UNIVERSITI PUTRA MALAYSIA

EFFECTS OF Chlorella vulgaris SUPPLEMENTATION ON DIETARY DIGESTIBILITY, INTESTINAL MORPHOLOGY AND MICROBIOTA, GROWTH PERFORMANCE AND SURVIVAL OF RED TILAPIA HYBRID (Oreochromis spp.)

AHMAD MUHAMMAD TALBA

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By

AHMAD MUHAMMAD TALBA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

November 2018

DEDICATION

This thesis is dedicated to my mother Hajjiya Maryam Umar Suleiman, my wife Maryam Abubakar Umar and my son Ahmad Muhammad Talba.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair : Professor Dato' Mohamed Shariff Mohamed Din, PhD
Faculty : Veterinary Medicine

While the global aquaculture production is increasing, outbreak of diseases and the stagnation of wild fish catch which is the main source of protein to the aquaculture feed industry are the major impending drawbacks. There are several claims on the roles of microalgae in fish nutrition and health, with limited information on the effects of its supplementation on dietary digestibility as well as an in-depth effect on fish health. To explore these; a feeding trial using *Chlorella vulgaris* was carried out. The study was aimed at investigating the effects of *C. vulgaris* supplementation on the dietary digestibility and its effects on intestinal function, morphology and microbiome alongside growth performance and haemato-biochemical parameters. A commercial diet (base diet) was supplemented with *C. vulgaris* at 0, 1, 3, and 5% kg^{-1} (control, *C. vulgaris* supplemented feed I, II and III, respectively). A total of 84 red hybrid tilapia (105 ± 7 g, mean ± SE) were randomly distributed into 12 tanks (three plicate per treatment group) with 21 fish in each treatment group (7 fish/tank). The fish were fed the supplemented diet for a 21-day feeding trial. Faeces were collected twice daily, which was analysed at the end of the experiment. For the second phase of the experiment, the design described above was used. Four hundred and fifty-six tilapia were randomly distributed as described earlier. Each experimental feed was fed to the appropriate treatment group of fish for 12 weeks, with sampling carried out at 30 days intervals. The result of digestibility trial showed that *C. vulgaris* supplementation at all the levels (1, 3, and 5%), significantly improved the apparent digestibility coefficient values for dry matter and protein which was also associated with increase in supplementation. At the end of the second phase experiment, the results of performance trial showed a positive influence of the supplementation and duration of feeding on growth, feed utilization and haemato-biochemical parameters. Simultaneous and significant influence of supplementation and duration was observed on weight gain, average growth rate, mean daily feed intake total

plasma protein, albumin, globulin, red blood cell counts and packed cell volume. Similarly, simultaneous and significant influence of supplementation levels and duration of feeding was noted in the intestinal morphology, alongside an increase in the inducible nitric oxide synthase (iNOS) in fish fed *Chlorella* supplemented feed. Following challenge trial with *Streptococcus iniae,* significantly higher cumulative survival was seen in fish fed diets supplemented with *Chlorella*. Next generation sequencing of the 16S rRNA biomarker gene V3-V4 region of the gut microbiota revealed that 97.65% of the bacteria belonged to five phyla; the usobacteria (53.84 %), Bacteroidetes (33.99%), Proteobacteria (5.56%), Verrucomicrobia (3.04%) and Firmicutes (1.22%). Both the control and treatment groups appeared to have similar microbial community structural composition and core gut microbiome. Whereas, the intestinal microbiota of fish fed treatment diets had microbial biomarkers dominated by *Firmicutes* as opposing to the *Cyanobacteria* of the control fish. Therefore, the current study revealed the influence of *Chlorella vulgaris* supplemented feed on fish gut microbiota, intestinal health and growth performance of red hybrid tilapia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN PENAMBAHAN *Chlorella vulgaris* TERHADAP PENGHADAMAN PEMAKANAN, MORFOLOGI USUS DAN MIKROBIOTA, PRESTASI PERTUMBUHAN DAN KEMANDIRIAN TILAPIA MERAH HIBRID (*Oreochromis* spp.)

Oleh

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Dikala pengeluaran akuakultur global semakin meningkat, ketepuan hasil tangkapan ikan liar yang menjadi sumber protein utama industri ini dan ancaman wabak penyakit masih lagi menjadi masalah utama. Keadaan ini mengancam perkembangan industri dan memerlukan beberapa pendekatan untuk mengurangkan kesannya. Mikroalga adalah makanan semulajadi ikan, kaya dengan nutrien penting dan biomolekul lain yang berkeupayaan untuk menambah pemakanan dan kesihatan ikan.

