UNIVERSITI PUTRA MALAYSIA

COMPREHENSIVE METABOLITE PROFILE OF Chlorella vulgaris AND METABOLIC SIGNATURE OF ITS IMMUNOSTIMULATING EFFECT IN NILE TILAPIA [Oreochromis niloticus (Linnaeus, 1758)]

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By

HAMZA AHMED PANTAMI

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

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DEDICATION

This thesis is dedicated to my late parents, my wife Habiba, my children; Ahmad, Nana Hafsat, Nana Aisha and the soon expected unborn child.

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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Chairman : Professor Khozirah binti Shaari, PhD
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Chlorella vulgaris is a green, single-celled, spherical freshwater microalga belonging to the family *Chlorellaceae* of the division *Chlorophyta*. *C. vulgaris* is currently used as food supplement and medicinal agent due to its carotenoids, chlorophylls and proteins content. *Chlorella vulgaris* is incorporated in fish feed as a growth and immune stimulant. Tilapia is one of the most important cultured fish species with numerous advantages, contributing more than USD 10 billion to the world's seafood market annually. Despite the important features of tilapia, disease outbreaks tend to cause massive mortalities in fish farms which impose serious negative impact to economies worldwide. Most tilapia mortalities were reported as a resultant stress due to overcrowding coupled with bacterial infection. *Streptococcus agalactiae* is one of the most virulent bacteria causing most of tilapia mortalities recently. The use of immunostimulants like microalgal supplementation in fish feed holds a promising future in mitigating tilapia fish disease. Nevertheless, the common amounts used (≥12%) in this practice is considered uneconomical by fish farmers, considering the high price of microalgal biomass procurement. In addition, scientific reports regarding the association of metabolites with pathways involved in the stimulation of immune system of tilapia fish (*Oreochromis niloticus*) exerted by consumption of *C. vulgaris* are still limited. Hence, the main aim of this study was to investigate the immune-stimulating role of bioactive metabolites from *C. vulgaris* on the tilapia fish at lower concentrations using proton nuclear magnetic resonance (^{1}H-NMR) metabolomics approach. The first part of the study dealt with the characterization of the metabolite composition of the cultured samples of *C. vulgaris* via ^{1}H-NMR, gas chromatography mass spectrometry (GCMS) lipid analysis and liquid chromatography mass spectrometry (LC-MS/MS) analysis. ^{1}H-NMR data obtained from six different organic solvent extracts were used to establish the general metabolite profile and their variation was determined via multivariate data analysis (MVA) and complemented with mass spectrometric data. LC-MS/MS analysis led to the profiling of carotenoids

