contributed RM201 million to Malaysia economy (Annual Fisheries Statistics, 2000-2020). However, whiteleg shrimp aquaculture is susceptible to many diseases, which might be a reason behind the drop in economic value between the years 2014 and 2020. One of the recent diseases that hit whiteleg shrimp aquaculture is the Acute Hepatopancreatic Necrosis Disease (AHPND), caused by the virulent strain of *Vibrio parahaemolyticus* (Li *et al.*, 2017). AHPND can cause 100% mortality of shrimp postlarvae (PL), 20 to 30 days after ponds stocking (Li *et al.*, 2017).

There are many strategies have been applied to control AHPND such as specific pathogen free (SPF) PL, better disease management and effluent management, phage therapy and use of chemical and antimicrobials treatments (Lakshmi *et al.*, 2013). However, these AHPND control strategies are not able to follow up with the intensive and superintensive culture of shrimp that will become more common (Lakshmi *et al.*, 2013). In the case of antibiotics, antibiotics resistant bacteria can pose threats to human health (Cabello, 2004). Control strategies such as vaccination and passive immunization are effective in reducing mortalities, however, the effect is not long-lasting (Lee *et al.*, 1997; Witteveldt *et al.,* 2004). Phytobiotic, plant extract administered through intraperitoneal injection is efficient, but expensive and can induce stress (Jana *et al.*, 2018). Phytobiotic is dose-dependent and ineffective if correct dosage is not administered (Jana *et al.*, 2018).

Therefore, alternative control strategy such as probiotic is often used. Probiotic is widely used in aquaculture due to its disease control properties. However, probiotic might cause transfer of antibiotic resistance gene to environmental microbes. According to Doron and Snydman (2015), lactic acid bacteria (LAB) contain plasmids with antibiotic resistance gene to tetracycline, erythromycin, chloramphenicol or linco-samide, macrolide, streptomycin, and streptogram. Prebiotic that is used with probiotic to form synbiotic may change the physiology of gastrointestinal tract, and support pathogens (Amenyogbe *et al.*, 2020; Huynh *et al.*, 2017). Rapid development of prebiotic and synbiotic results in lack of careful assessment, strict enforcement and regulation of the production and utilization of the products, and could compromise quality (Amenyogbe *et al.*, 2020). Heat activation on probiotic to produce paraprobiotic causes cells to become rough and coarse, thus reducing adhesion to the gut wall, which then reducing the beneficial effect of the product (Choudhury and Kamilya, 2019). Therefore, another alternative disease control strategy known as postbiotics are currently being explored.

Postbiotics are soluble metabolic products secreted by living bacteria or released after bacterial lysis, that could benefit the host physiologically (Aguilar-Toalá *et al.*, 2018). Postbiotics have clear chemical structures, safety dose parameters, long shelf life and unlike probiotics, there are no antibiotic gene transfer to environmental microorganism, no maintenance of bacteria viability during manufacturing and storage process, much stable throughout different environmental variables, and much safer with potential development of controlled and standardized methods of postbiotics application (Aguilar-Toalá *et al.*, 2018; Phister *et al.*, 2004; Shenderov, 2013; Shigwedha *et al.*, 2014).

However, application of postbiotics in aquaculture field is still limited, with research has only been done in poultry agriculture (Kareem *et al.*, 2016; Kareem *et al.*, 2017). Therefore, this research is conducted with the first objective is to evaluate the antibacterial effect of postbiotic derived from bacteria isolate from the gastrointestinal tract of whiteleg shrimp against aquaculture bacterial pathogen. Second objective is to determine the effect of physicochemical factors on the antibacterial properties of postbiotic. The third objective is to identify the component of postbiotic that is effective against the aquaculture bacterial pathogen. The fourth objective is to examine the toxicity of postbiotic on brine shrimp. This research will bring insight on antimicrobial treatment using postbiotics as novel disease control strategy in aquaculture.

CHAPTER 2

LITERATURE REVIEW

2.1 Whiteleg Shrimp Farming in Sabah, Malaysia

Whiteleg shrimp aquaculture is a very important industry in Sabah. Currently, Sabah has the highest production of whiteleg srimp in Malaysia (Annual Fisheries Statistics, 2000-2020). From 2013 to 2014, the production of whiteleg shrimp in Sabah has reported a hike by 38%, with value of RM321.08 million in 2014 (Borneo Post, 2015). Sabah is keen in developing whiteleg shrimp aquaculture, with four companies, Sunlight Inno Sdn Bhd, KB Aquaculture, Pegagau Aquaculture Sdn Bhd and QL Aquamarine Sdn Bhd had invested RM1.12 billion in setting up shrimp farms in Pitas, Kota Belud, Tawau and Kudat respectively (Borneo Post, 2015). The four farms are expected to produce 110, 870 metric tonnes of whiteleg shrimp by 2020, with RM1.5 billion contribution to Gross National Income (Borneo Post, 2015). As of 2020, production of whiteleg shrimp in Sabah has contributed RM201 million to Malaysia economy, which is the highest contribution among all the states (Annual Fisheries Statistics, 2000-2020).