Walaupun ada terdapat beberapa penjelasan umum tentang peranan mikroalga terhadap tahap nutrisi dan kesihatan ikan, namun maklumat tentang penghadamannya di tahap pemberian tambahan serta kesan mendalam terhadap kesihatan masih lagi terhad. Untuk meneroka keadaan ini, kajian percubaan pemakanan menggunakan Chlorella vulgaris telah dijalankan. Kajian ini bertujuan untuk menyiasat kadar penghadaman diet *C. vulgaris* suplemen keseluruhan dan kesannya pada fungsi usus, morfologi dan mikrobiom, selari dengan kadar pertumbuhan dan parameter hemato-biokimia.

Penggunaan diet komersial (diet asas) yang ditambah dengan C. vulgaris pada 0, 1, 3 dan 5% kg-1 telah digunakan (kawalan dan untuk setiap ujian diet I, II dan III). Sebanyak 84 ekor ikan tilapia merah hybrid (105 + 7g) secara rawak ditempatkan di dalam 12 tangki (tiga replikasi untuk setiap kumpulan ujian) iaitu sebanyak 21 ekor ikan setiap kumpulan ujian selama 21 hari ujian pemakanan. Tinja ikan dikumpulkan dua kali sehari untuk dianalisa di akhir ujikaji. Untuk fasa kedua ujikaji, rejimen diet seperti yang tersebut di atas telah digunakan. Sebanyak empat ratus lima puluh ekor ikan tilapia secara rawaknya telah dibahagikan sama seperti yang dijelaskan di atas. Setiap diet diberikan kepada

kumpulan ujian tertentu selama 12 minggu dan persampelan dijalankan pada setiap 4 minggu.

Hasil daripada ujian penghadaman menunjukkan tambahan C. vulgaris suplemen pada semua tahap (1, 3 dan 5%), dengan ketara meningkatkan nilai ADC untuk bahan kering dan protein yang juga berkait secara langsung dengan peningkatan pemberian makanan tambahan ini. Hasil ujikaji terhadap prestasi menunjukkan kesan ketara pada pertumbuhan, penggunaan makanan dan parameter hemato-biokimia. Daripada pemerhatian, terdapat kesan yang ketara di antara makanan tambahan dan tempoh ujikaji terhadap pertambahan berat badan, purata kadar pertumbuhan, jumlah plasma protein, globulin dan jumlah kiraan leukosit. Sama juga, terdapat kesan ketara pada tahap pemberian makanan tambahan yang berbeza dan jangkamasa ujikaji terutamanya pada morfologi usus, selari dengan peningkatan pendorongan nitrik oksida synthase (iNOS) dalam diet ikan yang mengandungi makanan tambahan Chlorella.

Lanjutan daripada ujian cabaran dengan Streptococcus iniae, menunjukan ikan yang yang diberi makanan tambahan Chlorella secara ketara memiliki kadar kehidupan dan daya ketahanan penyakit yang tinggi. Penjujukan generasi berikut 16S rRNA gen penanda bio rantau V3-V4 mikrobiota perut menunjukkan bahawa 97.65% bakteria terkandung dalam 5 filum; Fusobakteria (53.84%), Bakteriodete (33.99%), Proteobakteria (5.56%), Verrucomikrobia (3.04%) dan Fermikutes (1.22%). Kedua-dua kumpulan kawalan dan ujian memiliki komuniti mikrobial yang hampir sama, komposisi struktur dan mikrobiom teras perut. Sedangkan, mikrobiota usus ikan yang diberikan makan diet ujian memiliki penanda bio mikrobial yang didominasi oleh Firmikutes berbanding Cynobakteria pada kumplan ikan kawalan. Oleh itu, kajian ini telah menjelaskan kesan-kesan positif tambahan mikro Chlorella pada mikrobiota perut ikan, kesihatan usus dan prestasi pertumbuhan ikan tilapia merah hibrid.

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I certify that a Thesis Examination Committee has met on 22 November 2018 to conduct the final examination of Ahmad Muhammad Talba on his thesis entitled "Effects of *Chlorella vulgaris* Supplementation on Dietary Digestibility, Intestinal Morphology and Microbiota, Growth Performance and Survival of Red Tilapia Hybrid (*Oreochromis* spp.)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the (insert the name of relevant degree).