comprising of violaxanthin, neoxanthin, lutein, β-carotene, vulgaxanthin I, astaxanthin and antheraxanthin along with other metabolites such as amino acids, vitamins, and chlorophylls. The metabolite compositions indicated that *C. vulgaris* could serve as a good source of dietary nutrients. Fatty acid profile of *C. vulgaris* revealed that it was rich in omega-6,7,9 and 13 fatty acids, with dominance of omega-6 fatty acids (>60% of the total fatty acids). Besides, the main fatty acids were those of C_{16}-C_{18} (>92%), suggesting high potential for utilization of *C. vulgaris* in biodiesel production. In addition, 48 lipids were putatively identified via Molecular Networking of the LC-MS/MS data which include glycosphingolipids, phosphoethanolamines, phosphoserines and phosphocolines. The second part of the study determined the safety of *C. vulgaris* concentration as a fish feed at 500 mg/kg body weight (bw) by assessing several immune parameters. The immune parameters assessed were serum lysozyme activity (SLA), serum bactericidal activity (SBA), phagocytosis activity (PHA), respiratory burst activity (RBA) and lymphocyte proliferation (LP). There was no mortality recorded nor sign of organ lesion observed throughout the assessment period. The immune parameters assessed at 5%, 2.5%, 1.25% and 0.625% showed significant improvement in PHA, RBA and LP with 1.25% (125 mg/kg bw) been the best dose for future use economically. The third part of the present study correlated the immune parameters with the primary metabolite composition of the serum and spleen of the tilapia fish using ^{1}H-NMR metabolomics. Metabolites such as choline, glucose, riboflavin, stearic acid, linolenic acid, leucine, histamine, proline, glycine, and alanine levels in tilapia fish fed with *C. vulgaris* exhibited improvements compared to the control tilapia fish, with reduction in the level of glutamic acid. The linoleic acid metabolism had the highest impact factor, followed by riboflavin metabolism, *D*-glutamine and *D*-glutamate metabolism and starch and sucrose metabolism, with impact factors of 1.00, 0.50, 0.50 and 0.39, respectively. *Chlorella vulgaris* incorporation into fish diet at a concentration of 125 mg/kg bw showed to be considerably enough in improving tilapia immune parameters and was used for the prophylaxis studies in the last part of the research study for economic considerations. The last part of the study showed that the immune stimulation by *C. vulgaris* was sustained even after challenging of the tilapia fish with a virulent bacterium, *Streptococcus agalactiae* concentration of 10^{8} $CFUmL^{-1}$ for 7 days. This finding agreed with the 90% survival rate of the fish fed with *C. vulgaris* (CVC) as compared to the 13% survival rate of the control fish (CF) and significantly higher metabolite fold changes in CVC in comparison with CF. This research provides scientific evidence for utilization of *C. vulgaris* as an immune stimulant for tilapia fish. On the other hand, ^{1}H-NMR metabolomics is a sound methodology for the identification of biomarkers in immune system-stimulated tilapia fish. To the best of our knowledge, the present study was the first investigation that proposed the biomarkers along with associated pathways involved in immune stimulation of tilapia fish consuming *C. vulgaris*. The findings could pave a way for development of efficient strategies in mitigating fish diseases in aquaculture practice.

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

PROFIL METABOLITE KOMPREHENSIF *Chlorella vulgaris* DAN MAKLUMAT METABOLIK DENGAN KESAN IMMUNOSTIMULASI DI NILE TILAPIA [*Oreochromis niloticus* (Linnaeus, 1758)]

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Chlorella vulgaris ialah mikroalga air tawar berbentuk sfera, bersel tunggal dan berwarna hijau yang tergolong dalam keluarga Chlorellaceae di bahagian Chlorophyta. *C. vulgaris* kini digunakan sebagai makanan tambahan dan agen perubatan disebabkan kandungan karotenoid, klorofil dan proteinnya. *Chlorella vulgaris* digunakan sebagai satu bahan dalam makanan ikan untuk merangsang system tumbesaran dan imun. Tilapia adalah salah satu spesies ikan berbudaya yang paling penting dengan banyak kelebihan, menyumbang lebih dari USD 10 bilion untuk pasar makanan laut dunia setiap tahun. Walaupun terdapat ciri penting ikan nila, wabak penyakit cenderung menyebabkan kematian besar-besaran di ladang ikan yang memberikan kesan negatif yang serius kepada ekonomi di seluruh dunia. Sebilangan besar kematian tilapia dilaporkan sebagai tekanan yang disebabkan oleh kesesakan yang banyak ditambah dengan jangkitan bakteria. *Streptococcus agalactiae* adalah salah satu bakteria yang paling banyak menyebabkan kematian tilapia baru-baru ini. Penggunaan imunostimulan seperti suplemen mikroalga dalam makanan ikan memiliki masa depan yang menjanjikan dalam mengurangkan penyakit ikan nila. Walaubagaimanapun, jumlah *C. vulgaris* yang biasa digunakan dalam amalan ini (≥12%) dianggap tidak ekonomik oleh penternak ikan, memandangkan harga perolehan biojisimnya yang tinggi. Di samping itu, kajian saintifik mengenai hubungkait metabolit dengan laluan yang terlibat dalam rangsangan sistem imun ikan tilapia (*Oreochromis niloticus*) kesan daripada pengambilan *C. vulgaris* adalah masih kurang. Oleh yang demikian, matlamat utama kajian ini ialah untuk mengkaji sebatian bioaktif dalam *C. vulgaris* yang berperanan merangsang sistem imun ikan tilapia pada kepekatan yang lebih rendah menggunakan pendekatan metabolomik resonans magnet nuklear proton (^{1}H-NMR). Bahagian pertama kajian mencirikan komposisi metabolit sampel kultur *C. vulgaris* melalui ^{1}H-NMR, analisis asid lemak spektrometri jisim-kromatografi gas (GC-MS) dan analisis spektrometri jisim serentak-kromatografi cecair (LC-MS/MS). Data ^{1}H-NMR enam ekstrak pelarut organik yang berbeza

