Cinnamon Essential Oil (EOCIN) Functional Diet: Effect on Growth Performance and Health Status of *Penaeus vannamei* in Super-Intensive Tank Culture

Padilah Bakar [1*], Rohaiza-Asmini Yahya [1] and Kua Beng Chu [2]

[1] *National Fish Health Research Centre, 11960 Batu Maung, Penang, Malaysia*

[2] *Fisheries Research Institute, 11960 Batu Maung, Penang, Malaysia*

[*]*Corresponding author: padilah@dof.gov.my*

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Abstract

A field trial was conducted to assess the application of cinnamon essential oil (EOCIN) in the super-intensive culture of white shrimp (*Penaeus vannamei*). The objective of this study is to determine the efficacy of EOCIN on growth performance and prophylaxis in prevention against two common diseases in farmed shrimps, specifically acute hepatopancreatic necrosis disease (AHPND) and *Enterocytozoon hepatopenaei* (EHP). The EOCIN dose regime, set at 1.5% (v/w kg feed), was administered for 14 days in two series of application. The experiment was carried out in two replicates for the EOCIN and control involving four (4) tank cultures, 34 m in diameter with estimation of 300,000 pieces of shrimps per tank at stocking density of 300 pieces/m^2 in a single cycle of culture production. An increased in weight gain of shrimp from EOCIN group was recorded compared to control, particularly in grow-out shrimps at DOC40-56 after completion of 28-days of EOCIN application. Shrimp Specific Growth Rate (SGR) showed a compensatory growth of 4.5856% day^{-1} from the EOCIN group compared with control, 4.0586% day^{-1}. Average daily weight gain for EOCIN (0.2328 g/day) was higher than control (0.1733 g/day), with survival rates of 80% and 75%, respectively. The FCR for EOCIN was lower (1.503) compared to control (2.014). The EOCIN additive regime used in this study improved the growth performance of white shrimps in particular, prevention against AHPND and control low prevalence of EHP infection detected in the early culture.

Keywords: EOCIN, feed additive, growth, health, white shrimp

Introduction

For decades, humans rely on natural ingredients from herbal and plants for remedy in treatment of various health problems in humans and animals. Commercial essential oils such as cinnamon, garlic, lemongrass, lime and lemon are well-known with their broad range of

antibacterial, antiviral and antifungal properties and as such can be utilised as antimicrobial agents (Tariq et al., 2019; Purkait et al., 2020). Essential oils have also been shown to inhibit the growth of drug-resistant microbial strains which makes it even more challenging to be treated by conventional antibiotics (Tariq et al., 2019). Essential oils are regarded as GRAS (generally regarded as safe) grade chemicals by the U.S. Food and Drug Administration (FDA), hence, continuous treatment as preventive measures or supplement reduces the likelihood of resistance towards drug/biochemical and less side effects of toxicity issues while utilizing organic compound for a long period of time.

Research and development studies on antimicrobial effects of herbal/plant extracts and their uses (benefits and treatment) to cultured aquatic animals are still at the infant stage. Currently, few commercial essential oils known to contain antimicrobial properties have been used as feed additive and appetite enhancer to improve growth performance and prevent disease outbreak in fish and shrimp culture (Burt, 2004; Ghafoor, 2020; Bandeira Junior et al., 2022). Study has shown that cinnamon and clove oil did not show any cytotoxic effect at dosage level from 1000 to 2000 μg mL^{-1} (Purkait et al., 2020). Such results evince that cinnamon and clove oils, used separately, or in combination, are a potential source of safe and effective natural antibacterial, antifungal and antioxidant blend in animal-feed and other pharmaceutical products (Purkait et al., 2020).

The *in vitro* antibacterial effect of essential oil has been reported against bacteria isolated from fish, such as *Citrobacter freundii* and *Aeromonas hydrophila* (Öntaş et al., 2016; Snuossi et al., 2016; Majolo et al., 2017). Supplementation of 1 mL of cinnamaldehyde per kg of diet in Nile tilapia fingerlings improved the feed conversion ratio (FCR) (days 46–60) and increased body weight gain and feed intake (days 61–75) (Amer et al., 2018). In another study, an improvement in growth performance was reported in white shrimps (*P. vannamei*) culture supplemented with plant derived essential oils blend in diet and a lower mortality rate when challenged against *Vibrio parahaemolyticus* bacteria causing acute hepatopancreatic necrosis disease (AHPND) compared to the positive control (unsupplemented) which received no treatment (Hoa et al., 2023). The *Vibrio* spp. count in the hepatopancreas of shrimps fed with 2 g kg^{-1} of the essential oil blend was marked lower compared to that of the control.

