REQUIREMENTS AND EFFECTS OF VITAMIN C AND E ON NON-SPECIFIC IMMUNE RESPONSES AND DISEASE RESISTANCE IN HYBRID GROUPER *Epinephelus fuscoguttatus* × *E. lanceolatus*

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DECLARATION

I hereby declare that the materials in this thesis are my own, except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Hybrid grouper of *E. fuscoguttatus* × *E. lanceolatus* is popular in Southeast Asia due to their high growth rate, good price, and demand in the live fish trade market. Despite the advantages, there is very limited information on the nutritional requirements of the hybrid grouper and so far, there is no information on their vitamins C and E requirements. Vitamins C and E are well known for their function in boosting the immune responses in fish and improve fish growth. Bacteria especially *Vibrio harveyi* is one of the pathogens that cause a disease outbreak in marine fish species. Therefore, the present study was carried out to determine the vitamins C and E requirements and their effects on non-specific immune responses and disease resistance against *V. harveyi* in hybrid grouper. In Trial 1, eight experimental diets containing different vitamin C (0, 12, 24, 47, 76, 95, 156, and 303 mg/kg) levels in the form of L-ascorbic acid (AA) were fed to triplicate group of fish with initial weight of 7.7±0.1g and cultured in flow-through seawater system for 10 weeks. The results show that the highest final body weight (FBW), body weight gain (BWG) and specific growth rate (SGR) were achieved by the fish fed diet supplemented with 156 mg AA/kg. In this study, supplementation of vitamin C did not significantly affect the survival of fish. However, fish groups fed with the diet of less than 95 mg AA/kg show skeletal deformities (fusion, lordosis, kyphosis, and scoliosis). In Trial 2, eight experimental diets containing different vitamin C (0, 18, 45, 76, 142, 241, 377, and 768 mg/kg) levels in the form of L-ascorbyl acid 2-polyphosphate (C2PP) were fed to a triplicate group of fish with an initial weight of 10.4±0.1g. After 14 weeks of the feeding trial, the results show highest FBW, BWG, SGR were achieved by the fish fed diet supplemented with 18 mg C2PP/kg. In Trial 3, seven experimental diets containing different vitamin E (10, 34, 61, 122, 232, 416, and 815 mg/kg) levels in the form of alpha tocopherol acetate were fed to a triplicate group of fish with an initial weight of 7.74±0.1g for 10 weeks. The results show that the highest FBW, BWG, and SGR were achieved by the fish fed diet supplemented with 110 mg vitamin E/kg. Higher level of vitamin E supplementation (815 mg/kg) is needed to reduce thiobarbituric acid reactive substances (TBARS) in fish tissues and enhance disease resistance when infected with *V. harveyi*. In Trial 4, fish were fed with six experimental diets containing different levels of C2PP [no supplementation (-), optimum (+), and high (++)] and vitamin E [no supplementation (-), low (↓), and optimum (+)]. Significant lower survival and growth performance were observed in fish fed no C2PP and vitamin E supplementation diet (-C-E: -ve Control). Meanwhile, fish fed higher level of C2PP and no vitamin E supplementation (++C-E) (contained 271.8 C2PP and 13.4 vitamin E mg/kg, respectively) shows comparable survival, growth, and disease resistance to the fish fed diet supplemented with optimum level of C2PP and vitamin E (+C+E: +ve Control) (contained 18.3 C2PP and 814.8 vitamin E mg/kg, respectively). In conclusion, a combination of 18.3 mg/kg C2PP and 814.8 mg/kg vitamin E or 271.8 mg/kg C2PP and 13.4 mg/kg vitamin E dietary supplementation is recommended to produce healthy fish with optimum growth. Additionally, a higher supplementation level of vitamin C is able to spare vitamin E in the diet for hybrid grouper.