digunakan untuk mengetahui profil metabolit umum *C. vulgaris* dan variasi ekstrak-ekstrak ini ditentukan melalui analisis data pelbagai pembolehubah (MVA) dan dilengkapi dengan data spektrometri jisim. Analisis LC-MS/MS menunjukkan profil karotenoid yang terdiri daripada violazantin, neozantin, lutein, β-karotena, vulgazantin I, astazantin dan antherazantin bersama-sama dengan sebatian lain seperti asid amino, vitamin dan klorofil. Komposisi metabolit menunjukkan bahawa *C. vulgaris* ialah sumber nutrisi yang baik. Profil asid lemak *C. vulgaris* mendedahkan bahawa ia kaya dengan asid lemak omega-6,7,9 dan 13, dengan dominasi asid lemak omega-6 (> 60% daripada jumlah asid lemak). Selain itu, asid lemak utama ialah C_{16}-C_{18} (> 92%), justeru mencadangkan penggunaan *C. vulgaris* dalam penghasilan biodiesel. Di samping itu, 48 sebatian lemak telah dikenalpasti melalui Rangkaian Molekular data LC-MS/MS, yang merangkumi glycosphingolipids, phosphoethanolamines, phosphoserines dan phosphocolines. Bahagian kedua kajian menentukan keselamatan *C. vulgaris* sebagai makanan ikan pada kepekatan 500 mg/kg berat badan (bw) dengan penilaian beberapa parameter sistem imun. Parameter sistem imun yang dinilai ialah aktiviti serum lisozim (SLA), aktiviti bakterisida serum (SBA), aktiviti fagositosis (PHA), aktiviti pecah pernafasan (RBA) dan proliferasi limfosit (LP). Tiada kematian atau tanda luka organ direkodkan sepanjang tempoh penilaian. Parameter sistem imun yang dinilai pada 5%, 2.5%, 1.25% dan 0.625% menunjukkan peningkatan yang ketara dalam PHA, RBA dan LP dengan 1.25% (125 mg / kg bw) adalah dos terbaik untuk penggunaan masa depan secara ekonomi. Bahagian ketiga kajian ini mengaitkan parameter imun dengan komposisi metabolit serum dan limpa ikan tilapia menggunakan metabolomik ^{1}H-NMR. Metabolit seperti kolin, glukosa, riboflavin, asid stearik, asid linolenik, leusin, histamin, prolin, glisin, dan alanin dalam ikan tilapia yang diberi makan *C. vulgaris* menunjukkan peningkatan berbanding ikan tilapia kawalan, dengan pengurangan tahap asid glutamik. Metabolisma asid linoleik mempunyai faktor kesan tertinggi, diikuti dengan metabolisma riboflavin, *D*-glutamin dan metabolisma *D*-glutamat dan metabolisme kanji dan sukrosa, dengan faktor kesan masing-masing 1.00, 0.50, 0.50 dan 0.39. Penggabungan *Chlorella vulgaris* ke dalam diet ikan pada kepekatan 125 mg / kg bw terbukti cukup baik dalam meningkatkan parameter imun tilapia dan digunakan untuk kajian profilaksis di bahagian terakhir kajian penyelidikan untuk pertimbangan ekonomi. Bahagian terakhir kajian menunjukkan bahawa rangsangan imun oleh *C. vulgaris* telah dikekalkan walaupun selepas cabaran ikan tilapia dengan bakteria *Streptococcus agalactiae* kepekatan 10^{8} $CFUmL^{-1}$ selama 7 hari. Dapatan ini bertepatan dengan 90% kadar survival ikan tilapia yang diberi makan *C. vulgaris* (CVC) berbanding kadar survival 13% ikan kawalan (CF) dan perubahan lipatan metabolit yang lebih tinggi dalam CVC berbanding CF. Penyelidikan ini memberikan bukti saintifik penggunaan *C. vulgaris* sebagai perangsang imun bagi ikan tilapia. Selain itu, metabolomik ^{1}H-NMR ialah satu kaedah yang baik untuk mengenalpasti biomarker dalam ikan tilapia yang dirangsang sistem imunnya. Dalam amatan kami, kajian ini merupakan penyiasatan pertama yang mencadangkan biomarker bersama-sama dengan laluan yang berkaitan dengan rangsangan imun ikan tilapia yang memakan *C. vulgaris*. Penemuan ini dapat membuka jalan bagi pembangunan strategi yang cekap dalam mengurangkan penyakit ikan dalam amalan akuakultur.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