In the aquaculture industry, cinnamon (*Cinnamomum zeylanicum*), is one of the oldest medicinal plants widely used globally, where it is used in the form of powder or essential oil (El-Hack et al., 2020). Essential oil extracts possess good antimicrobial effects against *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Listeria monocytogenes, Staplylococcus aureus* (Purkait et al., 2020). In another report, it is reported that essential oils are effective against *V. parahaemolyticus, Escherichia coli, Staphylococcus epidermidis* and *Enterococcus faecalis* (Chang et al., 2001) as well as promoting the stimulating effect on the digestive system (Ertas et al.,

2005). Hence, these herbs and their extracts received more attention as growth promoter alternatives.

AHPND in Malaysia was first detected in 2011 and has been linked with high mortalities of cultured white shrimps (*P. vannamei*). Following the outbreaks from 2011 to 2014, a disease surveillance program was implemented by the Department of Fisheries Malaysia (DOF) with active involvement of farmers and other stakeholders to monitor and control disease outbreaks in shrimp farms. A study on the status of AHPND in 2019/2020 showed that State of Pahang, among the major producers of white shrimp culture, had the highest prevalence of AHPND (23%), followed by Perak (11%), Sarawak (7%) and Kedah (1%) (Padilah et al., 2022). The disease is known to be endemic in nature due to the presence of disease agent *V. parahaemolyticus* and other *Vibrio* spp. carrying virulent plasmid and *PirA/B* toxin genes in the aquatic environment. Presently, AHPND has been under control through management approach of creating healthy shrimps fed with vitamins and/or other health supplement diet, as well as good management of water quality in the pond culture.

Countries in Southeast Asia such as China, Vietnam, Thailand and Malaysia have suffered from *Enterocytozoon hepatopenaei* (EHP) outbreaks during the last decade and eventually, have also had AHPND outbreaks every now and then (Aranguren et al., 2017). EHP causes growth retardation and increased size variability, and in more advanced stages, the infected shrimp have soft shells and exhibit lethargy, reduced feeding and empty midguts. It is possible that EHP favours the establishment of AHPND and other bacterial diseases, such as Shrimp Hepatopancreatic Necrosis (SHPN) and White Faeces Syndrome (WFS). The strong association was found between cases of SHPN and EHP which suggests that EHP increases shrimp susceptibility to infection by *Vibrio* spp. (Aranguren et al., 2017). EHP infection disrupts the cells of the hepatopancreas tubules and allows *Vibrio* spp. to colonize the sloughed cells and the exposed basement membrane leading to the state of illness or infections (Aranguren et al., 2017).

In the late 2000s, shrimp farmers implemented more intensive practices known as super-intensive farming technologies, driven mainly by the search for greater shrimp farm productivity. The super-intensive system for white shrimp, *P. vannamei*, as one that uses lined ponds, raceways, or tanks for stocking densities of over 150 shrimp per m^2 during growth and applies a significant level of technology. While commercial intensive shrimp systems in Asia have shown good results (Taw, 2017), the commercial economic viability of super-intensive systems has not yet been proven. The challenges in intensive shrimp systems include prevalence of diseases, feed efficiency, energy use, and genetic selection in terms of the organism and environmental, social, and welfare issues for the farmers (Villarreal & Juarez, 2022). Diseases were responsible for a significant shrimp production decline from 2009 to

2014, when around 1 million tons were lost, mainly in Asia and Latin America. White spot syndrome virus (WSSV/WSD), AHPND, EHP, and WFS, have impacted the industry with substantial economic losses (Shinn et al., 2018), so that producers look for more controlled intensive systems with higher biosecurity in an effort to mitigate this risk.

Feeding shrimps with functional diets such as mixed essential oils and herbs can be an advantageous strategy to mitigate infectious diseases, consequently, reduce the rise of antibiotic resistance problems of pathogenic bacteria towards treatment and build an environmentally friendly and sustainable shrimp aquaculture. Diet supplemented with a mixture of essential oils from thyme and cinnamon significantly improved the resistance of *P. vannamei* towards AHPND (Cabanyero et al., 2023). Survival of shrimps challenged with *V. parahaemolyticus* increased when they were fed with essential oils-enriched diet for 4 to 5 weeks. This feeding strategy also reduces the percentage of 'carrier' after induced infection or in the stressful situation (Cabanyero et al., 2023).