ABSTRAK

KEPERLUAN DAN KESAN VITAMIN C DAN E KEPADA IMUN TIDAK SPESIFIK DAN KETAHANAN PENYAKIT BAGI KERAPU HIBRID *Epinephelus fuscoguttatus × E. lanceolatus*

Kerapu hibrid Epinephelus fuscoguttatus × E. lanceolatus adalah terkenal di Asia Tenggara kerana kadar pertumbuhannya yang cepat, mempunyai harga dan permintaan yang tinggi dalam pasaran ikan hidup. Di sebalik kelebihan-kelebihan ini, maklumat mengenai keperluan nutrisi kerapu hibrid adalah sangat terhad dan setakat ini, tidak ada maklumat mengenai keperluan vitamin C dan E mereka. Vitamin C dan E terkenal dengan fungsi mereka dalam meningkatkan tindakbalas respon imun dalam ikan dan meningkatkan pertumbuhan ikan. Bakteria terutamanyaVibrio harveyi adalah salah satu patogen yang menyebabkan wabak penyakit kepada spesies ikan laut. Oleh itu, kajian ini dijalankan untuk menentukan keperluan vitamin C dan E serta kesannya terhadap tindakbalas respon imun yang tidak spesifik dan ketahanan penyakit terhadap V. harveyi dalam kerapu hibrid. Dalam Percubaan 1, lapan diet percubaan yang mengandungi vitamin C yang berlainan (0, 12, 24, 47, 76, 95, 156, dan 303 mg/kg) dalam bentuk asid L-askorbik (AA) diberi makan kepada kumpulan triplikat ikan dengan berat permulaan sebanyak 7.7 ± 0.1g dan dikultur dalam sistem air laut mengalir selama 10 minggu. Keputusan kajian ini menunjukkan berat badan akhir (FBW), pertambahan berat badan (BWG) dan kadar pertumbuhan spesifik (SGR) yang tertinggi dicapai oleh ikan yang diberi makanan tambahan 156 mg AA/kg. Dalam kajian ini, penambahan vitamin C tidak memberi kesan signifikan kepada kemandirian ikan. Walau bagaimanapun, kumpulan ikan yang diberi kurang daripada 95 AA/kg menunjukkan kecacatan kerangka (fusion, lordosis, kifosis, dan skoliosis). Dalam Percubaan 2, lapan diet percubaan yang mengandungi vitamin C yang berlainan (0, 18, 45, 76, 142, 241, 377, and 768 mg/kg) dalam bentuk L-askorbil 2-polyphosphate (C2PP) diberi makan kepada triplikat kumpulan ikan dengan berat permulaan 10.4 ± 0.1g. Selepas 14 minggu, keputusan menunjukkan FBW, BWG, SGR yang tertinggi dicapai oleh ikan yang diberi makan tambahan 18 C2PP/kg. Dalam Percubaan 3, tujuh diet percubaan mengandungi vitamin E yang berbeza (10, 34, 61, 122, 232, 416, and 815 mg/kg) dalam bentuk asetat tokoferol diberi makan kepada kumpulan triplikat ikan dengan berat permulaan 7.74 ± 0.1g selama 10 minggu. Keputusan menunjukkan FBW, BWG dan SGR dicapai oleh ikan yang diberi tambahan 110 mg vitamin E/kg. Tambahan vitamin E yang tinggi (815 mg/kg) diperlukan untuk mengurangkan bahan reaktif asid thiobarbiturik (TBARS) dalam tisu ikan dan meningkatkan ketahanan terhadap penyakit apabila dijangkiti dengan V. harveyi. Dalam Percubaan 4, ikan diberi enam jenis diet percubaan yang mengandungi tahap C2PP [tiada tambahan (-), optimum (+), dan tinggi (++)] dan vitamin E [tiada tambahan (-), rendah (↓), and optimum(+)] yang berbeza. Ketahanan hidup dan prestasi pertumbuhan adalah rendah secara signifikan dalam ikan yang tidak diberi tambahan C2PP dan vitamin E (-C-E: -ve Control). Sementara itu, ikan yang diberi tambahan C2PP yang lebih tinggi dan tiada tambahan vitamin E (++C-E) (masing-masing mengandungi 271.8 C2PP dan 13.4 vitamin E mg/kg) menunjukkan kemandirian, pertumbuhan, dan ketahanan terhadap penyakit yang setanding dengan ikan yang diberi tambahan C2PP yang optimum dan vitamin E yang optimum (+C+E: +ve Control) (masing-masing mengandungi 18.3 C2PP dan 814.8 vitamin E

mg/kg). Kesimpulannya, kombinasi makanan tambahan 18.3 mg/kg C2PP dan 814.8 mg/kg vitamin E atau 271.8 mg/kg C2PP dan 13.4 mg/kg vitamin E adalah disyorkan untuk menghasikan ikan yang sihat dan tumbesaran yang optimum. Di samping itu, tambahan vitamin C yang lebih tinggi mampu menjimatkan vitamin E dalam diet untuk kerapu hibrid.