^{1}H-NMR	Proton Nuclear Magnetic Resonance Spectroscopy
d	Doublet
dd	Doublet of doublet
m	Multiplet
DAD	Diode Array Detector
ESI	Electrospray Ionisation
g	Gram
mg	Milligram
mL	Millilitre
HMBC	Heteronuclear Multiple bond Correlation
HSQC	Heteronuclear Single bond Correlation
HCA	Hierarchical Cluster Analysis
HPLC	High Performance Liquid Chromatography
Hz	Hertz
LC_{50}	Lethal Concentration at 50 Percent
L	Litre
LC-MS	Liquid Chromatography-Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
m	Multiplet
m/z	Mass to charge ratio
MeOH	Methanol
Et	Ethanol
Ea	Ethyl acetate
Ch	Chloroform
Hex	Hexane
50%Et	50% ethanol
Aq	Aqueous

MVDA	Multivariate Data Analysis
^{o}C	Degrees Celsius
PC	Principal Component
PCA	Principal Component analysis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares-Discriminate Analysis
OPLSD-DA	Orthogonal PLS-DA
ppm	Part per million
s	Singlet
SIMCA	Soft Independent Modelling of Class Analogy
UV	Ultraviolet
UV/VIS	Ultraviolet/visible
VIP	Variable Importance in the Projection
δ	Chemical shift in ppm
µL	Microlitre
WHO	World Health Organisation
ANOVA	Analysis of Variance
CID	Collision Induced Dissociation
NaOD	Sodium Deuterium Oxide
D_2O	Deuterium Oxide
CD_3OD	Methanol-d_4
KH_2PO_4	Potassium Dihydrogen Phosphate
K_2HPO_4	Dipotassium Hydrogen Phosphate
2D	Two Dimensional
HMDB	Human Metabolome Database
KEGG	Kyoto Encyclopedia of Genes and Genomes

CHAPTER 1

INTRODUCTION

1.1 Background

Algae are the organisms that possess chlorophyll(s), having thallus that does not differentiate into roots, stem or leaves. They can use the light energy and carbon dioxide for the photosynthetic biomass production with higher efficiency compared to plants. There are over 300,000 species of microalgae, of which around 30,000 of them have been documented. They live in complex natural habitats, making them able to adapt rapidly in different extreme conditions like variable salinity, temperature, nutrients and UV–irradiation (Tomaselli, 2004).