A field trial on oral diet of EOCIN in *P. vannamei* culture was carried out to determine the efficacy of EOCIN dose-regime used on the growth performance and health status of white shrimp culture in super-intensive tank system, in particular against AHPND and EHP.

Materials and Methods

Field trial and sampling size

White shrimps were cultured at stocking density of 350 pieces per m^2 in 4 large round PVC tank, 34 meter wide with capacity of 1000 tonnes of water and estimation of 300,000 pieces of post larvae (PL-10) per tank at initial stocking. The study was carried out in two replicates for control (2) and EOCIN (2). The control groups were fed with a normal commercial based diet whereas the EOCIN group were fed with EOCIN mixed in feed pellet at 1.5% v/w kg of feed (3% body weight) for 14 days in two series of application at the age day of culture (DOC)1-14 and DOC40-54. A total of 210 white shrimps were sampled from the post larvae shrimps (PL-10) before stocking (n=10), the control (n=100) and EOCIN (n=100) with 6 times of sampling throughout the 56 days of culture. The second sampling was carried out after completion of the 14 days of EOCIN application from EOCIN group (2 tanks, n=20) and control (2 tanks, n=20) at day of culture-16 (DOC16, total, n=40), followed by sampling of shrimps before the second application of EOCIN started at DOC40 (n=40), during treatment, DOC45 (n=40), DOC47 (n=40) and DOC56 (n=40) after completion of treatment. Sampling of shrimps were carried out in the morning before feeding. Sample of hepatopancreas tissues was fixed in absolute alcohol for PCR analysis of AHPND and EHP, whereas the presence of bacteria in the hepatopancreas organs of shrimp was isolated using direct inoculation method on the culture media.

EOCIN treatment regime and preparation in feed pellet

The feeding regime of EOCIN was given at 1.5% v/w kg feed (3% BW) for 28 days in two series of applications of 14 days regime with 25 days interval between them. The first feeding of EOCIN was given for 14 days continuously right after the admission of post larvae shrimps into the super-intensive tank culture (DOC1-14) followed by the second regime of treatment at DOC40-54. Around 1.5 mL of EOCIN was mixed in 50 mL of sterile water and sprayed evenly onto 15 kg of commercial based feed pellet (prawn feed grower) before feeding to the shrimps. The control shrimps were fed with a commercial pellet-based diet throughout the study and the EOCIN group shrimps were fed with EOCIN mixed pellet from DOC1-14 and DOC40-54 whereas for other days, they were fed with normal commercial based diet. The shrimp was fed four times per day at 7-7:30 am, 10:30-11:00 am, 1:30-2:00 pm and 5:00-5:30 pm. The composition of a commercial prawn feed grower pellet used in the study is shown in Table 1.

Table 1. The composition of commercial prawn feed grower pellet.

No.	Composition of grower pellet	Percent (%)
1.	Crude Protein (min)	40-44%
2.	Lipid (fat)	6%
3.	Moisture (max)	12%
4.	Crude fibre (max)	3%
5.	Ash (max)	15%

CTAB-DTAB DNA extraction protocols

A method described in the manual for IQ2000™ AHPND/EMS Toxin 1 was adhered to (GeneReach Biotech. Corp. Taiwan). Briefly, sampled of 10 PLs and hepatopancreas tissue of shrimps from DOC16 onward, weighing about 15-30 mg in the 1.5 mL tube were grinded until all tissues are thoroughly mixed and dissolved into 600 µl DTAB solution. The tissues in a tube was incubated at 75 ^{o}C for 5 minutes, which was proceeded to cool down to room temperature, vortexed briefly, then added with 700 µl of chloroform. It was then vortexed again, with the mixture centrifuged at 12,000 x g for 6 minutes. About 200 µl of the upper aqueous phase was transferred into a new tube, then added with 100 µl CTAB solution and 900 µl deionized water, vortex briefly and incubated at 75 ^{o}C for 5 minutes. The sample was cooled down to room temperature, then centrifuged at 12,000 x g for 10 minutes. The supernatant was discarded, the pellet was re-resuspended with 150 µl dissolving solution, followed by incubation at 75 ^{o}C for 15 minutes, cooled down to room temperature and centrifuged at 12, 000 x g for 5 minutes. The clear solution was transferred into a new tube containing 300 µl of 95% ethanol, vortex briefly and centrifuged at 12,000 x g for 5 minutes. The supernatant was discarded, the pellet was washed with 200 µl of 75% ethanol, centrifuged at 12,000

x g for 3 minutes. After the disposal of the supernatant, the DNA pellet was dried and dissolved in 100-200 µl of deionized water or TE buffer. The DNA sample was stored at -20 °C until use for further analysis.