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LIST OF ABBREVIATIONS

ACE	Abundance-based coverage estimator
ADC	Apparent Digestibility Coefficient
AGR	Average growth rate
AG	Albumin/globulin ratio
ALT	Alanine aminotransferase
AOAC	Association of Official Analytical Chemists
AST	Aspartate aminotransferase
BHIA	Brain heart infusion agar
C1	Control group (0% *C. vulgaris* kg^{-1} of feed)
CAT	Catalase
CGF	Chlorella growth factor
CF	Condition factor
CO_2	Carbon dioxide
CRD	Completely Randomised Design
DAPI	4′, 6-diamidino-2-phenylindole dihydrochloride
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EE	Ether extract
EFSA	European Food Safety Authority
FAO	Food and agriculture organisation
FCR	Feed conversion ratio

FER	Feed efficiency rario
GC	Goblet cells
GH	Growth hormone
GHR	Growth hormone receptor
GSH	Glutathione
GLM	General linear model
H&E	Haematoxylin and eosin
Hb	Haemoglobin content
Hcl	Hydrochloric acid
HSI	Hepatosomatic index
H_2SO_4	Sulphuric acid
IACUC	Institutional Animal Care and Use Committee
ID	Indicator in diets
IEL	Intraepithelial leucocyte
IF	Indicator in faeces
IFN-γ	Gamma interferon
IGF I	Insulin-like growth factor I
iNOS	Inducible nitric oxide synthase
IL	Interleukin
ILPL	Intralamina propria leucocytes
Ig	Immunoglobulin
ITIS	Integrated taxonomic information system
KW	Kruskal-Wallis rank test
LAB	Lactic acid bacteria

LEfSE	Linear discriminata anaysis effect size
LDA	Linear discriminate analysis
LPL	Lamina propria leucocyte
LPS	Lipopolysaccharides
MCHC	Mean Corpuscular Haemoglobin Count
MCV	Mean Corpuscular Volume
MDFI	Mean Daily Feed Intake
mRNA	Messenger RNA
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
ND	Nutrient or energy in diet
NF	Nutrient or energy in faeces
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween 20
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PCV	Packed cell volume
pH	Hydrogen ion concentration
QIIME	Quantitative insight into microbial ecology
RBC	Red bood cells
RBF	Round bottom flask
RDP	Ribosomal database project
RNA	Ribonucleic acid

rRNA	Ribosomal RNA
SCC	Sodium copper chlorophyllin
SGR	Specific growth rate
T1	Treatment group 1 (1% *C. vulgaris* kg^{-1}feed)
T2	Treatment group 2 (3% *C. vulgaris* kg^{-1}feed)
T3	Treatment group 1 (5% *C. vulgaris* kg^{-1} feed)
TAG	Triacylglycerol
TER	Trans-epithelial electric resistance
TNFα	Alpha- Tumor necrotizing factor
Tp	Total serum protein
UPM	Universiti Putra Malaysia
USFDA	United States Food and Drug Administration
US$	United states dollar
V3-V4	Hypervariable regions 3 and 4
Vd	Villus density per segment
Vh	Villus height
Vp	Villus perimeter
VSI	Viscerosomatic index
Vw	Villus width
WBC	White blood cells
WG	Weight gain

CHAPTER 1

INTRODUCTION

1.1 Background

Tilapia is among the widely cultured species of fresh water fish in the world. Cultured tilapia accounts for more than 75% of the world production, and is projected to surpass *Cyprinids* production in years to come (Hasan and Chakrabarti, 2009). Tilapia production is receiving attention in recent times. Several factors have contributed to the increased interest in tilapia production, such as their ability to rapidly and easily adapt to varying aquaculture conditions and also due to the high attractiveness and moderately stable market costs (Ng & Romano, 2013). China is the largest producer of farmed tilapia, with over 1.2 million metric tons in 2009, followed by Indonesia with over 250,000 metric tons in 2009, while the USA are the major importers by receiving up to 91.8% of global exports. The global tilapia trade has skyrocketed from US$1.7 billion in 2000 to US$5.0 billion in 2010 with an anticipated dramatic increase in couple of years (Ng & Romano, 2013).

In recent years, the use of microalgae in life sciences has received increasing attention due to their diverse phytoconstituents with different chemical structures and functions (Skulberg, 2004). Microalgae as feed additive enhances growth and feed utilization of many cultured fish spp., it promotes response to stress, proper dietary protein utilization, tolerance to starvation, physiological activity and disease resistance (Hasan and Chakrabarti, 2009). Dietary supplementation of *Chlorella* improves survivability, growth rate, immunity and activity of digestive enzymes of some fish (Bai *et al*, 2001; Xu *et al.*, 2014). Microalgae play a vital role in stabilizing water quality by utilization and elimination of by-products derived from aquatic animals (Khatoon, Yusoff, Banerjee, Shariff, & Mohamed, 2007; Nurul, Fatimah, Srikanth, & Mohamed, 2016).