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LIST OF ABBREVIATIONS

AA	-	Ascorbic acid
AAPP	-	Ascorbic acid polyphosphate
ACP	-	Alternative complement pathway
ADS	-	Absence of deficiency signs
AKP	-	Alkaline phosphatase
ALP	-	Alkaline phosphatase
ALT	-	Alanine aminotransferase
AMP	-	Ascorbic acid monophosphate
ANOVA	-	Analysis of Variance
ANS	-	Antioxidant status
AOAC	-	Association of Official Analytical Chemists
AST	-	Aspartate aminotransferase
BA	-	Bactericidal activity
BWG	-	Body weight gain
C2D	-	L-ascorbyl-2-glucose
C2MP-Ca	-	L-ascorbyl-2-monophosphate-Ca
C2MP-Na	-	L-ascorbyl-2-monophosphate-Na
C2MP-Mg	-	L-ascorbyl-2-monophosphate-Mg
C2PP	-	L-ascorbyl-2-polyphosphate
C2S	-	L-ascorbyl-2 sulphate
C6P	-	L-ascorbyl palmitate
CAT	-	Catalase activity
CF	-	Condition factor
CL	-	Crude lipid
CP	-	Crude protein
DR	-	Disease resistance
FAO	-	Food of Agriculture of United Nation
FBW	-	Final body weight
FCR	-	Feed conversion ratio
FE	-	Feed efficiency;
FI	-	Feed intake
FM	-	Fishmeal
GLU	-	Glucose
Hb	-	Haemoglobin
HPLC	-	High performance liquid chromatography
HSI	-	Hepatosomatic index
IBW	-	Initial body weight
Ig	-	Total immunoglobulin
LZM	-	Lysozyme activity
MBS	-	Maximum body storage
MDA	-	Malondialdehyde
MKS	-	Maximum kidney storage
MLS	-	Maximum liver storage
MMS	-	Maximum muscle storage
MPO	-	Myeloperoxidase
ND	-	Not determined
NPU	-	Net protein utilization

PA	-	Phagocytic activity
PCV	-	Packed cell volume
PER	-	Protein efficiency ratio
RBC	-	Red blood count
SGR	-	Specific growth rate
SOD	-	Superoxide dismutase
SPSS	-	Statistical Package for the Social Sciences
TAS	-	Tolerance against stress
TBARS	-	Thiobarbituric acid reactive substances
TCHOL	-	Total cholesterol
TP	-	Total protein
TRIG	-	Triglyceride
VSI	-	Visceral somatic index
WBC	-	White blood count

LIST OF SYMBOLS

<	-	Less than
≥	-	Same or more than
%	-	Percentage
°C	-	Degree Celcius
cm	-	Centimeter
et al.	-	And others
g	-	Gram
gL^{-1}	-	Gram/liter
HKD	-	Hong Kong Dollar
kcal	-	Kilocalorie
Kg	-	Kilogram
kJ^{-1}	-	Kilojoule
L min^{-1}	-	Liter/minute
L	-	Liter
mg L^{-1}	-	Miligram/liter
mg	-	Milligram
mm	-	Milimeter
ppm	-	Part per million
ppt	-	Part per thousand
RM	-	Ringgit Malaysia
rpm	-	Rate per minute
sp.	-	Species
μL	-	Microliter
v/v	-	Volume/volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

The grouper aquaculture in Southeast Asia started in the late 1970s using the wild caught seed for grow-out culture (Tookwinas, 1989). As the wild catches of grouper keep declining while global demand continues to rise, aquaculturist starts to produce grouper through artificial breeding and succeeded to produce full-cycle aquaculture of groupers in the early 90's. Although the grouper breeding and rearing techniques are improving from time to time, the farmers are still facing shortage of seed supply, due to mass mortality that generally occurred during the larval fish stages (see Liao and Leaño, 2008). One of the progresses made by aquaculturist to tackle this problem is through hybridization with the objective of transferring and/or combines desirable traits between fish species. In 2006, Borneo Marine Research Institute (BMRI) of Universiti Malaysia Sabah had successfully produced the hybrid grouper of tiger grouper (*Epinephelus fuscogutattus*) x giant grouper (*E. lanceolatus*). Since then, this fish has become one of the most economically important aquaculture species in Southeast Asia, especially in China as a results of its high egg hatching rate and larval survival, faster growth, higher tolerance to environmental variations and greater resistance against diseases than the parental species (Arrokhman *et al.*, 2017; Bunlipatanon and U-taynapun, 2017; Koh *et al.*, 2016; Mustafa *et al.*, 2013). They are also an ideal candidate species for intensive aquaculture because of their adaptability in a crowded environment (Harikrishnan *et al.*, 2011).