Polymerase chain reaction (PCR): Reagent and amplification protocol for AHPND

Pre-mix reagent (12.5 µl) and IQzyme DNA Polymerase (2U/µl; 0.5 µl) was prepared for a total of 13 µl/reaction per test. Samples was run with 2 positive controls (10^3 and 10^2) and 1 template control (NTC; deionized water or yeast tRNA). The reaction condition for thermocycler was set as the following: 94 °C 20 sec; annealing 62 °C 20 sec; 72 °C 30 sec, repeat 35 cycles, 72 °C 30 sec final extension; 20 °C 30 sec at the end of the final cycle.

PCR EHP

Method described by Jaroenlak et al. (2016) was used for detection of spore wall protein gene-specific for EHP (SWP-PCR). The primer sequence for EHP was: First PCR: 1F-TTGCAGAGTGTTAAGGGTTT; 1R-CACGATGTGTCTTTGCAATTTTC; Nested PCR primer; 2F-GCTGTTTGTCTCCAACTGTATTTGA; 2R-TGGCGGCACAATTCTCAAACA and the amplification protocol for EHP was according to the following: **First PCR**; 94 °C 2 min; 94 °C 20 sec, annealing 60.2 °C 20 sec; 72 °C 30 sec; repeat 35 cycles; Final extension 72°C 4 min; **Nested PCR**: 94 °C 2 min, annealing 58.3 °C 20 sec, 72 °C 30 sec; final extension 72 °C 4 min; 20 °C 30 sec at the end of the final cycle.

Electrophoresis, gel staining and image documentation

A 2% agarose gel was prepared by adding 2 g of agarose gel with 100 ml TBE buffer in a wide mouth glass bottle. The mixture is then heated in the microwave oven for 1.5 to 2 minutes until it becomes hyaline which consequently leads to its cooling to 50 °C before pouring into the gel cast with the plastic comb and blockers. For electrophoresis, 5 µl of PCR product-loading dye mixture was loaded into each well with DNA molecular weight marker serve as a reference for PCR product size. Electrophoresis was run at a constant voltage of 100 V for 30 minutes and viewed under Bio-imaging system (Syngene, Cambridge, UK). For AHPND, negative sample showed only the 712 bp band (internal control) and AHPND plasmid negative whereas a band at 218 bp and 432 bp indicate a positive sample with toxin 1 and virulent AHPND plasmid. A band at 432 bp only (with or without 712 bp band) indicates AHPND plasmid with toxin 1 deletion, non-virulent. For EHP, positive sample was categorized into heavy and light infections based on detection of amplicon at 514 bp (heavy) or 148 bp (light).

Bacteria culture and isolation

Direct inoculation method was used for isolation of bacteria from the hepatopancreas organ. Biochemical analysis for identification of isolates was done following the method described by Austin & Austin (1999). Pure isolate from tryptone soya agar (TSA+ 1.5% NaCl) plate was identified by routine testing using Gram staining, oxidase, catalase, sulfur, indole, motility test (SIM) and the triple sugar iron (TSI) test. Colony morphology of *Vibrio* spp. was determined from pure isolate culture of bacteria on thiosulfate citrate bile salt agar (TCBS) and biochemical test for confirmation to genus and species using API 20NE (BioMérieux, Marcy IEtoile, France).

Calculation of growth parameters

To assess the growth performance of the shrimp in the feeding experiment, the following formulas are used:

a) Daily weight gain (DWG, g day^{-1}) = ($W_f - W_i$)/ t (rearing period, day)

$$DWG = \frac{Wf - Wi}{t} \quad \textbf{(Equation 1)}$$

where,

W_i = initial average weight (g)

W_f = final average weight (g)

t = rearing period (day)

b) Specific growth rate (SGR, % day^{-1}) = [Ln(W_f) − Ln(W_i)] * 100/t (rearing period, day)

where,

W_f = the final average weight (g)

W_i = the initial average weight (g) of shrimps during the rearing period (day).

t = rearing period (day)

c) Survival Rate (SR, %) = (final population of shrimp at harvest/initial population of post larvae stocked) x 100. The SR of *P. vannamei* shrimp is the survival rate during one cultivation cycle.