2.2 Diseases on Whiteleg Shrimp

Whiteleg shrimp is susceptible to various diseases, especially infectious diseases caused by viruses, bacteria, fungi and parasites. The major infectious viral diseases are yellow head disease, infectious myonecrosis disease, infectious hypodermal and haematopoietic necrosis disease, necrotising hepatopancreatitis disease, taura syndrome disease and white spot disease (Manual of Diagnostic Tests for Aquatic Animals, 2016). The major infectious bacterial diseases are luminous bacterial disease, shell disease and filamentous bacterial disease, and the major infectious fungal diseases are larval mycosis, black gill disease (Fusarium disease) and afaltoxicosis (red disease) (Lavilla-Pitogo *et al.*, 2000; Leaño, 2001). The major parasitic diseases are ciliate infestation, microsporidiosis and gregarine infestation (Lavilla-Pitogo *et al.*, 2000).

One of the recent and emerging infectious diseases that has affected whiteleg shrimp in Malaysia is the Acute Hepatopancreatic Necrosis Disease (AHPND) or previously known as Early Mortality Syndrome (EMS), which first appeared in China, and has spread to Malaysia in 2011 (Li *et al.*, 2017). AHPND is caused by specific virulent strains of *V. parahaemolyticus*, that cause 100% mortality to shrimp postlarvae (PL), 20 to 30 days after pond stocking (Li *et al.*, 2017). According to The Star (2013), AHPND has caused huge losses to local shrimp farmers in 2013, with a case in Johor where 100% of the shrimps in seven ponds died due to the infection. The occurrence of AHPND in Malaysia might be the main cause of general trend of production drop of whiteleg shrimp after 2014 in Malaysia, as shown by the top 3 producing states of whiteleg shrimp in Figure 2.1.

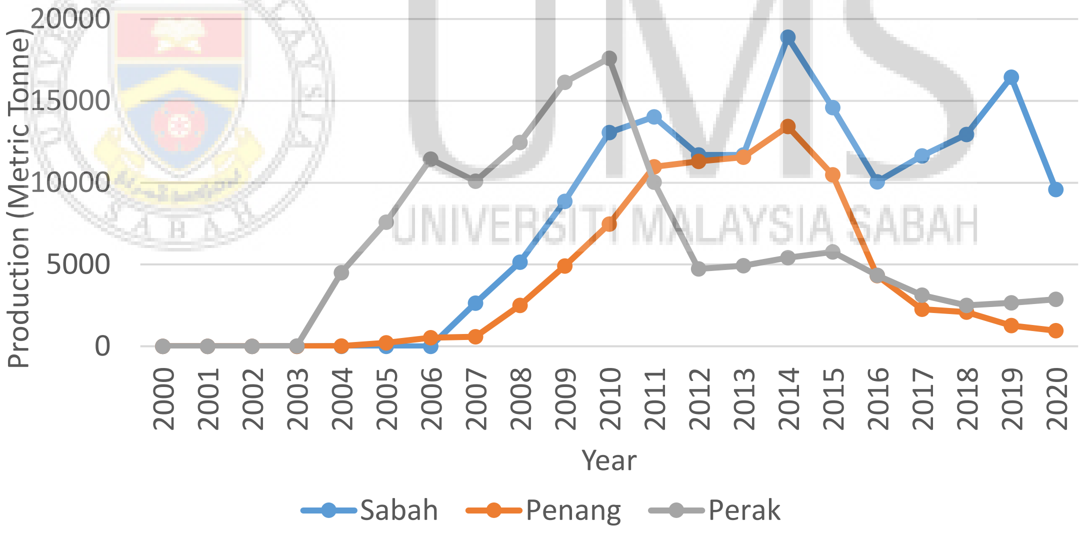

Figure 2.1: Top three whiteleg shrimp producing states in Malaysia from 2000 - 2020 in metric tonne.
Source : Annual Fisheries Statistics (2000-2020)

2.3 Challenges and Limitations of Control Strategies in Shrimp Aquaculture

The common strategy to control disease outbreak in shrimp are the use of Specific Pathogen Free (SPF) PL, disease management, effluent management, phage therapy, chemical and antimicrobial agents. Specific Pathogen Free (SPF) means the postlarvae (PL) is diagnosed for specific disease, and once it is certified free, then it will be supplied to farmers. Better disease and effluent management involve treatment of pond effluent and proper disposal of dead shrimps from the cultured ponds. Wastewater and effluents are allowed to settle in settlement ponds for a while before being released. Filter feeders such as tilapias and milkfish can be cultured in the settlement ponds to feed on suspended particles from wastewater and effluents. Additionally, effective microorganisms can be used as well to recycle the sludge and use it as fertilizer. Disposing dead shrimps from cultured ponds properly is important because improper disposal causes transmission of disease (Lakshmi *et al.*, 2013).

Phage therapy is a bacterial disease control strategy that uses bacteriophage. Once bacteriophage infects pathogenic bacteria, it lyses and reduces the survival of the pathogenic bacteria (Lakshmi *et al.*, 2013). Chemicals such as sodium hypochlorite, iodine and formalin are used as disinfectant (Lakshmi *et al.*, 2013; *Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). Formalin can be used as parasitic and fungal control agent in fish and shrimp as well, in accordance to dosage approved (Lakshmi *et al.*, 2013; *Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). However, formalin is a toxic substance that may cause occupational health hazards such as irritation to respiratory system, damage to brain and nervous system and cause blindness (*Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). Copper sulphate is used to control parasitic diseases such as ichthyophthiriasis and saprolegniasis, but must be according to approved dosage (*Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). Sodium thiosulphate can be used during culture system preparation, for neutralization of chlorine in water after disinfection (Lakshmi *et al.*, 2013; *Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). Pesticides or piscicides such as saponin, roteonone and organophosphate are commonly used as well (Lakshmi *et al.*, 2013; *Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). The common organophosphates used are dichlorvos and trichlorfon (dipterex, neguvo) (*Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals*, 2013). The