In intensive aquaculture system, the fish is usually fed with generic marine fish formulated diets and cultured in high stocking density. In such condition, the fish may not only fed with unbalance diets which can lead to several malnutrition deficiency signs such as slow growth and/or deformities but also directly exposed to poor handling, stress, and pathogen infection. Bacteria such as *Vibrio harveyi* is considered as one of the main pathogens affecting a wide range of marine fish species (Zhang and Austin 2000). It has been reported to cause mass mortalities in several marine aquaculture farms (Albert and Ransangan 2013; Ransangan and Mustafa, 2009; Sivaram *et al.,* 2004; Lee *et al.,* 2002; Yii *et al.,* 1997; Saeed, 1995) and eventually lead to losses in aquaculture production. Adequate nutrition supplementation is necessary to avoid or minimize the deficiency signs, maintain growth performance and sustain the good health of fish.

Concerning the earlier problems, several studies have been carried out on the nutritional aspects of the hybrid grouper by several authors (Jiang *et al.*, 2015, 2016; Rahimnejad *et al.*, 2015; Luo *et al.*, 2016; Firdaus *et al.*, 2016; Lim *et al.*, 2017; Faudzi *et al.*, 2018; Ismail *et al.*, 2018). So far, studies related to dietary protein, lipid, and carbohydrate requirement for optimum growth hybrid grouper were reported (Jiang *et al.*, 2015, 2016; Rahimnejad *et al.*, 2015; Luo *et al.*, 2016). Other than that, much less is known about the nutritional requirements of this species, especially on the micronutrients such as vitamins. It is evident that supplementation of vitamin C and vitamin E in fish feeds has been a successful method for improving growth performances, health and disease resistance in both marine and freshwater fish species. (Hamre *et al.*, 2011; 2001; Shiau and Hsu, 2002; Gao *et al.*, 2012).

Vitamin C is crucial for the growth performance, maintenance of physiological function, support skeletal development, and enhanced immunity and disease resistance in fish (Ai *et al.*, 2006; Darias *et al.*, 2011; Gao *et al.*, 2014; Chen *et al.*, 2015). Meanwhile, vitamin E is an essential nutrient which protects the lipid against peroxidation in a fish membrane. They also play a significant role in many physiological and biochemical activity, including growth, development, reproduction and immune response (Lu *et al.*, 2016; NRC 2011), as well as the oxidative stress (Gao *et al.*, 2012) of fish. It has previously been observed that there is an interaction effect between these two major antioxidant additives in fish nutrition. For instance, vitamin C has been reported to have the ability to regenerate and/or spare vitamin E in fish including Atlantic salmon *Salmo salar* (Hamre *et al.*, 1997), gilthead sea bream, *S. aurata* (Montero *et al.*, 1999), yellow perch, *Perca flavescens* (Lee and Dabrowski,

2003) and red sea bream *Pagrus major* (Gao *et al.*, 2012). Knowledge on nutrient interactions is important in the development of a balanced diet to support the optimal growth and health of the fish and at the same time producing feeds that is cost-effective. Since grouper including the hybrid, have been identified to require high protein in the diets, it is important to include other nutrients at their optimum levels to prevent waste and optimizing profit.

1.2 Importance of Study

Despite the emerging production and popularity of hybrid grouper in Southeast Asia, there appears to be little information on their vitamins C and E requirements. Both vitamins are essential in growth, skeletal health and boosting the immune responses in fish (Lin and Shiau, 2005a; Lin and Shiau, 2005b; Waagbø, 2010).

The lack of these vitamins may lead to serious issues in cultured fish including poor growth, low survival, poor feed utilization, skeletal deformities and the scurvy (Wilson and Poe, 1973; Li and Robinson, 1999). The generated information from this study will be very useful in the development of species-specific formulated feed which will significantly help the aquafeed industry to produce commercial feed for this hybrid ygrouper.