$$SR = \frac{Nt}{No} \times 100 \quad \textbf{(Equation 2)}$$

where,

SR = Survival rate (%)

Nt = Final population (individual)

N_o – Initial population (individual)

d) Feed conversion ratio (FCR) = feed given/fish weight gain

Feed conversion ratio (FCR) is a unit to desribe a certain feed amount to earn one kilogram of biomass. FCR was calculated using the formula below:

$$FCR = \frac{F}{[(Bt+Bm)-Bo]} \quad \textbf{(Equation 3)}$$

where,

FCR = Feed conversion ratio

F = Total feed amount (g)

B_t = Final biomass (g)

B_m = Deceased biomass (g)

B_o = Initial biomass (g)

Calculation of bacterial prevalence (%) and statistical analysis

The prevalence of bacteria identified from the white shrimps were calculated as mean prevalence percentage (%). The prevalence (P) was estimated by dividing the number of samples that tested positive (S_I) for the specific bacterial divide by the total number of samples x 100 from each sampling group (S_N), P= S_I/ S_N x 100. Descriptive statistics, independent t-test was used to describe the results of bacterial (n) isolated from EOCIN and control. Body weight gain was analysed as measurement on growth performance using IBM SPSS Statistics Version 25, (2017) for significant difference ($p \leq 0.05$) between the control and EOCIN fed group.

Results and Discussion

Growth performance and body weight measurement

Higher daily weight gain was recorded in white shrimps from the EOCIN group compared with the control from DOC40-56. The EOCIN treated shrimps had higher average final body weight gain (BW = 13.09±2.20) than the control (BW = 9.75±1.99), however, statistical analysis using independent Mann-Whitney U test showed no significant difference ($p=0.280$, $p>0.05$) in body weight gain between the two groups after 56 days of culture. The difference in average body weight gain between EOCIN and control are shown in Figure 1. Higher body weight gain was recorded from DOC40-56 from shrimps that received EOCIN diet, but the overall growth performance did not reach the optimum potential due to light infection of EHP that was detected in the post-larvae shrimps (PL10, 10%) and white shrimps from EOCIN group at day-16 of the culture (DOC16, 20%). Results of growth performance analysis of white shrimps between EOCIN treated group dan control are

shown in Table 2. EHP infection is known to cause slow growth and increase variation in size.

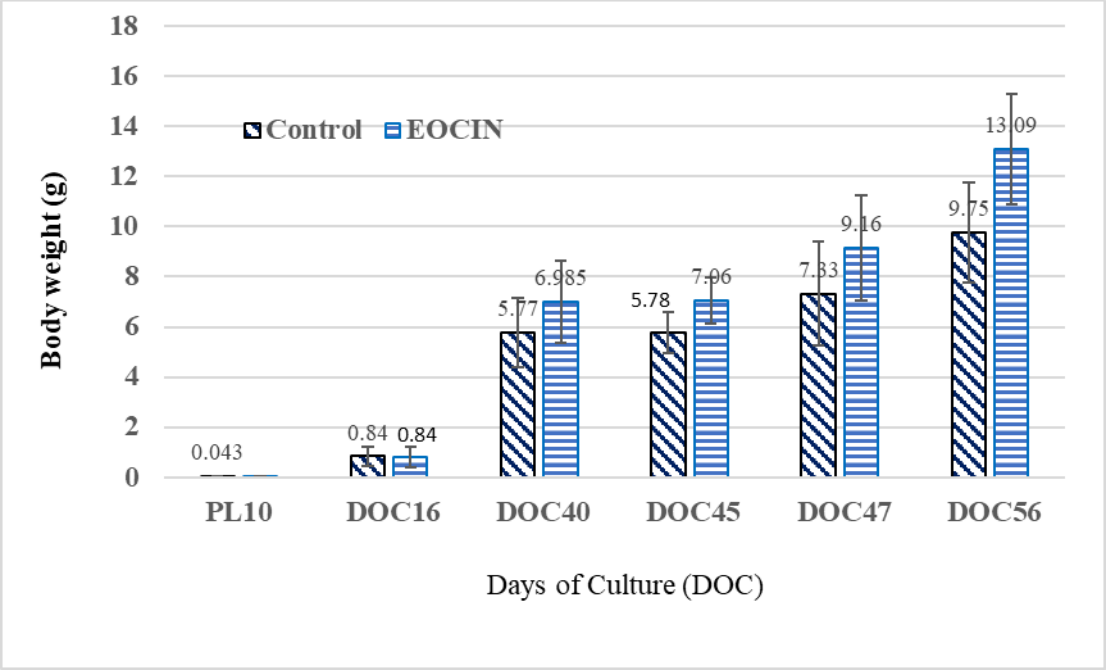

Figure 1. Comparison of body weight gain of *P. vannamei* culture between EOCIN treated group and control.