Chlorella vulgaris is a freshwater microalga, green color, spherical and single-celled, belonging to the family *Chlorellaceae* of the division *Chlorophyta*. It is currently used as a healthy food supplement of valuable contents and for medicinal purposes due to its carotenoids, chlorophylls and proteins content (Sharma et al., 2011). In addition, growth performance and total fish protein were both observed by using fish feed supplemented with *Chlorella* (Maliwat et al., 2016). It was also reported that *C. vulgaris* is a potential source of fatty acids due to its high lipid content (Varfolomeev & Wasserman, 2011).

Metabolomics is the most recent emerging 'omics' discipline that emphasizes more on the identity and quantity of the endogenous metabolites and measures the dynamic responses of the metabolome to various stimuli (Hounoum et al., 2015). This is because small variation in the body system results into great changes in the metabolite levels. Metabolome referred to the amplification of these changes, and is the most real reflection of biological status of the overall system (Booth et al., 2011). Metabolomics is related to metabolome, which in turn represents the small existing molecules of the cells of organisms. The approach is highly competent in the study and evaluation of changes in the small molecular metabolites composition of a biological system under various circumstances. Furthermore, globally, metabolomics is a well-accepted profiling tool for the detection of metabolites and metabolic pathways (Kaddurah-Daouk et al., 2008). Its additional value is in the biological regulation including the control and management of diseases and sheds light on medicine with the help of technological advancements, hence its rapid growth in biological and biomedical research areas. Many new ideas and insights provided by metabolomics-based researches on metabolites and their mechanism of action validated the platform to be further regarded as a leading platform for the study of diseases. Proton nuclear magnetic resonance (^{1}H NMR) is emerging as a leading analytical tool in metabolomics studies due to its large usage and the bulk number of compounds identified. Thus, ^{1}H NMR-based metabolomics can be used as a valid approach to assess the correlation between identified metabolites of an animal model and its biological parameters (Cevallos-Cevallos et al., 2009).

1.2 Statement of the problem

Tilapia is one of the most important cultured fish species worldwide, due to their exceptional fast growth rate and high tolerances towards various harsh environmental conditions (Zahran & Risha, 2014). Despite these important features of tilapia, various diseases find their way to attack and destroy them during culturing as a result of expansion and advancement of human activities, especially industrial. Most farmers revert to use antibiotics in fish disease treatment, but this practice is not completely a safe alternative. Increase in antimicrobial resistance keeps rising consequently leading to more fish mortality and pose threat to fish consumer's health. This is a major setback in aquaculture practices and requires immediate scientific attention to resolve the problem; it is believed that the introduction of microalgae as immunostimulants will replace other modalities used in disease mitigation in fish.

1.3 Justification of the study

Although *C. vulgaris* is one of the most studied microalga worldwide, nevertheless no research has been conducted to identify the correlation between the chemical biomarkers responsible for its immunostimulant property in tilapia sp. using metabolomics approach. Hence this research project reports for the first time the metabolic signatures of immunostimulant effects of *C. vulgaris* in tilapia *O. niloticus* via metabolomics approach.

1.4 Research objectives

The focus of the study is to obtain a deeper insight into the beneficial effects of the microalgae *C. vulgaris* as an immunostimulant for tilapia fish (*O. niloticus*). Towards realizing this goal, the study sets out to achieve the following objectives:

1. To comprehensively characterize the metabolome of *C. vulgaris* via metabolite profiling using NMR metabolomics, GCMS fatty acid analysis and LCMS/MS spectrometry.
2. To determine the biological and health effects of *C. vulgaris* on tilapia fish in a safety concentration study and evaluate the immunostimulant properties of *C. vulgaris* in the same model via immune and challenge assays.
3. To correlate the biological activity of *C. vulgaris* in tilapia to the metabolite's regulation via biomarker identification, and identify the biochemical pathways involved via metabolites association and pathway analysis.

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