1.3 Objectives

The general objectives of this study are to determine the vitamins C and E requirements of hybrid grouper, *E. fuscoguttatus* × *E. lanceolatus* and their effects on the non-specific immune responses. Specifically, the objectives are;

1. To determine the optimum vitamin C requirement and their effects on growth performance, survival, feed utilization, skeletal health, non-specific immune responses and disease resistance against *V. harveyi* in hybrid grouper, *E. fuscoguttatus* × *E. lanceolatus.*

2. To determine the optimum vitamin E requirement and their effects on growth performance, survival, feed utilization, non-specific immune responses and disease resistance against *Vibrio harveyi* in hybrid grouper, *E. fuscoguttatus × E. lanceolatus*.

3. To determine the interaction of vitamins C and E on growth performance, sparing effects, non-specific immune responses and disease resistance of the hybrid grouper juveniles

The hypotheses of this study are:

1. Supplementation of vitamin C in the diet can improve fish growth in general and maintain skeletal health of the fish. Meanwhile, different types of vitamin C use will affect the dietary vitamin C requirement of fish. High supplementation level of vitamin C can enhance non-specific immune responses in hybrid grouper.

2. Supplementation of vitamin E will improve the growth response of the hybrid grouper. Vitamin E can be accumulated in the fish body, while high supplementation level of vitamin E can protect fish against bacteria, *V. harveyi* infection.

3. Dietary vitamins C can spare vitamin E for growth, feed utilization, tissue oxidation, and disease resistance. High supplementation level of vitamin C can reduce the requirement of vitamin E in fish.

CHAPTER 2

LITERATURE REVIEW

2.1 Grouper Aquaculture

The grouper aquaculture in Southeast Asia has been growing rapidly in the last 14 years, increasing by approximately 400% from 2003 to 2016 (FAO, 2017). Most of the production came from Asia, with three countries accountable for an estimated 92% of global production, where China contributed 65%, Taiwan Province of China contributed 17% and Indonesia 11% of total production (Rimmer and Glamuzina, 2017). Figure 2.1 shows the grouper aquaculture production in Southeast Asia (by countries) from 2003 to 2016. Groupers are important fish species due to its high market demand especially in the live seafood market in Hong Kong, Taiwan, Singapore, and Malaysia (Yap, 2002).

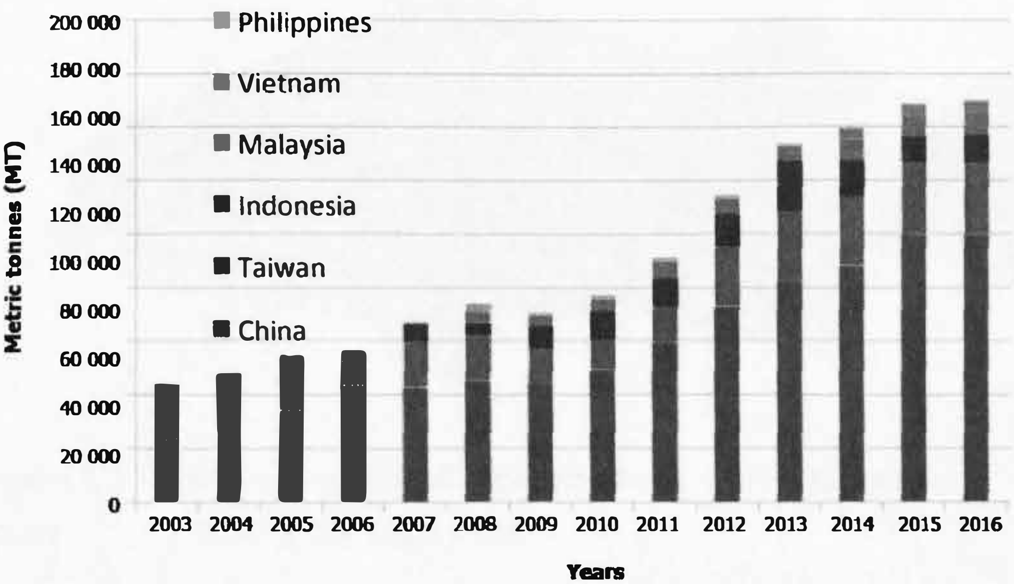

Figure 2.1 : Grouper production of Southeast Asia from year 2003 to 2016 in tones

Source : FAOSTAT Database