After completion of EOCIN treatment, The SGR values showed that the compensatory growth does occur in shrimps treated with EOCIN (4.5856% day^{-1}) compared to control (4.0586% day^{-1}) (Table 3). Average daily weight gain (DWG) for EOCIN (0.2328 g/day) were also higher than the control (0.1733 g/day). Shrimps treated with EOCIN showed higher feed efficiency (FCR, 1.503) although it is common to obtain the FCR values of 1.5 to 2.5 in *P. vannamei* culture. EHP infection was suspected by farmers when FCR was higher and DWG of cultured *P. vannamei* shrimp was below 0.35 g day^{-1}. Normal average daily gain for shrimp would be about 0.25g up to 0.3g/per day (Hoa et al., 2023). The control group had higher FCR (2.014), showed by lower body weight gain and increase variation in size (Figure 1) suspected due to EHP (infected PL) although the control shrimps was tested negative at DOC16-56.

FCR values vary with the production system and the type of feed used. FCR of 1.4-1.8 are generally obtained from intensive whiteleg shrimp farming and FCR of 1.8 is used by FAO Fisheries and Aquaculture department to compare with the existing efficient management (Briggs, 2006). FCR as low as 1.2 can be achieved when applying a superior quality of feed, yet many shrimp farmers experience FCR of higher than 2.2 (Chaikaew et al., 2019; ASEAN, 1998). Low FCR values can be ascribed by various reasons, such as the strict control of feeding in closed system (Dien et al., 2018), partial harvest and shorter period of culture for reaching harvest size (Thakur & Lin, 2003), and seasonal variation (Teichert-Coddington, Martinez & Ramírez, 2000). On the contrary, Lim (2010)

listed five common mistakes that lead to the high FCR: usual high-water temperature, overfeeding, over frequent feeding, fast water current from aerators, and insufficient aeration. A high FCR of 2.0 in the control group indicates that this farm requires better feed management strategy such as EOCIN supplement regime in diet to enhance shrimp appetite and their growth performance.

Table 2. Growth performance analysis of white shrimps between EOCIN treated group and control.

Day of culture (DOC)	**DOC1-56**	
Group	**Control**	**EOCIN**
1. Mean Initial weight	0.043±0.014	0.043± 0.017
2. Mean Final weight	9.75±1.99	13.09±2.20
3. Average daily weight gain (DWG)	0.1733	0.2328
4. Specific growth rate (SGR% day^{-1})	4.0586	4.5856
5. Survival rate (SR%)	75	80
6. Feed conversion ratio (FCR)	2.014	1.503

Table 3. The prevalence of AHPND and EHP in *P. vannamei* between control and EOCIN.

			EOCIN		**Control**	
Trial 1	**No of samples**	**Age**	**AHPND**	**EHP**	**AHPND**	**EHP**
1	10	PL10	0(0/10)	**10(1/10)**	0(0/10)	**10(1/10)**
2	40	DOC16	0(0/20)	**20 (4/20)**	0(0/20)	0(0/20)
3	40	DOC40	0(0/20)	0(0/20)	0(0/20)	0(0/20)
4	40	DOC45	0(0/20)	0(0/20)	0(0/20)	0(0/20)
5	40	DOC47	0(0/20)	0(0/20)	0(0/20)	0(0/20)
6	40	DOC56	0(0/20)	0(0/20)	0(0/20)	0(0/20)
Total	210	Prevalence (%)	0%	**10-20%**	0	**10%**

FCR values vary with the production system and the type of feed used. FCR of 1.4-1.8 are generally obtained from intensive whiteleg shrimp farming and FCR of 1.8 is used by FAO Fisheries and Aquaculture department to compare with the existing efficient management (Briggs, 2006). FCR as low as 1.2 can be achieved when applying a superior quality of feed, yet many shrimp farmers experience FCR of higher than 2.2 (Chaikaew et al., 2019; ASEAN, 1998). Low FCR values can be ascribed by various reasons, such as the strict control of feeding in closed system (Dien et al., 2018), partial harvest and shorter period of culture for reaching harvest size (Thakur & Lin, 2003), and seasonal variation (Teichert-Coddington, Martinez & Ramírez, 2000). On the contrary, Lim (2010) listed five common mistakes that lead to the high FCR: usual high-water temperature, overfeeding, over frequent feeding, fast water current from aerators, and insufficient aeration. A high FCR of 2.0 in the control group indicates that this farm requires better feed management strategy such as EOCIN supplement regime in diet to enhance shrimp appetite and their growth performance.