Feed supplements are substances and/or organisms added into fish feed at low-levels in order to improve fish performance and health and/or enhance feed quality and palatability (Barrows, 2000). In trying to improve fish performance nutraceuticals and functional feed are often used. Such supplements promote the fish growth performance, fish health and immunity, and improve physiological activities beyond the traditional feeds. Other options used as supplements in order to regulate and manage fish performance and health includes probiotics, immune- stimulants, prebiotics, enzymes, phytogenic substances, mycotoxin binders, hormones and organic acids. Probiotics are termed as live but non-pathogenic and nontoxic microorganisms that are void of damaging side-effects when administered to aquatic organisms at low-levels. The range of probiotics used for health promotion yeasts, Gram-negative and Gram-positive bacteria, bacteriophages and microalgae (Alemayehu, Geremew, & Getahun, 2018).

Chlorella is a unicellular green microalga with the highest level of chlorophyll among all plants (Raja, Hemaiswarya, Kumar, Ganesan, & Anbazhagan, 2011; Raja, Hemaiswarya, Venkatesan, & Isabel, 2014). The microalga has an interesting protein and amino acid profiles, carbohydrates, vitamin B (complex), C, and E, enzymes and some trace elements among others. At an optimal inclusion levels in fish diets, *C. vulgaris* is well accepted by many of the fish species so far studied (Enyidi, 2017).

1.2 Problem Statement

Among all the food commodities, fish is at the top of the list of the highly traded commodities all over the world. It is the vital export commodity for many of the developing countries and it accounts for almost half of the whole export commodities (FAO, 2014). Fish caters for about 17% of human animal protein intake globally. However, in some countries the percentage of animal protein sourced from fish is greater than 50 percent. Moreover, fish accounts for 44 to 70% of the total animal protein used in some countries in West Africa. Similarly in some countries in Asia the share ranges from 54 to 71% (FAO, 2014). Of all the figures mentioned, aquaculture caters for almost 50% of the fish supplied for human consumption. With the recent levelling off of the wild capture fisheries, it is projected that by 2030 aquaculture production will rise to 62% due a projected increase demand from the middle income economies (FAO, 2014).

Besides, consumption of fish by humans and/or its usage as major ingredient in aquaculture feeds is a matter of a great concern nowadays. This is because of an increasing tendencies of contamination of the fisheries product due to a corresponding increase in water pollution (FAO, 2014). Therefore, use of fishery products in formulating animal feeds and its subsequent consumption by farmed fish, may result in ingestion of poisonous or harmful substances (as there is limited or no control over the contaminants) like heavy metals, insecticide, pesticides, herbicides and veterinary drugs residues (FAO, 2014). Several attempts were made to reduce the mean fishmeal use in commercial tilapia feeds, with a projected decrease to 1% inclusion level by 2020 (Ng & Romano, 2013).

Proteins of plant origin such as wheat gluten and corn gluten meal have been used as fundamental components of aquaculture feeds in several studies (Vizcaino et al., 2014). Although, the anti-nutrient factors present in such plant proteins limit their usage, as they could cause a negative effect on growth performance and digestive enzyme activities of fish. This is in addition to the damaging effect of such diets on intestinal epithelial lining (Vizcaino et al., 2014). Therefore, supplementation of fish diet with organisms or substances that could alleviate such untoward effects would move the aquaculture production to the next level.

Microalgae could be used as a supplement in order to enhance fish performance. Microalgae are an integral part of aquatic food chain that is mostly used in raising aquatic

vertebrate and invertebrate at various stages of development (Borowitzka 1998). Microalgae is either used as whole feed or supplement in aquaculture production. A live microalgae enhances fish nutrition and maintain a better water quality (Brown, Jeffrey, Volkman, & Dunstan, 1997). Acceptance of microalgae as farmed fish feed supplement is gaining grounds due to their nutritional and health values, in addition to being source of pigment to the flesh and skin of fish (Brown et al. 1997). To explore the potentials of microalgae as a supplement that could alleviate the untoward effects associated with some feed ingredients *C. vulgaris* was used as a supplement in the present trial. We hypothesised that *C. vulgaris* supplementation and durations of feeding does not influence the fish health and performance. Therefore, the aim of the present study was to determine the effects *C. vulgaris* on growth performance, haemato-biochemical indices and intestinal health of red hybrid tilapia.

1.3 Objectives

- To determine the effects of *C. vulgaris* supplementation on nutrient/energy digestibility of fish feed in red hybrid tilapia.
- To determine the effects of *C. vulgaris* supplementation and duration of feeding on growth performance alongside haemato-biochemical indices of red hybrid tilapia.
- To determine the effects of *C. vulgaris* supplementation and duration of feeding on the intestinal epithelium of red hybrid tilapia, and effect of the diet on inducible nitric oxide synthase (iNOS).
- To determine the effects of *C. vulgaris* supplementation on structure, composition, microbial biomarkers and core gut microbiota of red hybrid tilapia.
- To determine the effects of *C. vulgaris* supplementation and experimental challenge with *Streptococcus iniae* on survival, pre and post challenged haemato-biochemical parameters of red hybrid tilapia.

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