Bacterial prevalence (%)

Gram negative bacteria were commonly isolated from hepatopancreas of white shrimps with highest prevalence in the control group consisting of *V. parahaemolyticus* (35-60%), *V. vulnificus* (40-60%) and *A. hydrophila/caviae* (20-26.7%). *V. parahaemolyticus* (15-20%) and *A. hydrophila* (10-15%) were isolated at much lower prevalence following EOCIN treatment at Day-16, Day-47 and Day-56 of culture. Although prevalence of *V. vulnificus* (10-13.3%) was much lower in EOCIN group, the result was inconsistent throughout the study since a high prevalence was detected at DOC56 (60%) after completion of 28 days of treatment (Figure 2). Other bacteria isolated were *Pasteurella multocida* (6.7-20%) and *Mannheimia haemolytica* (5%) with low prevalence.

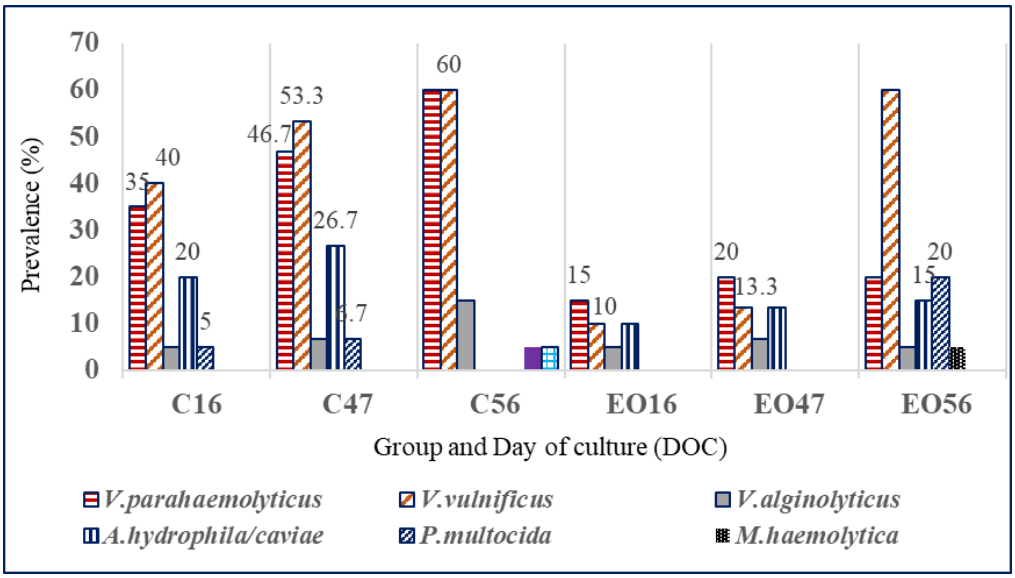

Figure 2. Bacterial prevalence from white shrimps in control (C) and EOCIN (EO) at different day of culture (DOC).

Analysis for the Levene test showed that p≥0. 05 (p=0.796), whereby the variance (*V. parahaemolyticus*) is not significantly different from each other in group, hence the homogeneity of the variance is met. Independent sample test showed the p-value of 0.03 (p≤0.05) indicating that there was a significant difference in *V. parahaemolyticus* prevalence isolated from EOCIN and control shrimps. Statistical analysis in the prevalence of other bacteria (*V. vulnificus, V. alginolyticus* and *A. hydrophila*) isolated from shrimps following EOCIN treatment showed no significant difference (p≥0.05) from the control.

In this study we determine the microbial isolated from the hepatopancreas of white shrimps to see their connections with the disease incidence, in particular AHPND. Estimation of bacterial prevalence is fundamental in determination and management of population health, disease dynamics and infections risk factors. It is known that members of *Vibrionaceae* are the natural pathogens of the aquatic marine environment. Microbiota of shrimp's digestive system which includes the stomach, hepatopancreas and intestine are closely related to the environmental condition, as well as host developmental stage and health status of shrimp culture (Cheung et al., 2015; Cornejo-Granados et al., 2018). High prevalence of *V. parahaemolyticus* opportunistic pathogen in the gut microbiota will increase the risk of the shrimps to infection following the colonization of bacteria on the epithelial cell walls. Thus, presence of *V. parahaemolyticus* in the hepatopancreas organ pose a high risk for the microbe to invade hepatopancreas cells under any stressful conditions. *Vibrio* spp., in particular *V. parahaemolyticus* pathogen carrying virulent factor 'Plasmid' and *PirA/B* toxin can cause AHPND which has a possibility of resulting in high mortality rates of susceptible shrimp aged below DOC30 (Lightner et al., 2012). EOCIN treatment regime at early culture (DOC1-14) intended to boost the immune function, prevent stress and reduce the prevalence of *V. parahaemolyticus*, hence, reduce the risk of AHPND whereas EOCIN treatment at juvenile/grow-out stage (DOC40-56) help to boost the growth performance.

The effectiveness of cinnamon essential oil as antimicrobial agent was studied by many researchers. Antimicrobial activities of EOCIN inhibit *Vibrio* spp. from colonizing in the hepatopancreas organ of white shrimp. Our study showed that EOCIN treatment regime used helps to control AHPND and EHP in the early stage of culture and juvenile shrimp. Minimum inhibitory concentration of EOCIN at 1400 µg mL^{-1} and minimum bactericidal concentration (MBC) at 1500 µg mL^{-1} able to suppress the biofilm formation and swarming motility of *V. parahaemolyticus,* thus indirectly affecting the virulence activities of this bacteria (Domínguez-Borbor et al., 2020).

Conclusion

EOCIN has a promising use in aquaculture. A holistic approach is required in relativity in evaluating the potential use of EOCIN as functional diet for shrimp, particularly towards the control of *V. parahaemolyticus* AHPND bacterium and microsporidia protozoa EHP. EOCIN regime in two

applications at 1.5% (v/w kg feed) for 14–28 days are proven effective to promote growth and improve health status of white shrimp culture. EOCIN improves the growth performance of white shrimps as shown by an increase in DWG and SGR %/day, increase survival rate and reduced FCR. The management approach during culture by using alternative therapeutics, such as EOCIN, coupled with improvements in water quality and regular fish health monitoring program may bring about reductions in bacteria and EHP in the aquatic culture system. EHP is the main problem encountered in many shrimp farms today. Disinfection of the tank culture or pond bottom treatment prior to culture can effectively kill all the spores of EHP and pathogenic bacteria. Low prevalence of EHP in the early stages of culture was managed with EOCIN treatment and improved farm management by practicing appropriate stocking density, partial harvest of culture, adoption and maintenance of hygienic practices, and implementing strict biosecurity measures to control the spread an infection. EOCIN treatment is proven to reduce the prevalence of *Vibrio* sp., particularly the colonization of *V. parahaemolyticus* in shrimp hepatopancreas tissues. Field trials carried out showed that EOCIN treatment regimens helped control AHPND and EHP in the post-larvae and juvenile stages if treated while at low prevalence.

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Appendices

Annex 1

Calculation of FCR

1) Control, FCR, 1: 2.014
Feed per day: 60.59 kg, Total carrying capacity: 2.654kg
Total feed: 4544.9 kg
Total product: 2255.9 kg
Partial harvest at DOC79 (76 pcs/kg), 691.9 kg;
DOC80 (76 pcs/kg), 1032 kg and DOC87 (70pcs/kg), 532 kg.

2) EOCIN, FCR, 1: 1.503
Feed per day: 48.5 kg, Total carrying capacity: 4.74kg
Total feed: 4273.9 kg
Total product: 2844 kg
Partial harvest DOC76 (70 pcs/kg); 770 kg
DOC78 (68 pcs/kg); 1042 kg and DOC88 (51 pcs/kg); 1032 kg

Annex 2

Levene's Descriptive statistics for *V. parahaemolyticus*

Parameter	EOCIN	Control
Mean	2.3333	4.3333
Standard Deviation	1.2111	1.5055
Standard Error	0.4944	0.6146
N	6	6

Independent Sample test

Levene's test for equality of variances				t-test for equality of means						
V. parahae-molyticus		F	Sig	t	df	Sig (2-tailed)	Mean difference	Standard error difference	95% confidence interval of the difference	
	Equal variances assumed	0.070	0.796	2.535	10	0.030	-2.000	0.7888	-3.7576	-0.2424
	Equal variances not assumed			2.535	9.561	0.031	-2.000	0.7888	-3.7686	-0.2314